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Rodgers, M.L.; Jones, E.R.; Klinkhamer, C.; Mahapatra, C.T.; Serafin, J.; Bosker, T.; ... ; Sepúlveda, M.S.

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Combined Effects of *Deepwater Horizon* Crude Oil and Environmental Stressors on *Fundulus grandis* Embryos

Maria L. Rodgers,[a] Elizabeth R. Jones,[a] Christopher Klinkhamer,[b] Cecon T. Mahapatra,[b] Jennifer Serafin,[b] Thijs Bosker,[c] Christopher Perkins,[d] Robert J. Griffitt,[a] Sylvain De Guise,[e] and Maria S. Sepúlveda[b,*]

[a]Division of Coastal Sciences, School of Ocean Science and Technology, University of Southern Mississippi, Ocean Springs, Mississippi, USA
[b]Department of Forestry and Natural Resources, Purdue University, West Lafayette, Indiana, USA
[c]Leiden University College/Institute of Environmental Sciences, Leiden University, Leiden, The Netherlands
[d]Center for Environmental Sciences and Engineering, University of Connecticut, Storrs, Connecticut, USA
[e]Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, Connecticut, USA

Abstract: In the present study, we examined how sensitivity to oil changes in combination with environmental stressors in *Fundulus grandis* embryos. We exposed embryos (<24 h post fertilization) to a range of high-energy water accommodated fraction (HEWAF) concentrations (0–50 parts per billion [ppb] total polycyclic aromatic hydrocarbons [PAHs]) made from Macondo crude oil in conjunction with various environmental conditions (temperature: 20 and 30 °C; salinity: 3, 7, and 30 practical salinity units [PSU]; and dissolved oxygen: 2 and 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 parts per million (ppm) dissolved oxygen regimes. For the 6 mg/L dissolved oxygen treatments, mortality and median lethal time (LT50) 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 having a significant impact on hatching in only the 25-7-6 and 30-30-6 groups at the greatest HEWAF exposure. Expression of *cyp1a* was up-regulated in most treatment groups versus the controls, with *cyp1c* 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. *Environ Toxicol Chem* 2018;9999:1–10. © 2018 SETAC

Keywords: Fish; HEWAF; Cytochrome p450s; Dissolved oxygen; Salinity; Survival; Temperature

INTRODUCTION

The 2010 *Deepwater Horizon* oil spill resulted in the release of approximately 5 million barrels (~800 million L) of crude oil into the Gulf of Mexico (McNutt et al. 2012). Oil from the spill was documented on 1773 km of Gulf Coast shoreline with 687 km of that shoreline remaining oiled 2 yr after the spill (Michel et al. 2013). The concentration of polycyclic aromatic hydrocarbons (PAHs) in coastal sediments immediately after the spill was reported to reach 178 000 parts per billion (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 Gulf of Mexico 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 Deepwater Horizon spill was shown to negatively impact *F. grandis* health including reduced hatching, increased mortality, and developmental abnormalities (Dubansky et al. 2013).

Although the effects of embryonic exposure to *Deepwater Horizon* oil have been examined, limited studies are available that 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 Gulf of Mexico because the shoreline that was oiled during the *Deepwater Horizon* 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 *Deepwater Horizon* spill) experience hypoxia

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* Address correspondence to mssepulv@purdue.edu
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(dissolved oxygen < 2 parts per million [ppm]; Engle et al. 1999). In these same estuaries, salinities range widely from < 5 practical salinity units (PSU) to > 18 PSU (US Environmental Protection Agency 1999). In addition to dissolved oxygen and salinity, temperature can also vary greatly in the shallow estuaries of the Gulf of Mexico and can range from 4 to 32 °C throughout the year (US Environmental Protection Agency 1999). As a result, fish living in these environments were not only exposed to the oil in the aftermath of the *Deepwater Horizon* spill but also to varying environmental stressors that may affect their response to oil exposure.

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

During the *Deepwater Horizon* 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 the present study was to simultaneously expose embryos of *F. grandis*, a representative estuarine fish, to both *Deepwater Horizon* 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 PAHs (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 elevated mortality.

MATERIALS AND METHODS

Animal model

Fundulus grandis was chosen as a model species for the present study. It is an abundant species along the Gulf of Mexico 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 d 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 females per 130-L aquarium. A total of 12 tanks were connected as part of an approximately 1900-L recirculating aquaculture system complete with mechanical, biological, and ultraviolet filtration (Pentair Aquatic Eco-Systems). Breeding pairs were fed to satiation with frozen chironomids and Purina Aquamax 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 during a 16:8-h light:dark photoperiod. Culture media was reconstituted salt water prepared using Fritz Super Salt Concentrate for a salinity of 15 PSU.

Experimental design

Spawntex Mats (15 × 20 cm; Pentair Aquatic Eco-Systems) were placed in breeding tanks each morning and collected 6 h later. Embryos were collected from a minimum of 10 sets of breeding pairs by submerging and gently tapping the spawning mats in culture media according to the methods described by Green (2013). Immediately after collection, eggs were examined under a dissecting microscope at 10 to 40× magnification to confirm viability. Embryos displaying cell cleavage and the formation of a biconvex lens structure, but not yet having 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 constant oxygen levels. A total of 6 embryos/vial with 4 replicates per treatment group were used. A multifactorial design that included different combinations of salinity (3, 7, and 30 PSU), temperature (20, 25, and 30 °C), dissolved oxygen levels (2 and 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 (dissolved oxygen, temperature, and salinity), embryo survival, and number of embryos hatched were recorded. Before media exchange, temperature was measured using a YSI Pro1020 meter, salinity was determined with a Pentair Vital Sine SR6 handheld refractometer, and dissolved oxygen was reported utilizing custom-made sensors previously developed in our laboratory 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) (Frontier Scientific), and 7 polystyrene pellets as described in Gao et al. (2016). The 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 time it was insoluble) before the start of experiments. Fluorescence readings were recorded and converted to ppm using a data logger and an optrode (Tau Theta Instruments). Temperature was maintained by placing all experimental units in an environmental chamber. Tests ended when all embryos had either hatched or died (~25–45 d). Hatched embryos were flash frozen in liquid nitrogen and stored at –80 °C for future molecular analyses.

HEWAF

High-energy water accommodated fraction stock (1:1) was prepared by mixing 2 g Macondo crude oil (supplied by British Petroleum) with 2 L reconstituted salt water of either 3, 7, or 30 PSU in a Waring CB15 commercial blender set on low speed 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 tests 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 Supplemental Data, Table S1.

HEWAF fluorescence

A Turner Designs 10-AU fluorometer was used to measure total fluorescence emitted by PAHs present in the HEWAF as a cost-effective approach for quantifying total PAH concentrations. According to 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 9100 *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. To create a calibration curve, 750-mL serial dilutions of HEWAF were prepared at each of the 3 salinities (3, 7, and 30 PSU; Supplemental Data, Figure S1), packaged in amber glass bottles, and shipped on ice overnight to the University of Connecticut Center for Environmental Sciences and Engineering for total PAH quantification.

Chemical analyses of HEWAF

Samples used to generate the fluorescence calibration curve were analyzed at the University of Connecticut for the alkyl and parent PAHs as well as alkanes. Water samples were extracted using solid-phase extraction. The Waters HLB SPE cartridge was conditioned with methanol; 200 mL of water were passed across the cartridge; and the target compound was eluted with acetonitrile. After extraction, samples were analyzed using an Agilent 6890 gas chromatograph equipped with a Restek Rxi-5Sil MS column (30 m) using splitless injection, coupled to a Waters Quattro Micro tandem mass spectrometer. 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 3 larvae in each of the 0, 3.13, 12.5, and 50 ppb HEWAF treatments and total RNA was extracted using QIAzol reagent (Qiagen) according to the manufacturer's protocol. Total RNA concentration was quantified via ultraviolet spectrophotometer (NanoDrop 1000; Thermo Scientific), then DNAse-treated to remove possible genomic DNA contamination (DNase I; Fermentas). Treated RNA (2.5 μg per reaction) was reverse transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit.

Quantitative real-time polymerase chain reaction (qPCR) assays were performed according to minimum information for publication of qPCR experiment guidelines (Bustin et al. 2009) to measure expression of cytochrome p450 transcripts (*cyp1a*, *cyp1c*) as biomarkers of PAH exposure (primers are listed in Supplemental Data, Table S2). Beta-*actin* was used as the assay reference gene because its expression was stable throughout development and was 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), 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 using the following conditions: initial template denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 10 s, primer annealing at 58 °C for 30 s, and product extension at 72 °C for 30 s. Bio-Rad CFX Ver 2.1 software was used for 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 Ver 11 software and are reported as mean ± standard error. Data were $n+1$ log transformed, tested for equality of variance (all groups passed), and tested 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 analysis of variance (ANOVA) and then a Dunnett's multiple comparisons test. Significant differences were considered at $p < 0.05$. To examine interactive effects among temperature, salinity, and PAHs, a 3-way ANOVA was performed (Supplemental Data, Table S4). To calculate

median lethal concentration (LC50) and median lethal time (LT50) values, generalized linear models were made in R's drc software package (drc, Ver 3.0.1; R, Ver 3.4.0).

RESULTS

No embryos survived the low dissolved oxygen (2 mg/L) conditions and all died soon after the start of the experiments; therefore, these data have been excluded from the remainder of the discussion, and further discussion will focus exclusively on the results of the exposures conducted under normoxia. We performed a 3-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 3-way interactive effect among the variables examined (Supplemental Data, Table S4); however, temperature and salinity in combination had a significant interactive effect on mortality (Supplemental Data, Table S4). Polycyclic aromatic hydrocarbon dosage significantly increased mortality when considered as an individual variable. It should be noted that to meet the assumptions necessary for the 3-way ANOVA, we had to exclude the 25-7-6 group and nominal PAH concentrations were used.

Water chemistry and exposure conditions

A summary of HEWAF and PAH concentration results is shown in Table 1 and Supplemental Data, Table S3. A high proportion of naphthalene and naphthalene homologues were present in the prepared HEWAF at all 3 salinities used in the tests. Fluorescence calibration curves, prepared as described in the *Materials and 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 1487 ppb and tended to escalate with increasing salinity. Variability in fluorescence was minimal over the course of the trials, which indicates that exposure to HEWAF was constant throughout the tests (Supplemental Data, Figure S2).

Temperature target values were achieved and maintained in all cases, with the exception of 2 instances where the 20 °C groups reached close to 25 °C for approximately 1 d (Supplemental Data, Figure S3A). For the normoxic conditions, dissolved oxygen levels were maintained near the 6 mg/L mark (Supplemental Data, Figure S3B).

Embryo hatching and mortality

Oil generally reduced hatching in a dose-dependent manner; nevertheless, statistically significant deviation from control values was observed in the highest dose for only 2 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 (>90%) than it was for exposures conducted at 30 °C (<70%). Salinity also influenced hatching rates. At 20 °C, increasing the salinity from 3 to 30 PSU reduced some of the effects of the oil exposure because the percentage 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 approximately 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 the 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 d longer to hatch compared with the low salinity group (3 PSU) when held at the same temperature. Mean time to hatch for controls at 20 °C and 3 PSU was 26.5 ± 0.6 d and increased to 36.5 ± 1.0 d at 30 PSU (Figure 2A). Trials conducted at 30 °C exhibited shorter hatching times compared with 20 °C. Increased salinity at 30 °C resulted in a substantial escalation in time to hatch (mean times to hatch for controls were 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 had

TABLE 1: Measured concentrations for all diluted HEWAF test solutions across conditions and treatments[a]

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	15.93	7.97	3.98	1.99	1.00
20-30-6 Measured concentrations	48.4	24.2	12.1	6.05	3.03	1.51
25-7-6 Measured concentrations	38.8	19.4	9.7	4.85	2.43	1.21
30-3-6 Measured concentrations	34	17	8.5	4.25	2.13	1.06
30-30-6 Measured concentrations	49.57	24.78	12.39	6.20	3.10	1.55

[a]All nominal concentration values are in ppb.
HEWAF = high-energy water accommodated fraction; ppb = parts per billion.

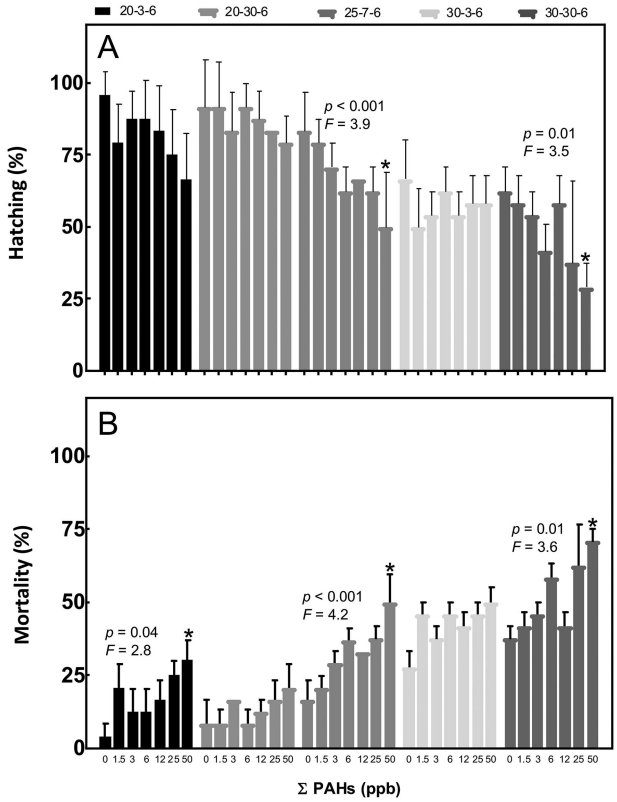

FIGURE 1: Mean ± standard error of *Fundulus grandis* embryo (A) hatching and (B) mortality for each condition in relation to polycyclic aromatic hydrocarbon (PAH) concentrations prepared as high-energy water accommodated fractions. Asterisks denote significant differences ($p < 0.05$) between PAH-dosed larvae and controls (one-way analysis of variance) within each condition. Note: the highest PAH dose in the 25-7-6 group is also significantly different from the lowest PAH dose. F = variance ratio; ppb = parts per billion.

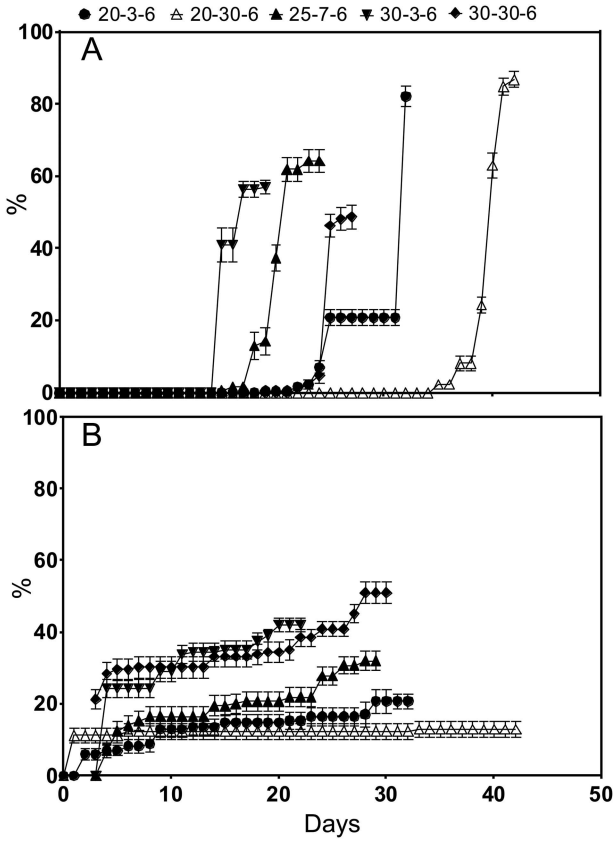

FIGURE 2: Mean ± standard error of percentage of *Fundulus grandis* (A) time to hatch and (B) time to death for each condition in relation to polycyclic aromatic hydrocarbon concentrations prepared as high-energy water accommodated fractions.

intermediate hatching rates with mean time to hatch of controls at 18.8 ± 0.5 d.

Similar to hatching, mortality increased in an approximate PAH dose-dependent manner, with significant differences from controls detected in the highest PAH dose for the 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 2 exposures conducted at 20 °C; 17% in the 25 °C exposure; and >30% in the 2 exposures conducted at 30 °C. At both 20 °C and 30 °C, there was an approximately 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 in the present study.

The impact of salinity on mortality mirrored the trend observed in the hatching data; increasing salinity in the 20 °C treatments diminished the effects of PAH exposure, whereas the opposite was true for the 30 °C treatments. For example, at 20 °C, boosting the salinity from 3 to 30 PSU nearly doubled the LC50 (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 LC50 was 76.20 ± 11.08 ppb for 30 PSU treatments, whereas the LC50 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 the embryos exposed to oil at 30-30-6; Figure 3A). The LC50 for the intermediate treatment (25-7-6) was 51.95 ± 3.07 ppb (Figure 3A).

The LT50 decreased with increasing temperature (Figure 3B). At the intermediate exposure conditions (25-7-6) the LT50 for the highest PAH concentration was 26.48 ± 4.18 d. 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 LT50 for the highest PAH concentration at 3 PSU was 16.82 ± 1.34 d, whereas at 30 PSU it was 18.38 ± 1.53 d. At the lower temperature, the LT50 was 40.39 ± 4.68 d at 3 PSU. No reliable LT50 was calculable for the exposures conducted at 20-30-6 because 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 with

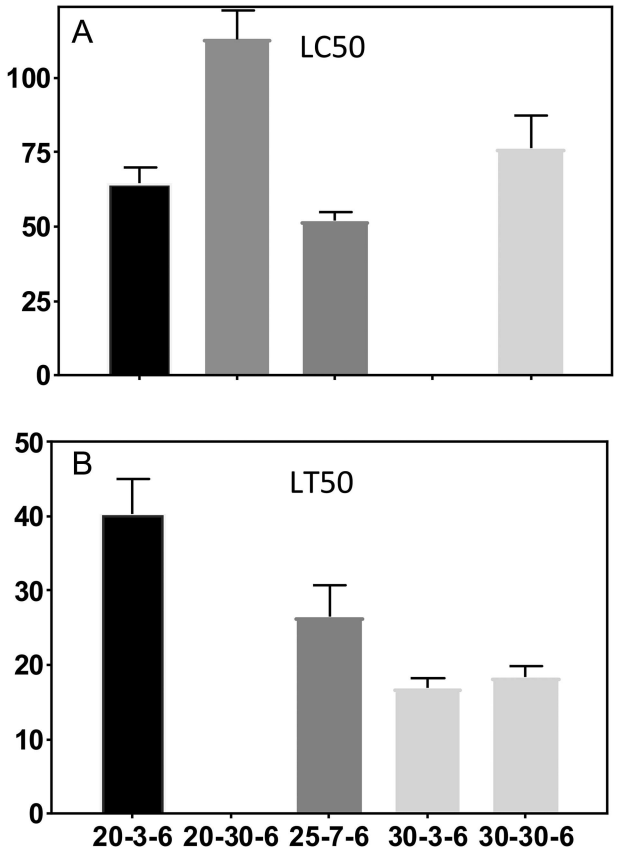

FIGURE 3: Mean ± standard error of (**A**) median lethal concentration (LC50) and (**B**) median lethal time (LT50). Note: the LC50 results for the 30-3-6 group and the LT50 outcomes for the 20-30-6 group were not able to be calculated. PAHs = polycyclic aromatic hydrocarbons; ppb = parts per billion.

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 a result of the PAHs causing overt toxicity, masking subtle changes in gene expression; Figure 4). Temperature also influenced the expression of *cyp1a* because fish exposed to 30 °C conditions exhibited greater expression (up to an ~900-fold increase) compared with those exposed to 20 °C conditions (up to an ~50-fold increase). High-energy water accommodated fraction concentration exposure also induced expression of *cyp1c*, although at much lower levels than *cyp1a* (Figure 5). In addition, induction of *cyp1c* expression appeared to be influenced by salinity because fish exposed to 30 PSU treatment exhibited higher expression values (up to an ~50-fold increase) than those exposed to 3 PSU (up to an ~12-fold increase). This effect generally corresponded to higher concentrations of PAHs present in HEWAF with increased salinity (Table 1 and Supplemental Data, Table 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 caused by hitting the upper limit of *cyp* transcript expression even at the lower HEWAF concentrations tested.

DISCUSSION

Although the mortality of all embryos under hypoxic conditions indicates that hypoxia is the major environmental driver of mortality in *F. grandis* embryos in the present study, 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 dosage may play a role in this interaction.

The effects of PAH exposure on fish embryos are well characterized and include increased mortality (Carls et al. 1999; Heintz et al. 1999; Carls and Thedinga 2010; Hedgpeth and Griffitt 2016), delayed or reduced hatching (Dubansky et al. 2013; Hedgpeth and Griffitt 2016), and developmental abnormalities (Carls et al. 2008; Dubansky et al. 2013; Incardona et al. 2014, 2013; Mager et al. 2014). Our data corroborate these findings because all the groups in the present study displayed an approximate PAH dose-dependent relationship for hatching and mortality data (Figure 1A and B). Our statistical analysis found no evidence of a full 3-way interactive effect among PAH dosage, temperature, and salinity; nevertheless, it should be noted that to meet the statistical assumptions necessary to perform a 3-way ANOVA, the 25-7-6 group was excluded and nominal PAH concentrations were used. The LC50 and LT50 outcomes imply some interactions among variables: changing the temperature from 20 to 30 °C decreases the LC50 at 30 PSU and sharply increases the LT50 at 3 PSU, indicating that exposure temperature can alter lethality of PAHs (Figure 3A and B). In addition, at 20 °C, elevating salinity from 3 to 30 PSU increases LC50 but changing the salinity at 30 °C has no effect on LT50 (Figure 3A and B). There is little literature regarding the interactive effects of these variables; nonetheless, our results are supported in early work by Linden et al. (1979), who found that a combination of suboptimal temperatures, salinity, and PAH dosage interacted to elevate embryonic mortality in *F. heteroclitus*, a congener of *F. grandis*.

The data from our control groups clearly show that even in the absence of PAHs, temperature and salinity act together to modulate mortality and hatching rates in *F. grandis* embryos. For example, control groups incubated at 20 °C exhibited increased time to hatching relative to intermediate conditions (25-7-6), whereas at 30 °C time to hatch decreased (Figure 2A). In each case, boosting 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 instance, Brown et al. (2011) found that in *F. grandis* embryos, temperature and salinity interactions significantly impacted time to hatching. The closely related species *F. heteroclitus* has shown shorter 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 the 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 LT50 data (Figure 3B) directly correspond with our hatching data to suggest that temperature and salinity interact

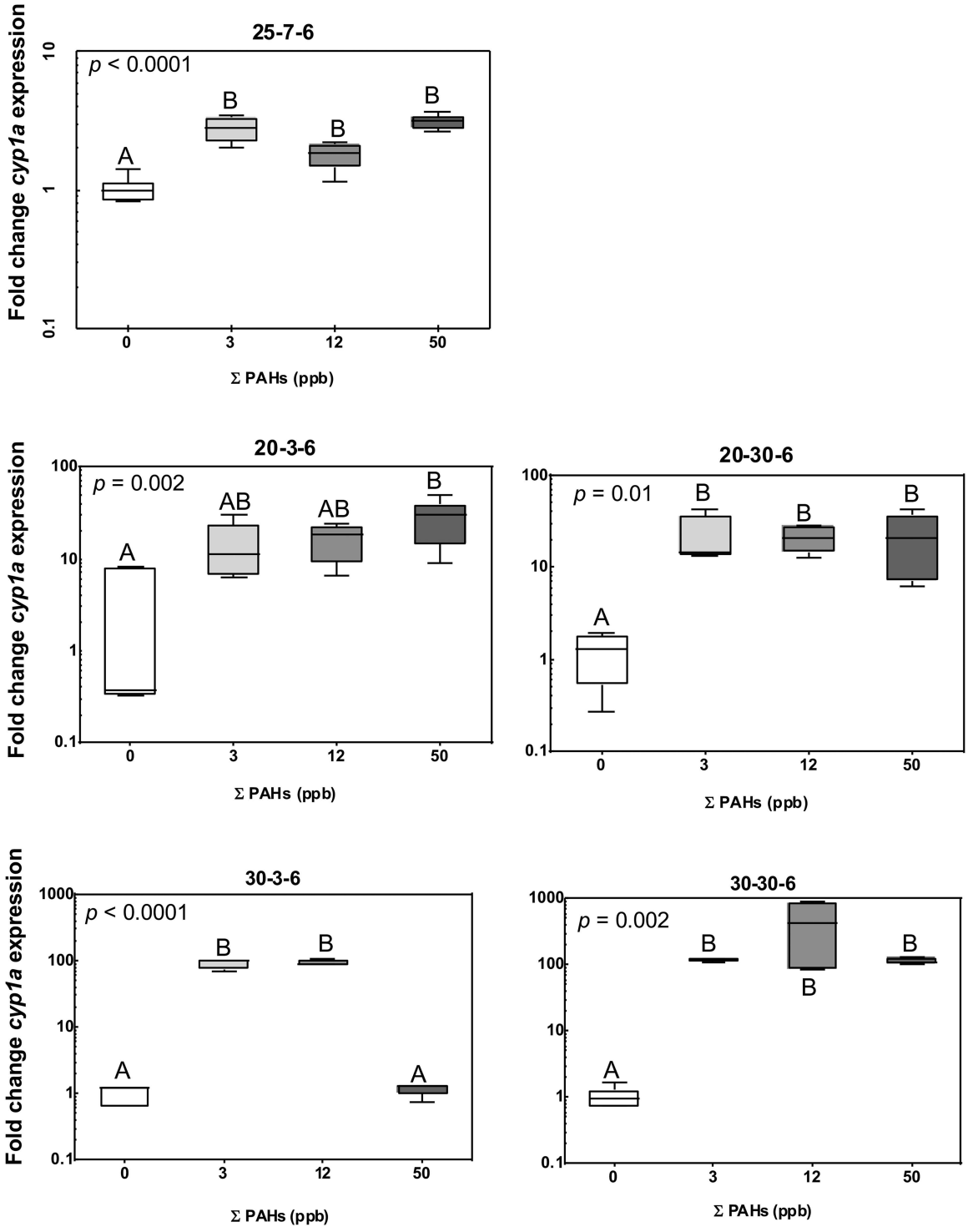

FIGURE 4: Fold change in *cyp1a* expression in *Fundulus grandis* whole larvae in relation to condition tested. Different letters depict significant differences ($p < 0.05$) between polycyclic aromatic hydrocarbon (PAH)-dosed larvae and controls (one-way analysis of variance). Note: graphs have different *y* scales. ppb = parts per billion.

to affect PAH-induced mortality. We found that LT50 values decreased with increasing temperature (from 20 to 25 to 30 °C). In addition to describing mortality, our LT50 values also correlated with *cyp* expression because the 2 groups with the lowest LT50 values (30-3-6 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 hatching 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 provides further support for the role of temperature as one of the primary influences on the effects measured in the present study.

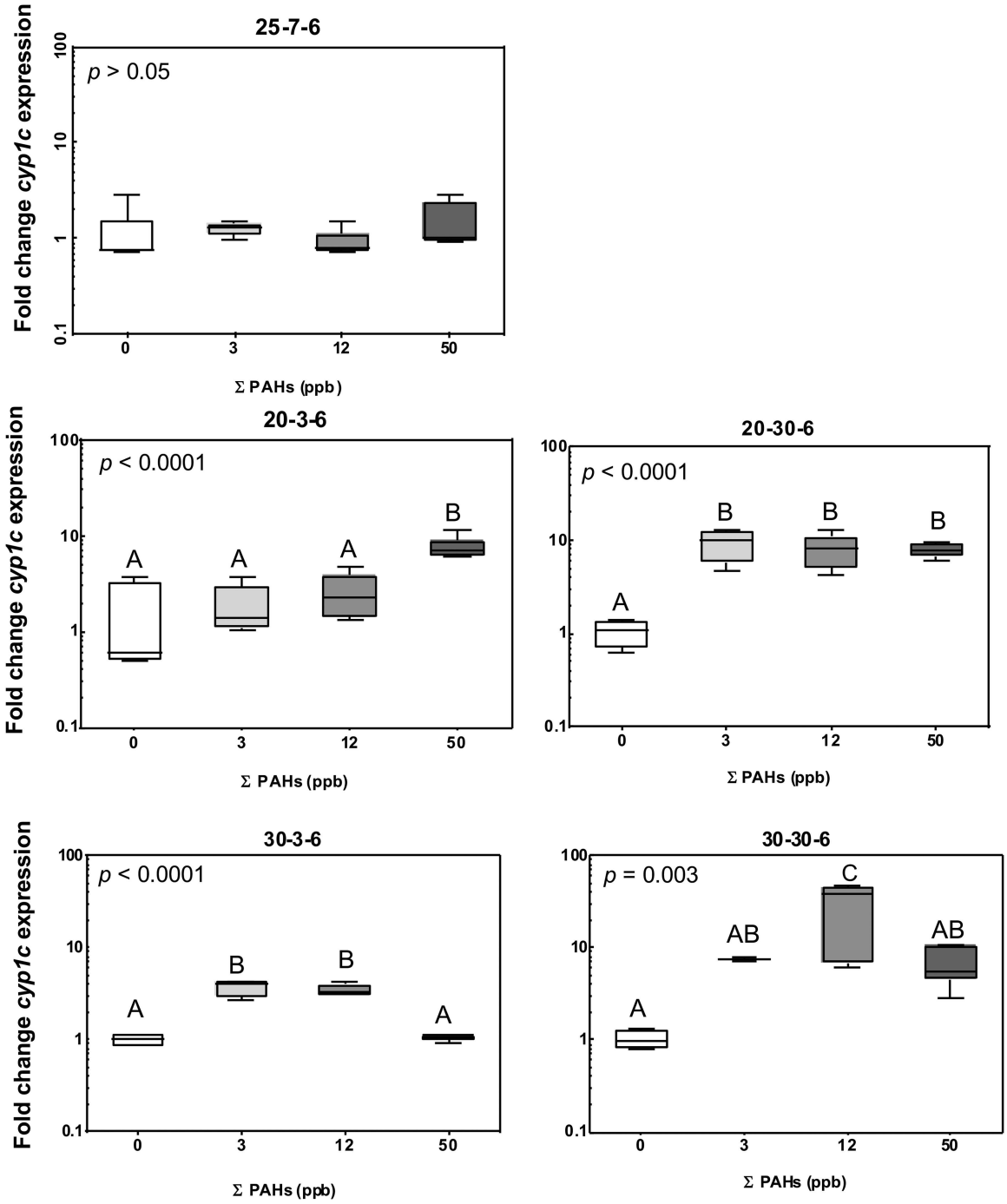

FIGURE 5: Fold change in *cyp1c* expression in *Fundulus grandis* whole larvae in relation to condition tested. Different letters indicate significant differences ($p < 0.05$) between polycyclic aromatic hydrocarbon (PAH)-dosed larvae and controls (one-way analysis of variance). ppb = parts per billion.

Overall, our *cyp1a* and *cyp1c* data are corroborated by previous research suggesting that exposure to *Deepwater Horizon* oil induces expression of *cyp* transcripts. Field studies conducted after the *Deepwater Horizon* incident 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 *Deepwater Horizon* crude oil (Crowe et al. 2014).

Although we found some evidence to suggest that temperature, salinity, and PAH exposure all interact to affect the endpoints of mortality and hatching, it is necessary to further investigate the combined effects of these stressors on sublethal endpoints or life stages not evaluated in the present study. For example, Dubansky et al. (2013) incubated *F. grandis* embryos in sediments oiled during the *Deepwater Horizon* oil spill and found no increase in mortality; however, they did find substantial developmental defects including cardiovascular abnormalities such as pericardial edema. Impacts to cardiovascular development after embryonic

PAH exposure appear to be a conserved response in teleosts because this effect has been documented in multiple taxa (Carls et al. 2008, 1999; Couillard 2002; Pollino and Holdway 2002; Incardona et al. 2005, 2009, 2013, 2014). As with cardiac defects, abnormal craniofacial development after 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 after hatching 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 on whether they are derived from polluted or pristine environments, resulting in differences in cardiac teratogenesis, *cyp1a* activity, and chromosomal damage (Oziolor et al. 2014).

Although statistically there was no evidence of a 3-way interaction among temperature, salinity, and PAH dosage, our LC50 values signal that some interaction exists among these variables that may be biologically relevant. In each condition set tested, elevating the temperature augmented both the LC50 and LT50 data, regardless of salinity. However, the actions of salinity were more nuanced; at 20 °C, increased salinity decreased mortality, whereas at 30 °C there was no effect of salinity on embryonic survival. The present study 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.4153.

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Data Availability—For data inquiries please contact M. Sepúlveda (mssepulv@purdue.edu).

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