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Combined effects of salinity, temperature, hypoxia, and *Deepwater Horizon* oil on *Fundulus grandis* larvae☆

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ABSTRACT

Oil spills have polluted the marine environment for decades and continue to be a major source of polycyclic aromatic hydrocarbons (PAHs) to marine ecosystems around the globe, for example during the 2010 *Deepwater Horizon* spill. Although the toxicity of PAHs to fish has been well studied, their effects combined with abiotic stressors are poorly understood. The goal of this study was to describe the combined impacts of crude oil and environmental stressors on fish larvae, a sensitive life stage. Gulf killifish (*Fundulus grandis*) larvae (< 24 h post-hatch) were exposed for 48 h to high energy water accommodated fractions (HEWAF; total PAHs 0–125 ppb) of Macondo oil from the *Deepwater Horizon* spill under different combinations of environmental conditions (dissolved oxygen 2, 6 ppm; temperature 20, 25, 30 °C; salinity 3, 10, 30 ppt). Even under optimal environmental conditions (25 °C, 10 ppt, 6 ppm) larval survival and development were negatively affected by PAHs, starting with the lowest concentration tested (~15 ppb). Hypoxia and high temperature each increased the adverse effects of HEWAF on development and mortality. In contrast, salinity had little effect on any of the endpoints measured. Importantly, expression of the detoxifying gene *cyp1a* was highly induced in PAH-exposed larvae under normoxic conditions, but not under hypoxic conditions, potentially explaining the enhanced toxicity observed under hypoxia. This work highlights the importance of considering how suboptimal environmental conditions can exacerbate the effects of pollution on fish early life stages.

1. Introduction

The sudden discharge of a large volume of crude oil can have severe environmental impacts (Carls et al., 1999, Monson et al., 2000; Peterson et al., 2003). In 2010, ~500 million L (3.19 million barrels) of crude oil were released in the Gulf of Mexico during the *Deepwater Horizon* (DH) oil spill (Beyer et al., 2016). Immediately following the spill, concentrations of polycyclic aromatic hydrocarbons (PAHs) or polycyclic aromatic compounds (PACs), major component of crude oil, in the surrounding area were found to be as high as 856 ppb (Wang et al., 2014). Four years after the spill, low-molecular weight PAHs (2–4 carbons rings) were still found in estuary sediments (230–380 ppb) (Wang et al., 2014). To date, several studies have assessed the adverse effects of PAHs on estuarine and marine fishes following the DH spill (e.g., Dubansky et al., 2013; Incardona et al., 2014; Whitehead et al., 2012). Observed effects include decreased survival, abnormal growth, damage to the cardiac system, and increased expression of the detoxifying gene *cyp1a* (e.g., Brette et al., 2014; Dubansky et al., 2013; Hicken et al., 2011, Johansen and Esbaugh, 2017). Overall, these studies have reported that PAH toxicity varies depending on life stage, with embryos being very sensitive (Cherr et al., 2017; Hicken et al., 2011; Pasparakis et al., 2016). However, there are limited data available on PAH effects on fish larvae immediately post hatch (yolk-sac fry), which is another very sensitive stage (Cherr et al., 2017; Couillard et al., 2005; Pasparakis et al., 2016).

In natural environments, exposure to oil occurs in combination with

☆ Nomenclature of genes follow those used for *F. grandis* or *F. heteroclitus* (https://www.ncbi.nlm.nih.gov/gene). Gene name abbreviation are given in lower case letters and as italics.

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various other environmental stressors. This is especially true in estuarine environments, due to their dynamic nature, experiencing drastic daily and seasonal changes in dissolved oxygen (DO), temperature, and salinity. This can result in additional stress on fish, potentially causing or exacerbating adverse effects. Previous studies have demonstrated important physiological consequences on fish early life stages when developing in the presence of environmental stressors, such as decreased survival, delayed growth and changes in time-to-hatch (Brown et al., 2012; Hoar and Randall, 1988; Ramee and Allen, 2016). Therefore, these environmental fluctuations can behave as natural stressors leading to synergistic, additive, or antagonistic effects in fish (Dasgupta et al., 2015, 2016; Pasparakis et al., 2016; Ramachandran et al., 2006). An example of this phenomenon is the co-exposure of fish to PAHs and hypoxia (DO < 3 ppm). PAHs are aryl hydrocarbon receptor (AhR) agonists (Anjos et al., 2011). The AhR, when activated in the presence of PAHs, binds to ARNT (AhR nuclear translocator) and subsequently induces expression of CYPA1A protein (cytochrome P4501A), a phase I enzyme that metabolizes organic toxicants. Hence, CYP1A1 has been recognized as a marker for PAH exposure in several studies (e.g. Dubansky et al., 2013). Under hypoxia, the hypoxia response protein HIF-1a (hypoxia inducible factor 1a) also binds to ARNT (Fleming et al., 2009). Hence, under hypoxia, there may be competition between the AhR and HIF-1a for the ARNT receptor. Accordingly, exposure of sheepshead minnow (*Cyprinodon variegatus*) to PAHs under hypoxia has been found to result in decreased induction of *cyp1a* (Dasgupta et al., 2016). This in turn can increase the developmental toxicity of PAHs (Wassenberg et al., 2005). Several mechanisms have been proposed to explain this, including an increased half-life of PAHs resulting in extended stimulation of the AhR pathway (Fleming and Di Giulio, 2011).

Studies on the joint effects of PAHs and suboptimal temperature or salinity on fish are scarce. Measured as increased expression of *cyp1a* or enzyme activity (EROD), interactive effects have been observed in adult fish exposed to high temperatures in combination with PAHs (Andersen et al., 2015). Increased temperature has also been associated with enhanced sensitivity to PAHs due to energy depletion after increased metabolism (Pasparakis et al., 2016). Higher salinities have been reported to decrease response to oil (Ramachandran et al., 2006; Lysenko et al., 2015), presumably because bioaccumulation of PAHs is decreased under high salinity (Neff and Anderson, 1981).

The goal of this study was to evaluate the combined effects of PAHs and sub-optimal environmental DO, temperature and salinity on the larvae of the Gulf killifish (*Fundulus grandis*). We tested the hypothesis that co-exposure of fish larva to PAHs in combination with suboptimal environmental conditions would result in enhanced toxicity. The estuarine Gulf killifish is commonly found inhabiting estuarine zones in the Gulf of Mexico and thus was impacted by the DH oil spill (Whitehead et al., 2012). They are an excellent laboratory model for toxicity studies that focus on early life stages as they are easy to breed and females are very fecund (Brown et al., 2012; Burnett et al., 2007; Crowe et al., 2014). In addition, mummichog (*F. heteroclitus*), a closely related species that inhabits the Atlantic coast of North America, has served as an ecotoxicological model for decades, resulting in a large number of studies describing *Fundulus* physiology and responses to contaminants (Burnett et al., 2007). To our knowledge, the present study is the first to simultaneously manipulate DO, salinity and temperature in combination with crude oil to examine their effects on fish larvae.

2. Materials and methods

2.1. Animal model and husbandry conditions

Broodstock fish were collected from estuaries along the coast of Mississippi in Guatier, Ocean Springs, Deer Island (off Biloxi) and Bay St. Louis in March 2015. Fish were held at least 14 days in quarantine before being shipped overnight to the Purdue Aquaculture Research Laboratory at Purdue University, West Lafayette, Indiana, USA. Fish were held in 130-L tanks with 2–3 females to each male in a Pentair Aquatic Eco-Systems (Palatka, FL, USA) setup consisting of 12 (70 cm × 48 cm × 32 cm) tanks connected as a part of a 1800-L recirculating aquaculture system complete with mechanical, biological, and UV filtration. The entire system was built inside an environmentally controlled room maintained at 25 ± 1 °C with 16L:8D photoperiod. Fish culture medium was reconstituted salt water prepared using Fritz SuperSalt Concentrate (Fritz Industries, Mesquite, TX, USA) for a target salinity of 10 ppt. Temperature and DO were measured using a YSI PRO1020 multi-parameter meter (YSI Incorporated, Yellow Springs, OH, USA) and kept between 25 and 26 °C and 5.5–6.5 ppm, respectively. Broodstock were fed *ad libitum* frozen chironomids each morning and frozen brine shrimp each evening (JEHM Co., Lambertville, NJ, USA). Embryos were collected 2–3 times per week from submerged coarse polyester filter mats and released by tapping the mats over the surface of additional culture medium following the methods described by Green (2013). Up to 40 embryos were then placed in floating mesh cylinders (15 cm × 15 cm) in the corresponding adult tanks from which they were collected and checked daily for hatched larvae.

Exposure of Larvae to High Energy Water Accommodated Fraction (HEWAF): *F. grandis* larvae < 24 h post-hatch (hph) were obtained from 8 spawning pairs. Larvae were chosen at random and placed into 125 mL glass jars filled completely with exposure water corresponding to each treatment group and sealed with Teflon-lined caps (Qorpak, Bridgeville, PA, USA). We exposed one larva per vial (n = 6 larvae per condition) for 48 h. Jars remained tightly closed for the entire experiment and larvae were not fed, since they were still relying on their yolk sac reserves.

The combined impacts of multiple stressors on larvae were tested using a factorial design consisting of six HEWAF concentrations (0, 15, 24, 31, 62, and 125 ppb) × two temperatures (20 and 30 °C) × two salinities (3 and 30 ppt) × two DO ppm levels (2 or hypoxic and 6 or normoxic ppm), resulting in a total of 48 unique exposure conditions. Throughout the manuscript, treatment groups are referred to by their temperature (°C), salinity (ppt), DO (ppm), and PAH concentration (ppb) (e.g, 30-3-6 refers to 30 °C-3 ppt- 6 ppm).

This experiment also included a “reference” group maintained at conditions that were previously reported as optimal (25-10-6) (Brown et al., 2011; Green, 2013; Ramee and Allen, 2016). If maintained at these optimal conditions, fertilized eggs hatch 13–15 days post-fertilization (dpf) (Green, 2013). In the present study, *F. grandis* fertilized eggs developed and hatched 8–10 dpf. Experiments were conducted between October and December of 2015.

HEWAF was prepared by mixing 1 g Macondo crude oil, supplied by British Petroleum, with 1 L of reconstituted saltwater in a Waring model CB15 commercial blender (Waring Lab, Stamford, CT, USA) for 30 using the protocol described in Forth et al. (2017). The blended liquid was then decanted into a separatory funnel and allowed to settle for 1 h. A 450-mL aliquot of the settled liquid was collected in a glass bottle and used immediately for exposures.

2.2. Tracking of water quality parameters and PAHs

Prior to filling vials with test water, we measured water temperature and DO using a YSI PRO1020 multi-parameter meter and salinity with a Pentair Vital Sine SR6 handheld refractometer. Low DO conditions were achieved by bubbling nitrogen gas through the exposure medium before the addition of HEWAF. During the experiment, DO was quantified using sensors read by touching the surface of the glass chamber with a fluorometer. Therefore, test vials did not need to be opened throughout the experiment, which was critical for maintaining and monitoring low DO (2 ppm). DO sensors were made by mixing 2 g of chloroform, 300 mg 5 μm TiO_2 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 with fluorescence characteristics that are a function of the oxygen level in the environment with an excitation λ_{max} = 392 nm and an emission λ_{max} = 650 nm. The mixture was applied to the bottom of each experimental jar and allowed to dry for at least 24 h before the start of experiments. We recorded and converted fluorescence readings to DO (ppm) using a Tau Theta data logger and optrode (Tau Theta Instruments, LLC, Boulder, CO). We then placed test vials in an environmental chamber during the exposure period and maintained temperature within ± 1 °C of the target temperature. Salinity was kept within ± 0.5 ppt of nominal by maintaining closed vials to avoid evaporation. The pH was measured using an OAKTON Instruments Waterproof pHTestr 10 (OAKTON Instruments, Vernon Hills, IL, USA) and maintained between 8.2 and 8.7 in all experiments. We quantified total ammonia nitrogen (TAN) concentration at the end of the exposure by collecting 1 mL of water from all jars with surviving larvae, preserving it with 1 µL of 1N HCl, and freezing at −20 °C. Samples were later analyzed using the salicylate method (Verdouw et al., 1978). Subsequent determination of unionized ammonia (NH_3) was calculated using the conversion table found in Francis-Floyd et al. (2009).

Fluorescence was quantified at 0, 24 and 48 h as an indirect measure of total PAHs using a Turner Designs AU-10 Fluorometer (Tuner Designs, San Jose, CA, USA). PAH molecules that are aromatic and/or contain conjugated double bonds fluoresce and can be quantified using this approach (Williams and Bridges, 1964). Fluorescence was measured at 0 h from freshly-mixed exposure solution, at 24 h from 2 to 3 jars without larvae (blanks), and at 48 h (termination of the experiment) from vials with surviving larvae. In accordance with the approach used by Greer et al. (2012), we collected 5 mL of exposure medium from each treatment group and added it to 5 mL of 100% ethanol in 50 mL centrifuge tubes. We then sonicated samples in a water bath for 3 min to minimize hydrocarbon adhesion to the container and centrifuged at 9100 *g* for 10 min to remove salt particles. After centrifugation, 5-mL aliquots were drawn from the sample and placed into a quartz cuvette for analysis in the fluorometer.

We quantified alkyls, alkanes and PAHs using liquid chromatography (LC)/mass spectrometry (MS)/MS at the Center for Environmental Sciences and Engineering, University of Connecticut. Samples were prepared in plain saltwater (blank) and 100% HEWAF (stock solution) at each of the three salinities (3, 10, and 30 ppt), packaged into amber glass bottles with Teflon lined caps, placed on ice, and shipped overnight. Temperature and dissolved oxygen were not controlled prior to chemical analyses. Upon arrival, samples were extracted using solid phase extraction using a Waters HLB SPE cartridge (Milford, MA, USA) conditioned with methanol and 200 mL of water passed across the cartridge. The target fraction 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. Typical quality control procedures were employed, including analysis of duplicate samples, method blanks (Blank), matrix spike duplicates, and laboratory control samples. We estimated exposure concentrations for each treatment group by using the total PAH concentration LC/MS/MS data obtained from each of the different stock solutions (see Tables S1 and S2). We assigned this concentration to fluorescence values that were obtained throughout the experiments for the different HEWAF treatment groups (0, 6.25, 9.5, 12.5, 25, and 50% HEWAF) for each of the salinities tested at the start of the experiment (0 h). These fluorescence values were averaged at 0 h across all experiments, and linear regressions (Fig. S1) were created to estimate PAH concentrations.

2.3. Endpoint assessment

At the end of the 48-h exposure period, we counted all surviving larvae and immediately photographed them on an Olympus SZ61 stereo microscope (Olympus Corporation, Waltham, MA, USA) using Olympus cellSens Entry 1.12 imaging software. We then measured standard length (SL) using ImageJ 1.46r software (Schneider et al., 2012).

All surviving larvae were frozen in liquid nitrogen and stored at −80 °C until they were processed for qPCR analysis. We quantified the expression of *cyp1a* and *hif1a* from whole larvae. *Cyp1a* was chosen because it has been routinely used as a biomarker of PAH exposure (Anjos et al., 2011; Dubansky et al., 2013). HIF1A was chosen because under hypoxia it competitively binds to ARNT, which may reduce availability of ARNT to the AhR pathway and therefore down-regulate *cyp1a* expression (Dasgupta et al., 2015). We extracted total RNA using an RNeasy Mini Kit (Qiagen, Hilden, Germany), which usually yielded 200–300 ng/µL RNA per sample. RNA was then treated with DNase-I (Thermo Scientific, Waltham, MA. USA) and converted to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific). We carried out quantitative real-time polymerase chain reaction (qPCR) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) following the thermal cycling protocol provided by the manufacturer, using a denaturation temperature of 94 °C and an annealing/extension/read temperature of 58 °C. We ran all qPCR in duplicate reactions on a Bio-Rad CFX Connect Real-Time PCR System using primers designed in Primer3Plus software using sequence data from the NCBI nucleotide database and ordered from Integrated DNA Technologies (Coralville, IA, USA).

Primer efficiency was ≥90% in all cases (see Table S3). The housekeeping gene used for all qPCR reactions was β-*actin*, since its expression was stable across all of the conditions tested, with a cycle threshold (C_t) value between 13 and 15 regardless of condition or PAH concentration. Cycle threshold relationships to expression levels (fold changes) were calculated using Pfaffl's method (Pfaffl, 2001).

The *hif1a* primer sequences (Table S3) were developed based on the sequence of *F. heteroclitus hif1a* (Accession # KR703588). Sometime later, a coding sequence of *hif1a* from *F. grandis* was published (Accession # KR703590) that shows that the region flanked by this primer set is identical in the two species. PCR reaction specificity was assessed by observation of a single peak during qPCR melt curve analysis and then by observation of a single band of the expected length (117 bp) upon agarose gel electrophoresis. To confirm the identity of this band, two separate sets of primers were used for two separate nested PCR reactions, in both cases using a subsample of the original PCR solution as the template for nested PCR. Based on the known sequence of *F. grandis hif1a*, nested reaction A used primers with the following sequences: F 5′-CAACAAGAACTCCCAGCCTCA-3′ and R 5′-CTGAGCACA AAGTTGACGCAC-3’. Nested reaction B used primers with the following sequences: F 5′- GCCACCGTCATCTACAACAAC-3′ and R 5′- GAGCACA AAGTTGACGCACAC-3’. In both cases, the nested PCR reaction produced a single product of the expected length (51 bp and 66 bp, respectively). Thus, the specificity of the qPCR primer set for *F. grandis hif1a* was confirmed.

2.4. Statistical analyses

We used R (R 3.2.1 Development Core Team, 2015) to analyze data for statistical differences. All data are reported as mean ± standard deviation. Assumptions of normality and equal variance were checked using Shapiro-Wilk's W-test. In some cases, the assumption of normality was violated (e.g., *cyp1a* expression) even after log transformation. However, generalized linear models (GLMs) are robust to minor violations of normality, so analyses were continued. Effects of environmental conditions on larvae size and gene expression were quantified using GLMs. The type of GLM used for each dependent variable was chosen based upon its distribution, which was determined using the

plot(density()) function in R. Degrees of freedom (DFs) varied due to mortality and/or qPCR data that were voided due to poor quality. Tukey tests were used to determine statistical differences or similarities amongst PAH concentrations for the mortality and standard length data. Welch's paired t-tests were chosen to test each parameter value against the parameter value from the "optimal" condition (25-10-6) and to account for unequal variances. The 25-10-6 samples were not used in the gene expression or standard length statistical analyses. These samples were excluded because they did not have the same salinity, therefore we could not compare them statistically. PAH levels were considered class variables. Interactive effects were graphically visualized and verified using the 'effects' package in R. Effects on survival was quantified using chi-squared tests. A statistically significant effect was declared at $p \leq 0.05$.

Dose-response curves were estimated using the R package 'drc' (Ritz et al., 2015). The models were fitted using the function *drm* with parameters type = "binomial" and fct = LL.2(). This function also estimates 95% confidence intervals.

3. Results

3.1. Environmental conditions

The PAH concentration levels were labeled according to the mean estimated concentration of PAHs at the beginning of the exposure period. In all conditions tested, fluorescence decreased over the first 24 h and continued to decrease to a lesser extent until the end of the 48-h experiment (Fig. 1). The average estimated PAH concentration over the 48-h time period was calculated for each treatment group, and is reported as mean ± SD: the 15 ppb treatment group decreased to a final concentration of 1 ± 2.9 ppb; 24 ppb group decreased to 1.1 ± 2.3 ppb; 31 ppb group decreased to 1.2 ± 1.5 ppb; 62 ppb group decreased to 5.5 ± 7.1 ppb; and 125 ppb group decreased to 19.5 ± 4.8 ppb.

Temperature and salinity were maintained throughout the experiments close to the nominal values (± 1 °C and ± 1 ppt, respectively). DO levels decreased (likely due to a combination of larval and microbial respiration) to 2.2 ± 0.6 ppm (mean ± SD) under normoxia and by 1 ± 0.8 ppm under hypoxia over the 48 h test (Fig. S2). Variation in DO was much greater under hypoxia. At the end of the experiment, total ammonia nitrogen levels (TAN) were ≤5 μM (= 0.09 ppm), with the exception of one group (30 °C-30 ppt-2 ppm) where it reached up to

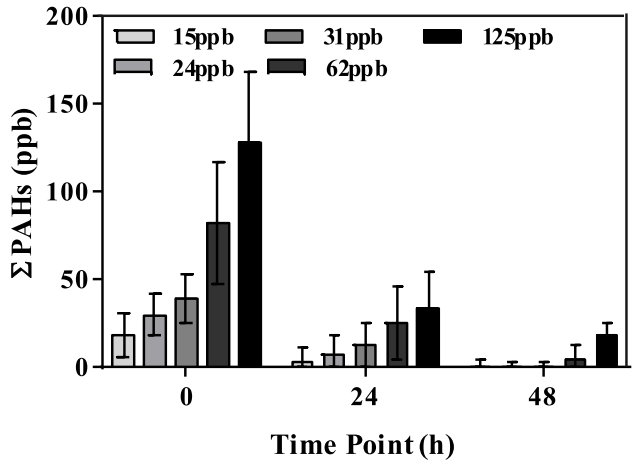

Fig. 1. Mean ± SD of estimated PAH concentrations over time across all conditions, by treatment group. PAH concentrations drastically decreased during the 48 h exposures. Concentrations in reference samples were all below the reporting limit. Sample sizes: 0 ppb −31 ppb n = 60; 62 ppb n = 48; 125 ppb n = 30. PAH concentrations were estimated using linear regressions from raw fluorescence values as described in Fig. S1.

10.8 μM (0.18 ppm) (Fig. S3). Based on our temperature and pH conditions, NH_3 levels in our study ranged from ~0.03 to 1.5 μM.

3.2. Mortality and development

The lowest mortality was observed under normoxia, at 20 or 25 °C in both salinity levels (Fig. 2). Larvae mortality increased in a PAH concentration-dependent manner under all conditions tested, under both normoxia and hypoxia (Table 1, Fig. S4). In most cases, exposure of larvae to PAHs under suboptimal environmental conditions resulted in higher mortality. Under hypoxia, the larvae began to exhibit mortality at lower concentrations of PAHs than under normoxia (Fig. 2, Table S4). Likewise, nearly complete mortality (80% or more) occurred under hypoxia at PAH concentrations less than or equal to those required to elicit similar mortality under normoxia. Most of the hypoxic treatment conditions led to PAH sensitivity similar to that observed in the most challenging normoxic treatment, 30-3-6. Furthermore, the larvae could not survive even 15 ppb PAHs under 30-30-2 conditions.

Hypoxia and high temperature significantly increased mortality (Tables 1 and S4). High salinity (30 ppt) increased mortality only when larvae were exposed under hypoxia (Fig. 2). The only mortality seen in larvae not exposed to PAHs was observed in the 30-30-2 group, in which two of the six fish died.

Larvae standard length was not a sensitive and consistent parameter (Fig. S4B). Effects were only statistically significant in the 31 ppb 20-30-6 group. Standard length was lower in hypoxic conditions but was not significantly related to temperature or salinity (Table 1).

3.3. Gene expression

All environmental factors tested significantly influenced *cyp1a* expression (Table 1). Under normoxia, there was an increase up to 30- or 40-fold in *cyp1a* expression in larvae exposed to PAHs. This increase in expression was reduced under high temperature (30 °C) as well as under high salinity (30 ppt); under both high temperature and salinity, the effects were roughly additive. PAH-dependent up-regulation of *cyp1a* was strongly suppressed under hypoxia (Fig. 3). Hypoxia resulted in a slight but significant induction of *hif1a* expression. High DO and high temperature slightly increased *hif1a* expression, and this effect was more pronounced at high salinity and hypoxia (Table 1). The *hif1a* expression levels were calculated by referencing Ct values to either within-group controls (i.e. PAH exposure vs control for each set of conditions) or to high DO controls (e.g. all samples in 20-30-2 vs. controls in 20-30-6) (Table 1). This was done to determine if there were differences in *hif1a* expression amongst 1) control vs. PAH exposure and/or 2) low DO vs. high DO.

4. Discussion

This is the first study to examine the effects of salinity, temperature, and DO combined with exposure to PAHs derived from crude oil on *F. grandis* larvae (0–48 hph). The strongest negative effects on survival and development were observed when larvae were exposed to PAHs under hypoxia and elevated temperature. Survival was decreased under high salinity (30 ppt) only when combined with hypoxia. Expression of the detoxifying gene *cyp1a* after PAH exposure, however, was significantly inhibited by hypoxia, high temperature and high salinity either alone or in combination. A lack of *cyp1a* induction under hypoxia and high temperature and salinity may have contributed to increased mortality in larvae exposed to PAHs.

4.1. Exposure conditions

PAH molecules are aromatic and contain conjugated double bonds that fluoresce and can be quantified using a fluorometer (Williams and Bridges, 1964). Therefore, with the exception of alkanes, which

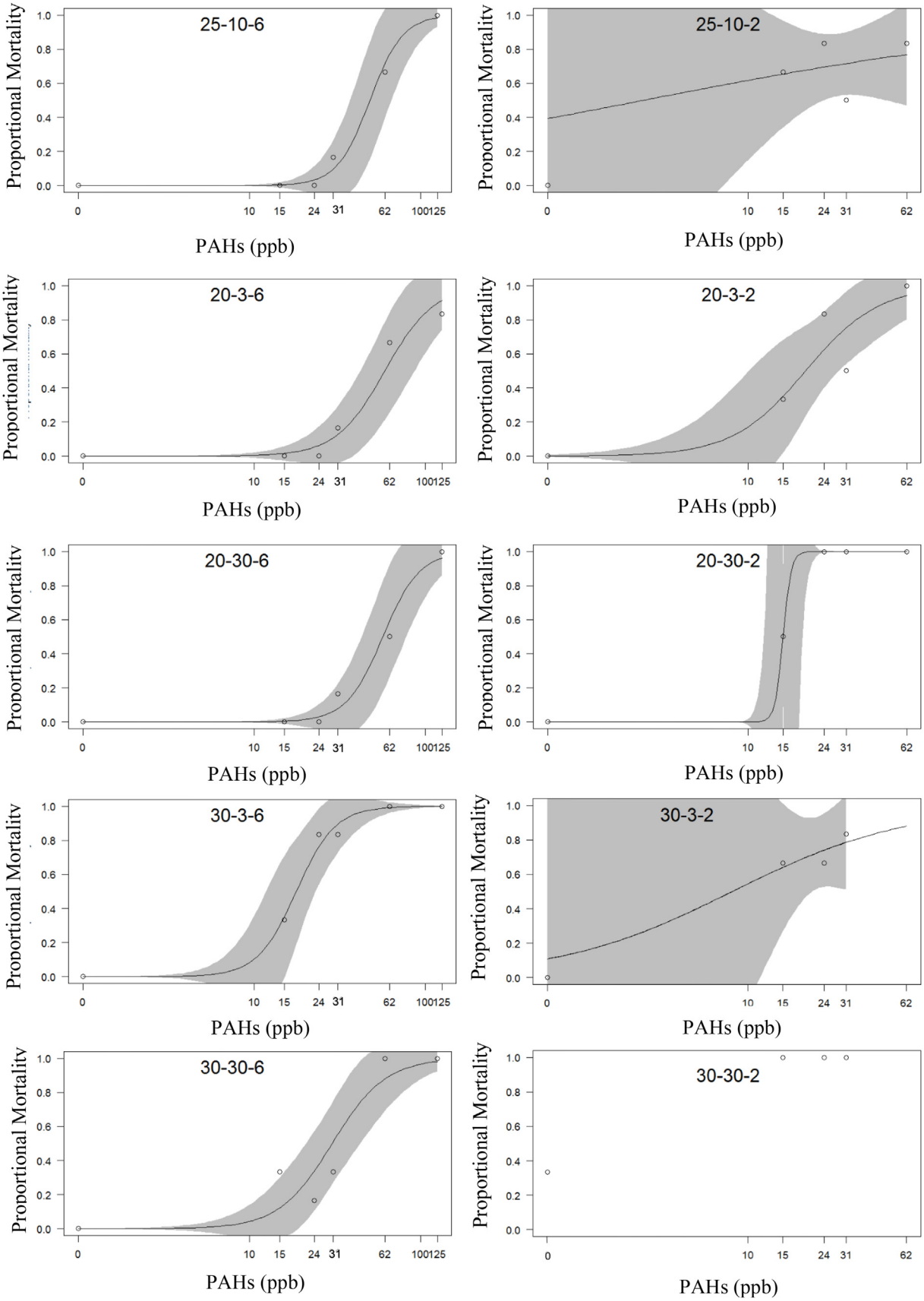

Fig. 2. Larva mortality after a 48-h exposure to polycyclic aromatic hydrocarbons (PAHs) under each tested condition. Conditions are defined as temperature [°C]—salinity [ppt]—DO [ppm]. Solid curves show the best fit logistic regressions and gray areas describe 95% confidence intervals. Curve and intervals could not be calculated for condition 30-30-2. This model was not a good fit for condition 25-10-2 at concentrations less than 15 ppb. N = 6 for each PAH concentration.

Table 1
Summary of statistical results of generalized linear models (GLM) and chi-square tests. Only significant interactions of independent variables are listed. F statistic (F) is given for Gaussian and gamma distributions, while a chi-square (χ^2) is provided for quasibinomial data.Degrees of freedom vary depending on number of samples submitted for qPCR based on RNA quality and non-inclusion of the 25 °C treatments for most of these analyses..

Effect	Distribution	DF	F or χ^2	P Value
Survival	Quasibinomial	159	χ^2	
[PAH]		5	19.886	< 0.001
DO		1	11.853	< 0.001
Temp		1	7.112	< 0.001
Salinity		1	6.958	0.243
DO x Temp		1	6.280	0.010
DO x Salinity		1	3.774	< 0.001
Standard Length	Gaussian	114	F	
[PAH]		1	4.063	0.003
DO		1	5.126	0.005
Temp		1	1.707	0.131
Salinity		1	2.053	0.339
***cyp1a* Expression**	Gamma	111	F	
[PAH]		4	238.834	< 0.001
DO		1	81.422	< 0.001
Temp		1	5.261	0.050
Salinity		1	4.111	0.043
***hif1a* Expression (compared to high DO)**	Gamma	117	F	
[PAH]		4	1.5705	0.501
DO		1	12.93	< 0.001
Temp		2	19.928	< 0.001
Salinity		1	12.759	0.032
DO x Temp		1	7.659	0.010
Temp x Salinity		1	11.201	0.001
***hif1a* Expression (compared to within-group controls)**	Gamma	117	F	
[PAH]		4	0.432	0.413
DO		1	0.679	0.258
Temp		1	7.013	0.002
Salinity		1	4.291	0.023

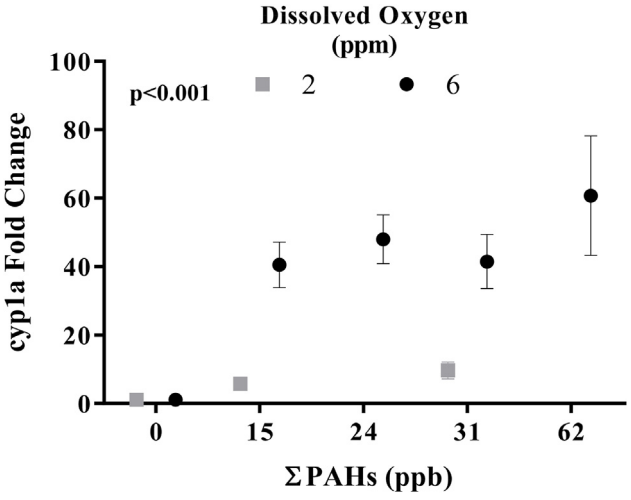

Fig. 3. *Cyp1a* fold change expression (mean ± SD) in relation to polycyclic aromatic hydrocarbon (PAH) concentration and dissolved oxygen levels (2 and 6 ppm), across all temperatures and salinities. Gene expression was significantly upregulated compared to controls for both normoxia and hypoxia, but the up-regulation was much higher under normoxia. Fold change mean values for controls were 1.11 and 1.15 for the 2 and 6 ppm dissolved oxygen levels, respectively. No larvae survived the 24 and 62 PAH ppb treatments under hypoxia. Sample sizes: Normoxia 0 ppb = 27; 15 ppb = 25; 31 ppb = 20; 62 ppb = 7. Hypoxia 0 ppb = 27; 15 ppb = 7; 31 ppb = 6.

constituted only ~4% of our HEWAF based on LC/MS/MS data, fluorescence measurements in this study are a good representation of total PAHs. Using fluorescence data, we observed a steep decline in PAHs by the end of the experiment (to ~3–15% of initial concentrations). Since experiments were carried out in closed containers, volatilization should not have been a major factor in this loss. However, PAHs are also photo-sensitive (Zhang et al., 2008) and are easily degraded by microbial activity (Ukiwe et al., 2013). Therefore, the ppb values used for reporting effects in this study are an over-estimation of the actual concentrations experienced by the larvae. Total ammonia values were ≤10 μM and NH_3 levels were ≤1.5 μM. Toxic effects of NH_3 are generally not observed until ~3 μM (Francis-Floyd et al., 2009; Mitchell and Tully, 2009). Hence, NH_3 in test vials over the 48-h experiments likely did not act as an additional stressor in this study.

5. Mortality and development

It could be argued that, since *F. grandis* experience frequent changes in temperature (Δ 28 °C), DO (Δ 5 ppm), and salinity (Δ 18 ppt) in the Gulf of Mexico (EPA, 1999), their sensitivity to oil exposure may be less influenced by suboptimal water quality conditions compared to other marine fishes. However, our results show that *F. grandis* larvae are equally or more sensitive to oil compared to other fishes and that sensitivity is increased when combined with suboptimal DO and temperature. A no observed effect level (NOEL) could not be determined for mortality in this study since significant mortality was observed at the lowest PAH concentration tested (15 ppb). In addition, larval body length was not a sensitive endpoint in this study, likely because of the short nature (48 h) of the experiments.

The sensitivity of fish embryos and larvae to PAHs is quite broad, with increased mortality ranging from 12,000 ppb in sheepshead minnows (*Cyprinodon variegatus)* (Dasgupta et al., 2015, 2016), to severe morphological malformations at 15 ppb in bluefin tuna (*Thunnus thynnus*) and amberjack (*Seriola lalandi*) (Cherr et al., 2017; Incardona et al., 2014), and increased mortality and morphological malformations at < 1 ppb in Pacific herring (*Clupea pallasii*) (Carls et al., 1999).

Several other studies on the toxicity of PAHs have been published on *Fundulus* species. Exposed adult *F. grandis* to PAHs from *Deepwater Horizon* oil at initial concentration of 7000 ppb for 12, 24, or 48 h under normoxia and saw no mortality, but did see a significant induction of *cyp1a* (Crowe et al., 2014). In another experiment, *F. heteroclitus* post-hatch larvae (< 24 hph) exposed to ~240 ppb PAHs from weathered Mesa light crude oil for 24 h under normoxia exhibited 22% mortality (Couillard et al., 2005). In a recently published study by our research group, LC_{50} values in *F. grandis* embryos exposed to PAHs from *Deepwater Horizon* oil from fertilization until hatch (~15–40 days) ranged from ~40 to 80 ppb, with no embryos surviving under hypoxia (Rodgers et al., 2018). In the present experiment with recently hatched larvae, mortality was observed at the lowest concentration tested. Therefore, life stage, length of exposure, and DO play a critical role in the sensitivity of *F. grandis* to PAHs.

A combination of high temperature, hypoxia, and PAHs resulted in the lowest survival. This is consistent with other studies showing that hypoxia and high temperatures are stressful conditions for developing embryos and larvae and that these conditions increase mortality in the presence of PAHs (Burggren and Blank, 2009; Dasgupta et al., 2016; Hoar and Randall, 1988). High salinity also increased mortality in combination with PAH exposure, but only under hypoxia and high temperature conditions. This stands in contrast to observations reported by Ramachandran et al. (2006). In that study, juvenile (8–10 weeks) *Oncorhynchus mykiss* and adult *F. heteroclitus* were exposed to crude oil (1–50 ppb) at varying salinities (0, 15, and 30 ppt) for 48 h. Those authors concluded that the lower salinities tested increased PAH solubility, concentration, and bioavailability and led to an increased *cyp1a* induction.

Considering all the survival results mentioned above, it was the 25-

10-6 combination that resulted in highest overall survival. It was apparent that poor water quality conditions (hypoxia, high temperature.) + PAHs had a significant influence on many of the endpoints measured when compared to optimal conditions + PAHs. Most significantly, it seems that hypoxia lowers the threshold dose at which PAHs lead to mortality at any salinity and temperature tested.

5.1. Gene expression

There was a significant increase in *cyp1a* expression in PAH-exposed larvae in all conditions tested. Expression was elevated to a similar degree in the lowest and highest PAH concentrations, suggesting the threshold for its up-regulation was exceeded. The use of this biomarker is well established as an indicator of exposure to PAHs (Anjos et al., 2011; Dasgupta et al., 2016; Dubansky et al., 2013; Vorrink and Domann, 2014; Whitehead et al., 2012). The threshold PAH concentration is species-specific and dependent on the individual PAH tested, and can range from 0.2 ppb in Pacific herring (exposed to "weathered oil", Incardona et al., 2015) to 10,000 ppb in sheepshead minnow (exposed to Southern Louisiana Crude oil, Dasgupta et al., 2016). In *F. heteroclitus* embryos, this threshold has been found to be as low as 1 ppb (Wassenberg et al., 2002, Wassenberg et al., 2004). However, the latter two studies exposed fish to a single PAH, benzo(a) pyrene). Our results would suggest that the *F. grandis* threshold value for *cyp1a* induction is also in the low ppb range, which would confirm the utility of this species as a sensitive sentinel for ecological impacts of oil in estuarine areas.

Hypoxia significantly decreased PAH-induced *cyp1a* up-regulation from ~30 to 40-fold–12-fold, but it had no significant effect on the expression of *hif1α*. Changes in *cyp1a* expression between normoxic and hypoxic conditions are consistent with other studies (Dasgupta et al., 2016; Fleming and Di Giulio, 2011). Under normoxia, the AhR protein binds PAHs and translocates to the nucleus to dimerize with ARNT transcription factor, resulting in the transcription of *cyp1a*. However, under hypoxia, HIF1α protein is degraded more slowly than under normoxia, making it more available for translocation to the nucleus, possibly leading to competition for ARNT binding (Vorrink and Domann, 2014). We therefore predicted that under hypoxia, PAH-induced expression of *cyp1a* would decrease and expression of *hif1α* would increase. However, while *cyp1a* exhibited the predicted pattern, we found that *hif1α* expression decreased slightly under hypoxia. Other studies have reported mixed results on the expression of this gene under hypoxia. One study found that a 4-h exposure to hypoxia (2 ppm) in sea bass (*Dicentrarchus labrax*) was enough to significantly up-regulate *hif1α* expression in liver (Terova et al., 2008). However, another study with grass carp (*Ctenopharyngodon idella*) found that the up-regulation of this gene was only observable in gills and kidneys after a 4-h exposure to extreme hypoxia (0.5 ppm), and expression had returned to normal at 96 h (Law et al., 2006). In Atlantic croaker (*Micropogonias undulates*) ovaries, 72 h of hypoxia exposure (1.7 ppm) caused an increase in *hif1a* expression (Rahman and Thomas, 2007). These results suggest that the expression and regulation of *hif1a* is likely species-, tissue-, and time-specific. We used whole larvae and therefore should have picked up on any marked changes in the organism. However, we may have missed the critical time point for *hif1a* induction.

In summary, hypoxia and high temperature increased the sensitivity of Gulf killifish larvae to PAHs. Importantly, larvae exhibited adverse effects at the lowest PAH concentration tested (15 ppb), which is well within ranges found in the Gulf of Mexico years after the *Deepwater Horizon* oil spill. Research on the response of fish larvae to multiple stressors is critical, but still in its infancy. This information can be used to help identify specific areas, including estuaries, that may be especially vulnerable to oil spill-related damage.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecoenv.2019.05.059.

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