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Environmental Toxicology and Chemistry

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**Abstract:** In this study, we examined how sensitivity to oil changes in combination with environmental stressors in *Fundulus grandis* embryos. We exposed embryos (< 24 hours post fertilization) to a range of High Energy Water Accommodated Fraction (HEWAF) concentrations (0-50 ppb total polycyclic aromatic hydrocarbons) made from Macondo oil in conjunction with various environmental conditions (temperature: 20, 30°C; salinity: 3, 7, 30 practical salinity units (PSU); DO: 2, 6 mg/L). Endpoints included mortality, hatching rates, and expression of cytochrome p450 1a and 1c (*cyp1a*, *cyp1c*) in hatched larvae. There was 100% mortality for all fish under the 2 ppm dissolved oxygen (DO) regimes. For the 6 mg/L DO treatments, mortality and mean lethal time were generally higher in the 30°C treatments versus the 20°C treatments. Oil increased mortality in fish exposed to the highest concentration in the 20-3-6 (°C-PSU-mg/L), 25-7-6, and 30-30-6 conditions. Hatching was driven by environmental conditions, with oil exposure only having a significant impact on hatching in the 25-7-6 and 30-30-6 groups at the highest HEWAF exposure. Expression of *cyp1a* was upregulated in most treatment groups versus the controls, with *cyp*1*c* expression exhibiting a similar pattern. These data suggest interactive effects among temperature, salinity, and PAHs, highlighting a need to further assess the effects of oil exposure under various environmental conditions. This article is protected by copyright. All rights reserved

**Keywords:** Fish, HEWAF, Cytochrome P450s, Dissolved oxygen, Salinity, Survival, Temperature

## INTRODUCTION

The 2010 Deepwater Horizon (DWH) oil spill resulted in the release of ~5 million barrels (~800 million L) of crude oil into the Gulf of Mexico (GoM) (McNutt et al. 2012). Oil from the spill was documented on 1,773 km of Gulf Coast shoreline with 687 km of that shoreline remaining oiled two years after the spill (Michel et al. 2013). The concentration of polycyclic aromatic hydrocarbons (PAHs) in coastal sediments immediately following the spill was reported to reach 178,000 ppb in certain locations with coastal seawater samples averaging 47 ppb (Sammarco et al. 2013). Oil concentrations at these coastal sites elicited genomic and physiological responses in resident GoM killifish (*Fundulus grandis*) as evidenced by expression of molecular biomarkers (cytochrome P450 (*cyp*) transcripts) and changes in gill histology (Whitehead et al. 2012). In particular, embryonic exposure to oiled coastal sediments from the DWH spill was shown to negatively impact *F. grandis* health including reduced hatching and increased mortality and developmental abnormalities (Dubansky et al. 2013).

While the effects of embryonic exposure to DWH oil have been studied, limited studies are available which assess the effects of PAH exposure throughout embryonic development in combination with additional environmental stressors in fish (Carls and Thedinga 2010). This area of research is especially important in the GoM, as the shoreline that was oiled during the DWH spill is comprised of estuarine habitats characterized by wide ranges of environmental conditions. For instance, 5 to 29% of estuarine areas of the Louisianan Province (some of which were impacted by oil after the DWH spill) experience hypoxia (Dissolved Oxygen, DO < 2 ppm) (Engle et al. 1999). In these same estuaries salinities range widely from <5 PSU to >18 PSU (U.S. Environmental Protection Agency 1999). In addition to DO and salinity, temperature can

also vary greatly in the shallow estuaries of the GoM and can range from 4-32°C throughout the year (U.S. Environmental Protection Agency 1999). As a result, fish living in these environments were not only exposed to the oil in the aftermath of the DWH spill, but also to varying environmental stressors that may affect their response to oil exposure.

Like oil, exposure to environmental stressors can impact the heath and survival of embryonic fish. *Fundulus grandis* is native to coastal salt marshes of the Atlantic and of Gulf of Mexico, and typically spawns from spring to fall months when salinities range from 5 – 39 PSU and temperatures from 19 – 24°C (Nordlie 2006; Brown et al. 2011, 2012). However, despite residing in habitats characterized by broad variability, sub-optimal environmental conditions can significantly impact reproductive success of this species. For example, Brown et al. (2011) demonstrated that *F. grandis* embryos reared in salinities of 0.4, 15, and 30 PSU exhibited decreased hatching compared to those reared at 7 PSU. High temperatures have also been shown to significantly decrease total length and total body depth of laboratory hatched *F. grandis* embryos (Brown et al. 2012). Dissolved oxygen levels, which fluctuate in estuaries inhabited by *F. grandis*, also impact embryonic development. Low DO levels during embryogenesis have been associated with increased mean hatching time in the closely related *Fundulus heteroclitus* (Dimichele and Taylor 1980).

During the DWH oil spill, fish exposed to oil were likely simultaneously experiencing stress from extreme temperature, salinity, and dissolved oxygen levels. Therefore, it is important to understand the combined impact of oil and environmental stressors on fish health, especially during the sensitive stage of embryonic development. The goal of this study was to simultaneously expose embryos of *F. grandis*, a representative estuarine fish, to both DWH oil and environmental stressors to determine under which environmental conditions embryos are

most sensitive to oil exposure. We hypothesized that increased stress as a result of extreme environmental factors would result in a greater uptake of PAH (as measured by expression of *cyp1* transcripts) and that this enhanced uptake would be correlated with adverse effects to embryonic health, measured as reduced hatching rates and increased mortality.

## MATERIALS AND METHODS

### *Animal model*

*F. grandis* was chosen as a model species for this study. It is an abundant species along the GoM shore and is easily cultured under laboratory conditions (Green 2013). In addition, *F. grandis* typically inhabits coastal marshes and estuaries, and is able to survive under a wide range of temperature, salinity (Green 2013) and oxygen levels (Nordlie 2006). Adult *F. grandis* specimens were collected from an uncontaminated site near Biloxi, Mississippi in March 2013 then held in quarantine for a minimum of 14 days at the Gulf Coast Research Laboratory of the University of Southern Mississippi and shipped overnight to the Aquatic Research Laboratory at Purdue University. Fish were held in sets of 1 male: 2 female per 130-L aquarium. A total of 12 tanks were connected as part of a ~1,900-L recirculating aquaculture system complete with mechanical, biological, and ultraviolet (UV) filtration (Pentair, Aquatic Eco-Systems, Apopka, FL, USA). Breeding pairs were fed to satiation with frozen chironomids and Purina Aquamax (Gray Summit, MO, USA) floating trout pellets (morning), and frozen brine shrimp and Purina Aquamax floating trout pellets (evening). The tank system was held inside of a walk-in environmental chamber and conditions were maintained at 25 ± 1˚C with a 16L:8D photoperiod. Culture media was reconstituted saltwater prepared using Fritz SuperSalt Concentrate (Mesquite, TX, USA) for a salinity of 15 PSU.

*Experimental design*

Spawntex mats (15 x 20 cm, Pentair, Aquatic Eco-Systems) were placed in breeding tanks each morning and collected six hours later. Embryos were collected from a minimum of 10 sets of breeding pairs by submerging and gently tapping the spawning mats in culture media following the methods described by Green (2013). Immediately after collection, eggs were examined under a dissecting scope at 10-40X magnification to confirm viability. Embryos displaying cell cleavage and the formation of a biconvex lens structure, but having not yet developed a discernible germ ring, were considered viable (Armstrong and Child 1965) and set aside for use in experiments.

For each experiment, live embryos were selected at random and transferred to 22 mL clear glass vials filled completely with exposure water corresponding to each treatment group and sealed with Teflon caps to maintain oxygen levels constant. A total of 6 embryos/vial with four replicates per treatment group were utilized. A multifactorial design that included different combinations of salinity (3, 7, 30 PSU), temperature (20, 25, 30 ˚C), dissolved oxygen levels (2, 6 mg/L), and oil concentrations (0-50 ppb of high energy water accommodated fractions, HEWAF) were tested for a total of 56 experimental conditions. Every 24 h, a 100% exchange of exposure media was performed, and water quality (DO, temperature, and salinity), embryo survival, and number of embryos hatched was recorded. Prior to media exchange, temperature was measured using a YSI PRO1020 meter (Yellow Springs, OH, USA), salinity was measured using a Pentair Vital Sine SR6 handheld refractometer (Cary, NC, USA), and DO was measured using custom-made sensors previously developed in our lab and read by touching the surface of the glass chamber with a fluorometer (Stensberg et al. 2014; Gao et al. 2016). Sensors were

made by mixing 2 g of chloroform, 300 mg 5-µm TiO2 particles, 13 mg platinum(II) meso-tetrakis(pentafluorophenyl)porphyrin (PtTFPP), and 7 polystyrene pellets as described in Gao et al. (2016). PtTFPP is an oxygen-sensitive dye that fluoresces in proportion to the oxygen level in the environment with an excitation $\lambda_{max}$ = 392 nm and an emission $\lambda_{max}$ = 650 nm. The mixture was applied to the bottom of each experimental glass vial and allowed to dry for at least 24 h (at which point it was insoluble) before the start of experiment. Fluorescence readings were recorded and converted to ppm using a Tau Theta data logger and optrode (Tau Theta Instruments, LLC, Boulder, CO). Temperature was maintained by placing all experimental units in an environmental chamber. Experiments ended when all embryos had either hatched or died (~25 – 45 days). Hatched embryos were flash frozen in liquid nitrogen and stored at -80˚C for future molecular analyses.

*High energy water accommodated fractions (HEWAF)*

HEWAF stock (1:1) was prepared by mixing 2 g Macondo crude oil (supplied by British Petroleum) with 2 L reconstituted saltwater of either 3, 7, or 30 PSU salinity in a Waring CB15 commercial blender set on low speed (Torrington, CT, USA) for 30 s. The blended liquid was then decanted into a separatory funnel and allowed to settle for 1 h. A 1-L aliquot of the aqueous phase was collected in amber glass bottles and stored at 4˚C for use in exposures and analysis. New HEWAF was prepared every 72 h throughout the duration of the experiments and serially diluted to reach the desired concentrations (nominal concentrations: 0, 1.56, 3.13, 6.25, 12.5, 25, and 50 ppb). Measured HEWAF concentrations for each condition and treatment group are shown in Table **S1**.

*HEWAF fluorescence*

A Turner Designs 10-AU fluorometer (San Jose, CA, USA) was used to measure total fluorescence emitted by PAHs present in the HEWAF as a cost-effective approach for quantifying total PAH concentrations. Following the method described by Greer et al. (2012), 3.5 mL samples of HEWAF were collected during water changes and added to 3.5 mL ethanol in glass scintillation vials. Samples were then sonicated for 3 min to minimize hydrocarbon adhesion to the container, and then centrifuged at 9,100 *g* for 10 min to remove salt particles (which could have interfered with fluorescence readings). A 5-mL aliquot was drawn from the sample and placed into a quartz cuvette for analysis. In order to create a calibration curve, 750 mL serial dilutions of HEWAF were prepared at each of the three salinities (3, 7, and 30 PSU, **Figure S1**), packaged in amber glass bottles, and shipped on ice overnight to the University of Connecticut Center for Environmental Sciences and Engineering (CESE) for total PAH quantification.

*Chemical analyses of HEWAF*

Samples used to generate the fluorescence calibration curve were analyzed at CESE for the alkyl and parent PAHs as well as alkanes. Water samples were extracted using solid phase extraction (SPE). The Waters HLB SPE cartridge (Milford, MA, USA) was conditioned with methanol, 200 mL of water was passed across the cartridge, and the target compound was eluted with acetonitrile. Following extraction, samples were analyzed using an Agilent (Norwalk, CT, USA) 6890 gas chromatograph equipped with a Restek (Bellefonte, PA, USA) Rxi-5Sil MS column (30 m) using splitless injection, coupled to a Waters (Milford, MA, USA) Quattro Micro tandem mass spectrometer (GC/MS/MS). All peaks were quantified against an internal standard and extraction efficiency was evaluated using multiple surrogate standards. Standard quality

assurance procedures were employed, including analysis of duplicate samples, method blanks (Blank), matrix spike duplicates, and laboratory control samples.

cyp1 *expression*

Tissue was pooled and homogenized from three larvae in each of the 0, 3.13, 12.5 and 50 ppb HEWAF treatments and total RNA was extracted using QIAzol reagent (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. Total RNA concentration was quantified via UV spectrophotometer (Nanodrop 1000, ThermoScientific, Wilmington, DE, USA), then DNAse-treated to remove possible genomic DNA contamination (DNase I, Fermentas Inc., Glen Burnie, MD, USA). Treated RNA (2.5 µg per reaction) was reverse transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).

Quantitative PCR (qPCR) assays were performed following MIQE guidelines (Bustin et al. 2009) to measure expression of cytochrome P450 transcripts (*cyp1a, cyp1c*) as biomarkers of PAH exposure (primers listed in **Table S2**). Beta-*actin* was used as the assay reference gene because its expression was stable throughout development and not affected by test conditions (data not shown). Reactions were performed in a 96 well plate with a 20 µL total reaction volume comprised of 10 µL of Master mix (iQ™ SYBR Green Supermix, Bio-Rad Laboratories, Hercules, CA, USA), 10 µM of forward and reverse primers, 100 ng of cDNA template, and nuclease-free water to fill the remaining volume. Three biological replicates were analyzed per condition, with each sample amplified in duplicate as separate reactions on the same plate. All reactions were performed on a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: initial template denaturation at 95°C for 3 min, 40 cycles of 95°C for 10 sec, primer annealing at 58°C for 30 sec, and product

extension at 72°C for 30 sec. Bio-Rad CFX 2.1 software was used for real-time qPCR data acquisition and analysis, and the relative expression of each target gene was calculated by normalization to the reference gene.

*Statistics*

All data were analyzed using SigmaPlot 11, and are reported as mean ± SEM. Data were n+1 log transformed, tested for equality of variance (all groups passed) and for normality using a Shapiro-Wilk test (all except the 30°C-30 PSU group passed). Means were compared across conditions using a one-way ANOVA followed by a Dunnett's multiple comparison test. Significant differences were considered at $P < 0.05$. To examine interactive effects among temperature, salinity, and PAH, a three-way analysis of variance (ANOVA) was performed (**Table S4**). In order to calculate $LC_{50}$ and $LT_{50}$ values, generalized linear models were made in R's drc package (drc version 3.0-1; R version 3.4.0).

**RESULTS**

No embryos survived the low DO (2 mg/L) conditions and all died soon after the start of the experiments; therefore, these data have been excluded from the remainder of this manuscript and further discussion will focus exclusively on the results of the exposures conducted under normoxia. We performed a three-way ANOVA to examine possible synergistic effects of combined temperature, salinity and PAH exposure on mortality and hatching under normoxic conditions. We found no significant three-way interactive effect among the variables examined (**Table S4**); however, temperature and salinity in combination had a significant interactive effect on mortality (**Table S4**). PAH dose significantly increased mortality when considered as an individual variable. It should be noted that in order to meet the assumptions necessary for the

three-way ANOVA, we had to exclude the 25-7-6 group and nominal PAH concentrations were used.

*Water chemistry and exposure conditions*

Summary of HEWAF and PAH concentration results are shown in **Tables 1** and **S3**. A high proportion of naphthalene and naphthalene homologues were present in the prepared HEWAF at all three salinities used in the experiments. Fluorescence calibration curves, prepared as described in the methods section, were used to estimate total PAH concentrations for each experiment. Estimations of total PAH concentrations in the HEWAF stock solution (1 g/L crude oil) prepared for the different conditions ranged from 956 to 1,487 ppb, and tended to increase with increasing salinity. Variability in fluorescence was small over the course of the experiments which indicates that exposure to HEWAF was constant over the course of the experiment (**Figure S2**).

Temperature target values were achieved and maintained in all cases, with the exception of two instances where the 20°C groups reached close to 25°C for approximately one day (**Figure S3A**). For the normoxic conditions, DO levels were maintained near the 6 mg/L mark (**Figure S3B**).

*Embryo hatching and mortality*

Oil generally decreased hatching in a dose-dependent manner; however, statistically significant deviation from control values was observed in the highest dose for only two of the conditions tested (25-7-6 and 30-30-6) (**Figure 1A**). Hatch percentage was higher for control organisms in exposures conducted at 20°C (greater than 90%) than it was for exposures conducted at 30°C (less than 70%). Salinity also influenced hatching rates; at 20°C, increasing the salinity from 3 PSU to 30 PSU reduced some of the effects of the oil exposure, as the percent

reduction in hatching success between the controls and highest PAH concentration was 30% at 3 PSU, but 12.5% at 30 PSU; however, this decline in hatching was not statistically significant. In contrast, when the exposures were conducted at 30°C, the 3 PSU exposed fish exhibited about a 20% reduction in hatching success when exposed to the highest concentration of oil, but a greater than 30% reduction in hatch success when the salinity was increased to 30 PSU. Hatch percentage for the control group in the intermediate 25-7-6 conditions was higher than controls in the 30°C groups and slightly lower, but similar, to the hatch percentage in 20°C groups.

Time to hatch decreased with increased temperature (**Figure 2A**). Salinity also impacted time to hatch with the high salinity group (30 PSU) taking approximately 10 days longer to hatch compared to the low salinity group (3 PSU) when held at the same temperature. Mean time to hatch for controls at 20˚C and 3 PSU salinity was 26.5 ± 0.6 d and increased to 36.5 ± 1.0 d at 30 PSU (**Figure 2A**). Experiments conducted at 30˚C exhibited shorter hatching times compared to 20˚C. Increased salinity at 30˚C resulted in a substantial increase in time to hatch (mean time to hatch for controls was 15.5 ± 1.0 d and 24.5 ± 1.0 d at 3 and 30 PSU, respectively). The experiment conducted at 25˚C and 7 PSU salinity had intermediate hatching rates with mean time to hatch of controls at 18.8 ± 0.5 d.

Like hatching, mortality increased in an approximate PAH dose-dependent manner, with significant differences from controls detected in the highest PAH dose for 20-3-6, 25-7-6, and 30-30-6 groups (**Figure 1B)**. Higher temperatures resulted in higher embryo mortalities: control embryo mortality was < 10% in the two exposures conducted at 20°C; 17% in the 25°C exposure; and > 30% in the two exposures conducted at 30°C. At both 20°C and 30°C, there was ~4% difference in control mortality between the 3 and 30 PSU groups, indicating that in the

absence of oil, temperature plays a more significant role in larval survival than salinity under the conditions tested herein.

The impact of salinity on mortality mirrored the trend observed in hatching data; increasing salinity in 20˚C treatments diminished the effects of PAH exposure, while the opposite was true for 30˚C treatments. For example, at 20°C, increasing the salinity from 3 to 30 PSU nearly doubled the $LC_{50}$ (64.64 ± 5.27 ppb at 20-3-6, but 113.05 ± 9.49 ppb at 20-30-6) (**Figure 3A**). At 30°C the $LC_{50}$ was 76.20 ± 11.08 ppb for 30 PSU treatments, while the $LC_{50}$ at 3 PSU was not calculable (although the mortality data presented in **Figure 1B** make it apparent that the embryos exposed at 30-3-6 exhibited less PAH-driven mortality than did the embryos exposed to oil at 30-30-6) (**Figure 3A**). The $LC_{50}$ for the intermediate treatment (25-7-6) was 51.95 ± 3.07 ppb (**Figure 3A**).

The median lethal time decreased with increasing temperature (**Figure 3B**). At the intermediate exposure conditions (25-7-6) the median lethal time ($LT_{50}$) for the highest PAH concentration was 26.48 ± 4.18 days. Unsurprisingly, the exposures conducted at 30°C exhibited mortality sooner than the exposures conducted at 20°C. When the exposures were conducted at 30°C, the median lethal time for the highest PAH concentration at 3 PSU was 16.82 ± 1.34 days, while at 30 PSU it was 18.38 ± 1.53 days. At the lower temperature, the $LT_{50}$ was 40.39 ± 4.68 days at 3 PSU. No reliable $LT_{50}$ was calculable for the exposures conducted at 20-30-6, as under these conditions the larvae had approximately 80% survival even under the highest PAH concentrations tested.

cyp1a *and* cyp1c *gene expression*

Expression of both *cyp1a* and *cyp1c* was induced by all HEWAF concentrations measured, indicating active uptake and metabolism of PAHs (**Figures 4** and **5**). For every

environmental condition tested, relative expression *of cyp1a* increased in fish exposed to low concentrations of HEWAF compared to controls and remained elevated in fish exposed to higher concentrations (with the exception of the highest concentration tested in the 30°C-3 PSU group, which is likely due to PAHs causing overt toxicity, masking subtle changes in gene expression) (**Figure 4**). Temperature also influenced the expression of *cyp1a,* as fish exposed to 30°C conditions exhibited greater expression (up to ~900 fold increase) compared to those exposed to 20°C conditions (up to ~50 fold increase). HEWAF exposure also induced expression of *cyp1c*, although at much lower levels than *cyp1a* (**Figure 5**). Additionally, induction of *cyp1c* expression appeared to be influenced by salinity, as fish exposed to 30 PSU treatments exhibited higher expression values (up to ~50 fold increase) than those exposed to 3 PSU (up to ~12 fold increase). This effect generally corresponded to higher concentrations of PAHs present in HEWAF with increased salinity (**Tables 1 and S3**). It should be noted that for both *cyp1a* and *cyp1c*, we saw lower overall induction in our intermediate conditions (25-7-6) than for any other conditions. Overall, we observed *cyp* expression levels topping out at a certain threshold rather than increasing monotonically, possibly due to hitting the upper limit of *cyp* transcript expression even at the lower HEWAF concentrations tested.

## DISCUSSION

While the mortality of all embryos under hypoxic conditions indicates that hypoxia is the major environmental driver of mortality in *F. grandis* embryos in this experiment, our data also suggest that temperature and salinity interact to affect the survival and hatching of *F. grandis* embryos exposed to oil, and that PAH dose may play a role in this interaction.

The effects of PAH exposure on fish embryos are well characterized and include increased mortality (Carls and Thedinga 2010; Carls et al. 1999; Heintz et al. 1999; Hedgpeth

and Griffitt 2016), delayed or reduced hatch (Dubansky et al. 2013a; Hedgpeth and Griffitt 2016), and developmental abnormalities (Carls et al. 2008; Dubansky et al. 2013a; Incardona et al. 2013, 2014; Mager et al. 2014). Our data corroborate these findings, as all groups in our experiment displayed an approximate PAH dose-dependent relationship for hatching and mortality data (**Figures 1A and B**). Our statistical analysis found no evidence of a full three-way interactive effect among PAH dose, temperature, and salinity; however, it should be noted that in order to meet the statistical assumptions necessary to perform a three-way ANOVA, the 25-7-6 group was excluded and nominal PAH concentrations were used. $LC_{50}$ and $LT_{50}$ results suggest some interactions among variables: changing temperature from 20°C to 30°C decreases the $LC_{50}$ at 30 PSU and sharply increases the $LT_{50}$ at 3 PSU, indicating that exposure temperature can alter lethality of PAHs (**Figures 3A and B**). In addition, at 20°C, increasing salinity from 3 PSU to 30 PSU increases $LC_{50}$, but changing the salinity at 30°C has no effect on $LT_{50}$ (**Figures 3A and B)**. There is little literature regarding the interactive effects of these variables, however our results are supported in early work by Linden et al. (1979) who found that a combination of suboptimal temperatures, salinity, and PAH dose interacted to increase embryonic mortality in *Fundulus heteroclitus*, a congener of *F. grandis*.

The data from our control groups clearly demonstrate that even in the absence of PAHs, temperature and salinity act together to modulate mortality and hatch rates in *F. grandis* embryos. For example, control groups incubated at 20°C exhibited increased time to hatch relative to intermediate conditions (25-7-6), while at 30°C time to hatch decreased (**Figure 2A**). In each case, increasing salinity from 3 to 30 PSU had the effect of prolonging time to hatching. Other research regarding the interactive effects of temperature and salinity (in the absence of PAH exposure) on fish hatching supports our findings. For example, Brown et al. (2011) found

that in *F. grandis* embryos, temperature and salinity interactions significantly impacted time-to-hatch. A closely related species, *F. heteroclitus*, has shown faster time to hatch rates under higher temperature conditions, and slightly longer time to hatch rates at higher salinities, regardless of temperature (Tay and Garside 1975). This same study observed that the highest total and highest viable hatch counts were at 20°C under any salinity regime tested (0-60 PSU), which is consistent with our findings that the 20°C treatments exhibit the highest hatching rates of any groups.

Our $LT_{50}$ data (**Figure 3B**) directly correspond with our hatch data to suggest that temperature and salinity interact to affect PAH-induced mortality. We found that $LT_{50}$ values decreased with increasing temperature (from 30°C to 25°C to 20°C). In addition to describing mortality, our $LT_{50}$ values also correlated with *cyp* expression, as the two groups with the lowest $LT_{50}$ values (30 3-3-6 and 30-30-6) were also the groups with the highest *cyp1a* expression levels (**Figure 4**), indicating heightened uptake of PAHs at elevated temperatures. Given that mortality and hatch were greatly influenced by temperature in our control groups, this enhanced uptake of PAHs at 30°C is likely an artifact of an overall increase of metabolism in *F. grandis* at heightened temperatures, and provide further support for the role of temperature as one of the primary influences on the effects measured in this study.

Overall, our *cyp1a* and *cyp1c* data are corroborated by previous research indicating that exposure to DWH oil induces expression of *cyp* transcripts. Field studies post-DWH have found increased expression of CYP1A protein in lamellae of *F. grandis* larvae at oiled versus non-oiled sites (Whitehead et al. 2012), as well as increases in *cyp1a* mRNA and protein expression in adults (Dubansky et al. 2013). These results have been confirmed in laboratory experiments, with increases in *cyp1a* found in adult *F. grandis* exposed to DWH crude oil (Crowe et al. 2014).

While we found some evidence to suggest temperature, salinity, and PAH exposure all interact to affect the endpoints of mortality and hatch, it is necessary to further investigate the combined effects of these stressors on sub-lethal endpoints or life stages not investigated herein. For example, Dubansky et al. (2013) incubated *F. grandis* embryos in sediments oiled during the DWH oil spill, and found no increase in mortality, but did find substantial developmental defects including cardiovascular abnormalities such as pericardial edema. Impacts to cardiovascular development following embryonic PAH exposure appear to be a conserved response in teleosts as this effect has been documented in multiple taxa (Carls et al. 1999, 2008; Couillard 2002; Pollino and Holdway 2002; Incardona et al. 2005, 2009, 2013, 2014). Like cardiac defects, abnormal craniofacial development following embryonic exposure to PAHs also appears to be a conserved effect in teleosts (Carls et al. 1999; Incardona et al. 2004; de Soysa et al. 2012; Greer et al. 2012). The prevalence of these developmental effects renders morphological endpoints following hatch ideal targets for future studies.

In addition to the inclusion of morphological endpoints, population and life stage differences in sensitivity to PAH exposure should be considered when designing future experiments. For example, *F. grandis* embryos have been shown to exhibit differential sensitivities toward PAHs depending upon whether they are derived from polluted or pristine environments, resulting in differences in cardiac teratogenesis, *cyp1a* activity, and chromosomal damage (Oziolor et al. 2014).

While statistically there was no evidence of a three-way interaction among temperature, salinity, and PAH dose, our $LC_{50}$ suggests that some interaction exists among these variables that may be biologically relevant. In each condition set tested, increasing the temperature increased both $LC_{50}$ and $LT_{50}$ data, regardless of salinity. However, the actions of salinity were more

nuanced; at 20°C, increased salinity decreased mortality, while at 30°C there was no effect of salinity on embryonic survival. Our work highlights the need to consider environmental variables when designing future experiments examining the effects of interactions between abiotic factors and PAHs.

*Supplemental Data*—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.xxxx.

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*Data Availability*—For data inquiries please contact Marisol S. Sepulveda (mssepulv@purdue.edu).

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Figure 1. Mean ± standard error of *Fundulus grandis* embryo hatching (A) and mortality (B) for each condition in relation to polycyclic aromatic hydrocarbon (PAH) concentration prepared as High Energy Water Accommodated Fractions. Asterisks denote significant differences ($p < 0.05$) between PAH-dosed larvae and controls (one-way ANOVA) within each condition. Note: the highest PAH dose in the 25-7-6 group is also significantly different from the lowest PAH dose.

Figure 2. Mean ± standard error of percentage of *Fundulus grandis* time to hatch (A) and time to death (B) for each condition in relation to polycyclic aromatic hydrocarbon (PAH) concentration prepared as High Energy Water Accommodated Fractions.

Figure 3. Mean± standard error of Lethal Concentration 50 ($LC_{50}$) (A) and Lethal Time 50 ($LT_{50}$) (B). Note that $LC_{50}$ results for the 30-3-6 group were not able to be calculated, nor were $LT_{50}$ results for the 20-30-6 group.

Figure 4. Fold change in *cyp1a* expression in *Fundulus grandis* whole larvae in relation to condition tested. Different letters denote significant differences ($p < 0.05$) between polycyclic aromatic hydrocarbon (PAH)-dosed larvae and controls (one-way ANOVA). Note that graphs have different Y scales.

Figure 5. Fold change in *cyp1c* expression in *Fundulus grandis* whole larvae in relation to condition tested. Different letters denote significant differences ($p < 0.05$) between polycyclic aromatic hydrocarbon (PAH)-dosed larvae and controls (one-way ANOVA).

**Table 1.** Measured concentrations for all diluted HEWAF test solutions across conditions and treatments.

| Condition | Nominal Concentration: 50 ppb | Nominal Concentration: 25 ppb | Nominal Concentration: 12.5 ppb | Nominal Concentration: 6.25 ppb | Nominal Concentration: 3.13 ppb | Nominal Concentration: 1.56 ppb |
|---|---|---|---|---|---|---|
| 20-3-6 Measured Concentrations: | 31.87 ppb | 15.93 ppb | 7.97 ppb | 3.98 ppb | 1.99 ppb | 1.00 ppb |
| 20-30-6 Measured Concentrations: | 48.4 ppb | 24.2 ppb | 12.1 ppb | 6.05 ppb | 3.03 ppb | 1.51 ppb |
| 25-7-6 Measured Concentrations: | 38.8 ppb | 19.4 ppb | 9.7 ppb | 4.85 ppb | 2.43 ppb | 1.21 ppb |
| 30-3-6 Measured Concentrations: | 34 ppb | 17 ppb | 8.5 ppb | 4.25 ppb | 2.13 ppb | 1.06 ppb |
| 30-30-6 Measured Concentrations: | 49.57 ppb | 24.78 ppb | 12.39 ppb | 6.20 ppb | 3.10 ppb | 1.55 ppb |

**Figure 1**.

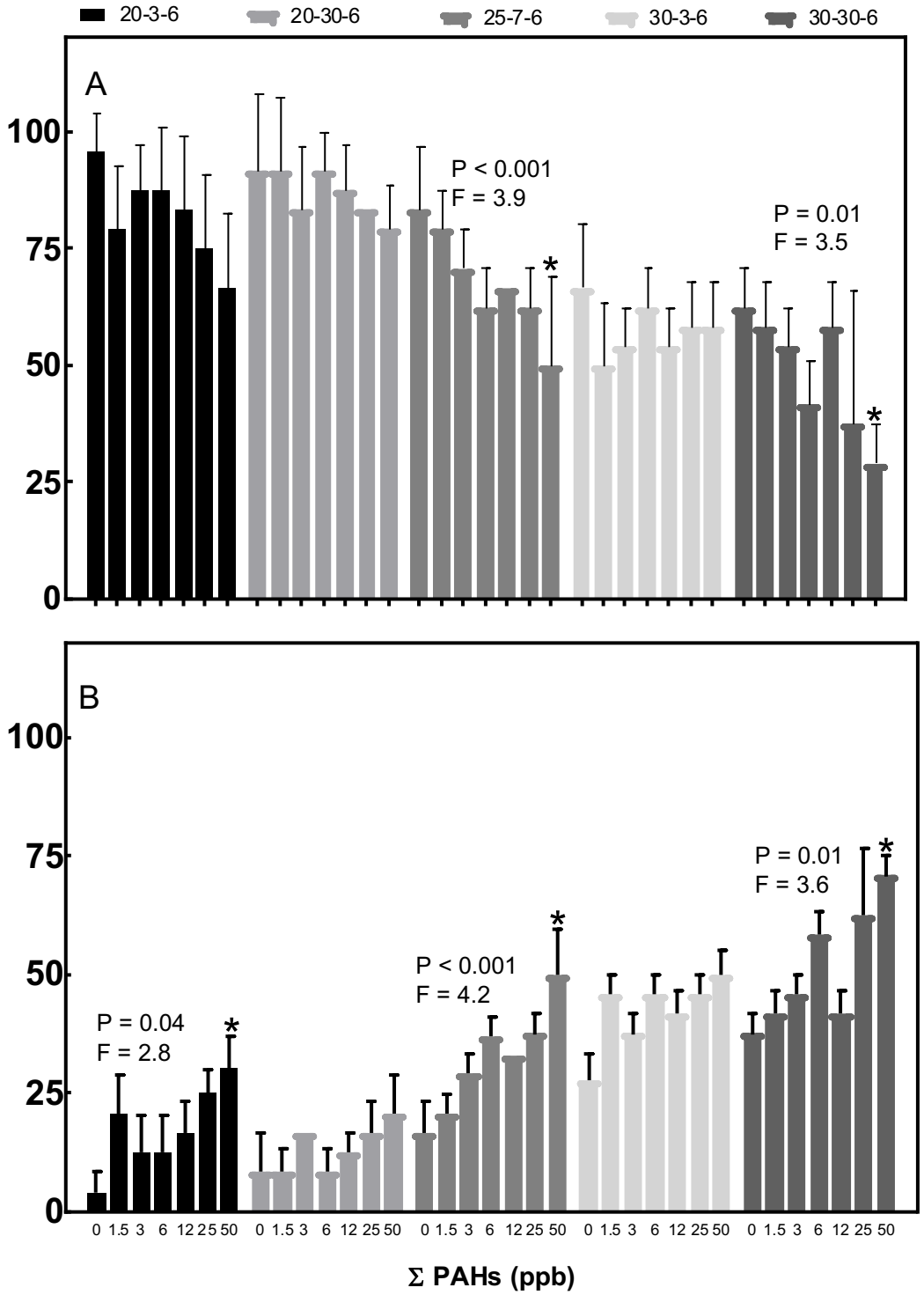

**Figure 2.**

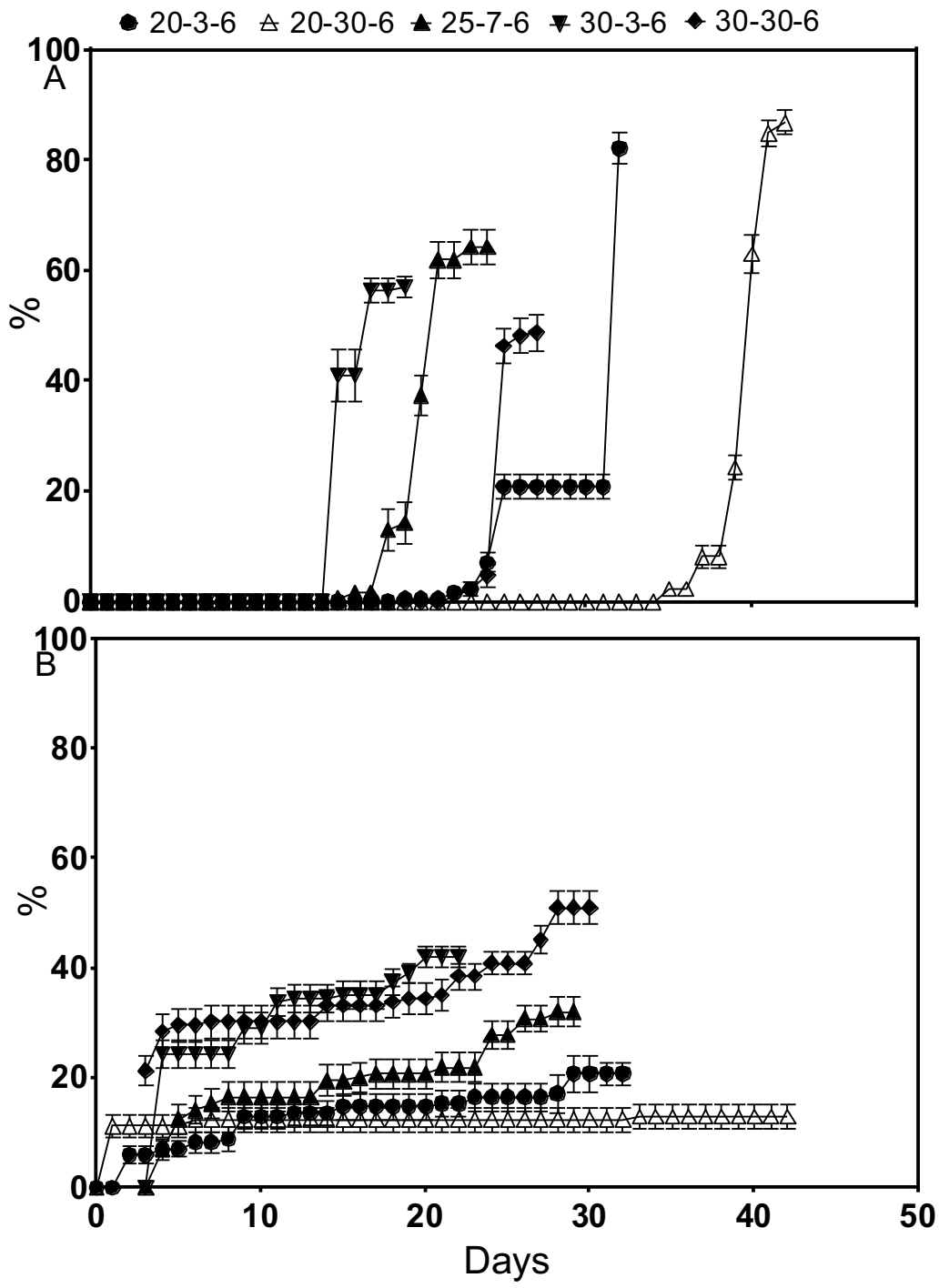

**Figure 3.**

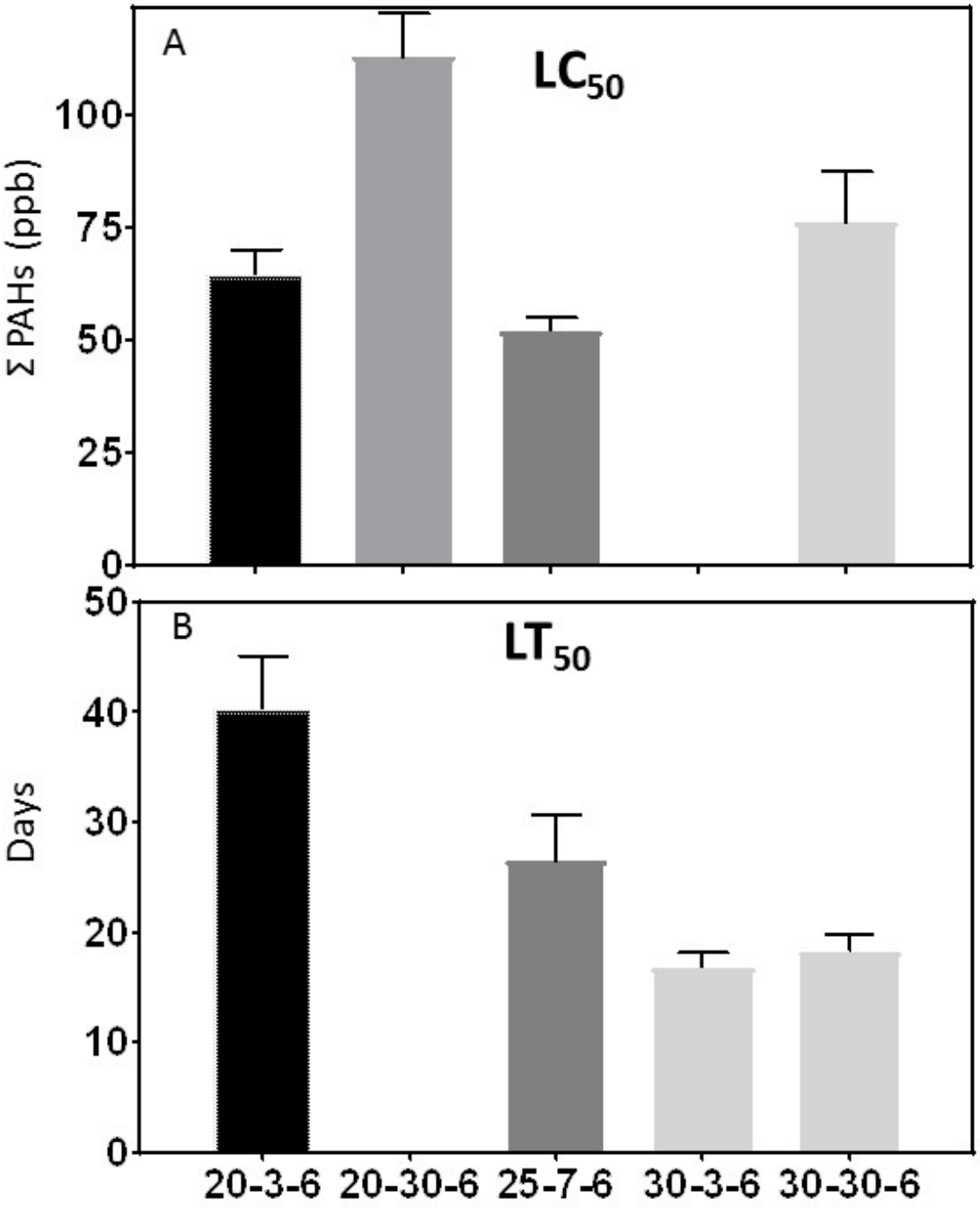

**Figure 4**.

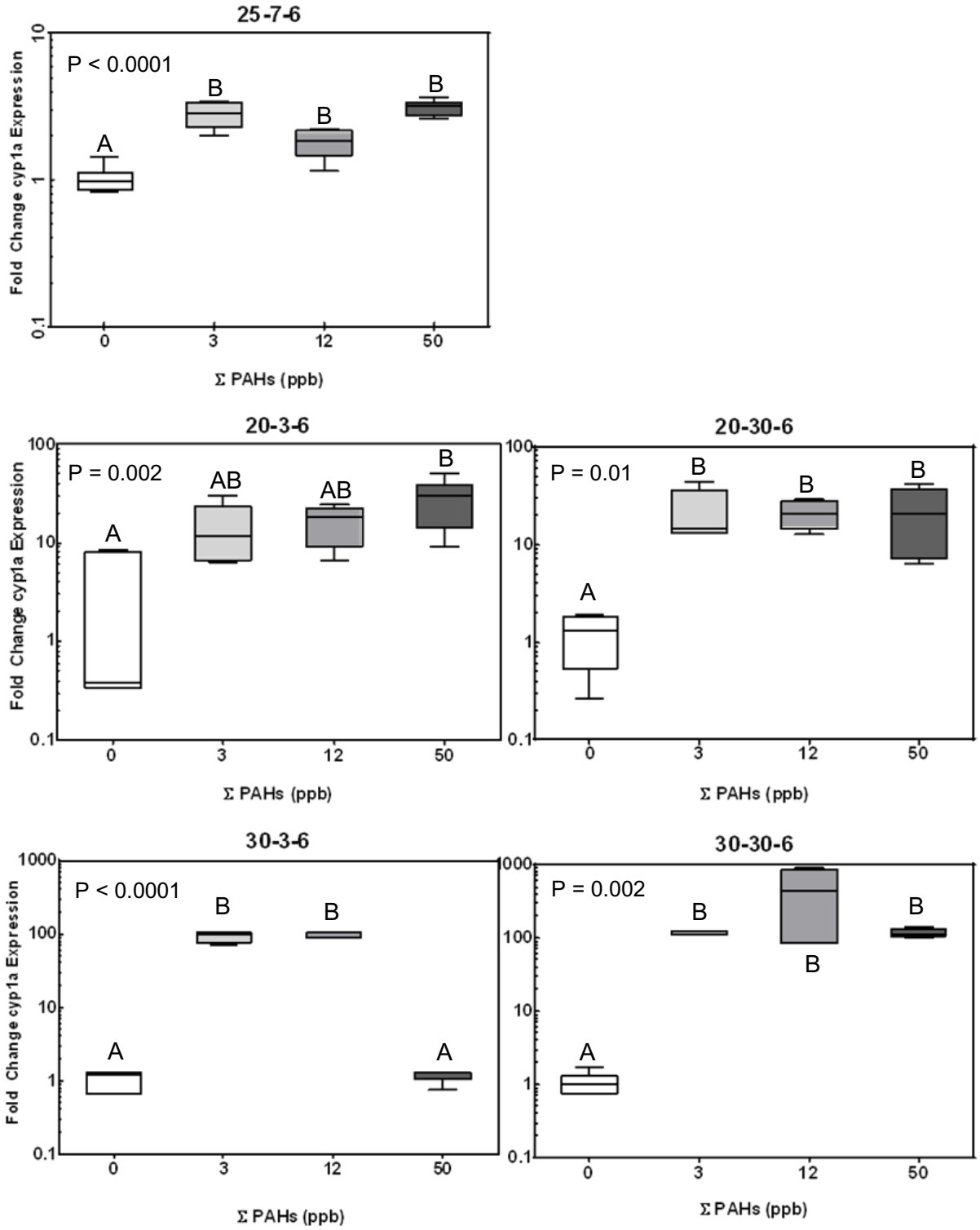

**Figure 5.**