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Exposure and Recovery from Environmentally Relevant Levels of Waterborne Polycyclic Aromatic Hydrocarbons from *Deepwater Horizon* Oil: Effects on the Gulf Toadfish Stress Axis

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Abstract: There is evidence that the combination of polycyclic aromatic hydrocarbons (PAHs) released in the *Deepwater Horizon* oil spill impairs the glucocorticoid stress response of vertebrates in the Gulf of Mexico, but the mechanisms are unclear. We hypothesized that inhibition of cortisol release may be due to 1) overstimulation of the hypothalamic–pituitary–inter-renal (HPI) axis, or 2) an inhibition of cortisol biosynthesis through PAH activation of the aryl hydrocarbon receptor (AhR). Using a flow-through system, Gulf toadfish (*Opsanus beta*) were continuously exposed to control conditions or one of 3 environmentally relevant concentrations of PAHs from *Deepwater Horizon* oil ($\sum PAH_{50} = 0–3\ \mu g\ L^{-1}$) for up to 7 d. One group of toadfish was then exposed to a recovery period for up to 7 d. No changes in corticotrophin-releasing factor mRNA expression, adrenocorticotropic hormone (ACTH), or pituitary mass suggested that overstimulation of the HPI axis was not a factor. The AhR activation was measured by an elevation of cytochrome P4501A1 (CYP1A) mRNA expression within the HPI axis in fish exposed to high PAH concentrations; however, CYP1A was no longer induced after 3 d of recovery in any of the tissues. At 7 d of recovery, there was an impairment of cortisol release in response to an additional simulated predator chase that does not appear to be due to changes in the mRNA expression of the kidney steroidogenic pathway proteins steroidogenic acute regulatory protein, cytochrome P450 side chain cleavage, and 11*β*-hydroxylase. Future analyses are needed to determine whether the stress response impairment is due to cholesterol availability and/or down-regulation of the melanocortin 2 receptor. *Environ Toxicol Chem* 2021;40:1062–1074. © 2020 SETAC

Keywords: *Opsanus beta*; Polycyclic aromatic hydrocarbons; Cytochrome P4501A1; Corticotropin-releasing factor; Adrenocorticotropic hormone; Cortisol; Steroidogenic acute regulatory protein; Cytochrome P450 side chain cleavage; 11*β*-hydroxylase

INTRODUCTION

The *Deepwater Horizon* oil spill occurred in April 2010 and resulted in the release of millions of gallons of crude oil into the northern Gulf of Mexico, making it the largest marine oil spill in US history (Camilli et al. 2012; McNutt et al. 2012; Reddy et al. 2012). The oil seeped from the well-head into the Gulf of Mexico continuously over several months and the combined impact of its release under high pressure at depth, natural weathering in relatively warm Gulf of Mexico water, and the use of a chemical dispersant resulted in oil slicks that had a high ratio of 2–3-ringed polycyclic aromatic hydrocarbons (PAHs; Reddy et al. 2012; Ryerson et al. 2012). The PAHs are a toxic component of any oil that has been found to have various physiological, morphological, and behavioral impacts on aquatic organisms (reviewed by Pasparakis et al. 2019). One of these physiological effects is interference with the vertebrate stress response (Thomas et al. 1980; DiBartolomeis et al. 1986; Thomas and Rice 1987; Hontela et al. 1992, 1995; Bestervelt et al. 1993; Girard et al. 1998; Hontela 1998; Wilson et al. 1998; Aldegunde et al. 1999; Aluru and Vijayan 2004, 2006; Kennedy and Farrell 2005; Oliveira et al. 2007; Gesto et al. 2008; Tintos et al. 2008; Schwacke et al. 2014; Reddam et al. 2017; Smith et al. 2017).

The vertebrate stress response is controlled by the hypothalamic–pituitary–adrenal (HPA) axis in mammals and the analogous hypothalamic–pituitary–inter-renal (HPI) axis in fish. When stress is perceived, corticotrophin-releasing factor (CRF) is released from the hypothalamus, which causes a release of adrenocorticotropin hormone (ACTH) from the pituitary.

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The ACTH then activates the melanocortin 2 receptor (MC2R) on mammalian adrenal or fish inter-renal cells, which causes conversion of cholesterol into cortisol through a process that requires the steroidogenic proteins: steroidogenic acute regulatory protein (StAR), cytochrome P450 side chain cleavage (P450scc), and 11*β*-hydroxylase (11*β*H). After biosynthesis, cortisol is then secreted into circulation and binds to glucocorticoid receptors (GRs) throughout the body to initiate various processes to promote survival from an environmental stressor or exerts negative feedback on the hypothalamus and pituitary, which inhibits further release of CRF, ACTH, and cortisol (Dinan 1996; Wendelaar Bonga 1997; Mommsen et al. 1999; Habib et al. 2001; Carrasco et al. 2003; Figure 1).

Only 3 studies have investigated the effects on stress of PAHs associated with the *Deepwater Horizon* oil spill (Schwacke et al. 2014; Reddam et al. 2017; Smith et al. 2017), Specifically, bottlenose dolphins (*Tursiops truncatus*) exposed to capture stress (Schwacke et al. 2014; Smith et al. 2017) and Gulf toadfish (*Opsanus beta*) kidneys after physiologically induced stress in vitro (Reddam et al. 2017) have been shown to have impaired release of cortisol, the main glucocorticoid stress hormone after exposure to *Deepwater Horizon* oil. However, over the past several decades, several studies have found that either PAH pollution in temperate environments or experimental exposure to individual PAHs can impair the stress response (Hontela et al. 1992, 1995; Aluru and Vijayan 2006). For example, studies have shown stimulation of the HPI axis and increases in cortisol as well as pituitary atrophy after PAH exposure in fish (Thomas et al. 1980; Thomas and Rice 1987; Hontela et al. 1992; Aldegunde et al. 1999; Aluru and Vijayan 2004; Kennedy and Farrell 2005; Oliveira et al. 2007; Gesto et al. 2008; Tintos et al. 2008). Other studies have measured the impact on the steroidogenic pathway of reduced plasma cortisol after PAH exposure (DiBartolomeis et al. 1986; Bestervelt et al. 1993; Aluru and Vijayan 2006; Gesto et al. 2008); such an impact might include increased ACTH release due to an environmental stressor but reduced sensitivity or internalization of MC2R (Girard et al. 1998; Hontela 1998; Wilson et al. 1998; Aluru and Vijayan 2004) and/or down-regulation in the steroidogenic pathway such as reduced cholesterol availability (McGruer et al. 2019) or down-regulation of StAR, P450scc, or 11*β*H, which leads to reduced cortisol release (Aluru and Vijayan 2004, 2006).

Based on these previous studies, we hypothesized that the impaired cortisol release in response to *Deepwater Horizon* oil exposure could be due to 1 of 2 proposed mechanisms. Our first hypothesis was that continuous exposure to PAHs associated with *Deepwater Horizon* oil is perceived as an environmental stressor that would cause chronic stimulation of the HPI axis, may cause pituitary fatigue or atrophy, and ultimately result in reduced cortisol release. Our second hypothesis was that impairment of cortisol release may be more directly due to PAH activation of the aryl hydrocarbon receptor (AhR) throughout the HPI axis, causing downstream changes in transcription of various genes such as cytochrome P4501A1 (CYP1A), which is used as a biomarker of AhR activation (Hahn 1998; Till et al. 1999; Sarasquete and Segner 2000; Billiard et al. 2002, 2004), as well as genes involved in steroid production within

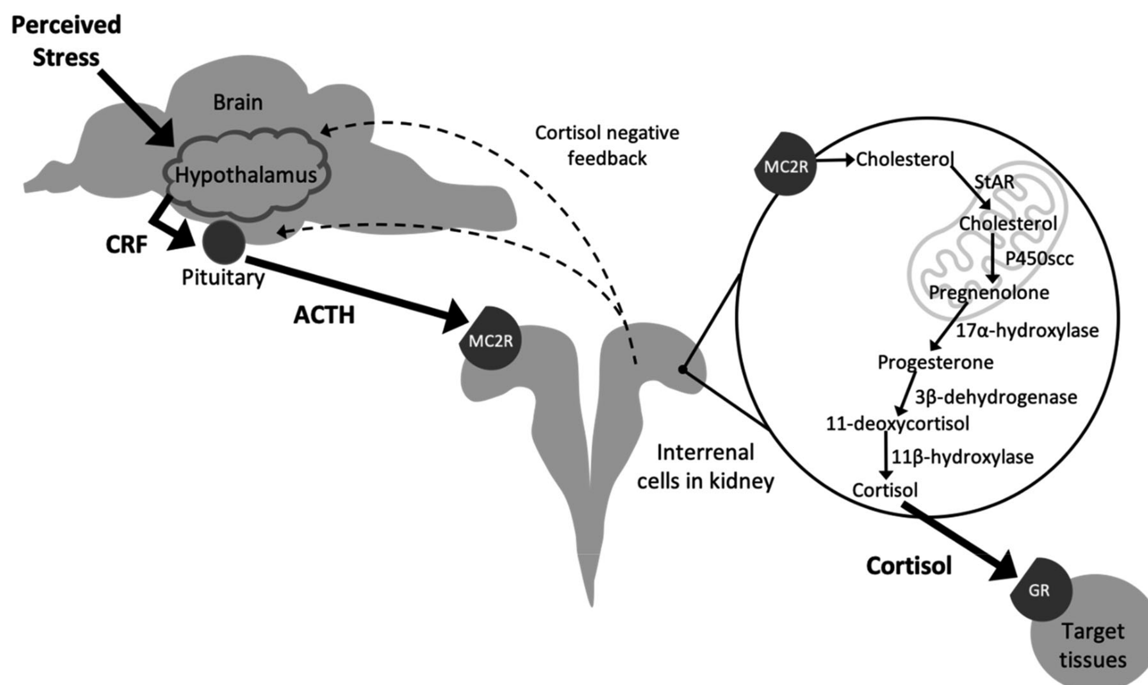

FIGURE 1: Diagram of the hypothalamic–pituitary–inter-renal (HPI) axis. Perceived stress causes a release of corticotrophin-releasing factor (CRF) from the hypothalamus, which triggers adrenocorticotrophic hormone (ACTH) release from the pituitary into the bloodstream. The ACTH then binds to melanocortin 2 receptor (MC2R) receptors on the interrenal cells of kidneys, which initiates the steroidogenic pathway to produce and release cortisol into the blood. Cortisol then binds to glucocorticoid receptors (GRs) on target tissues or can have negative feedback on the hypothalamus or pituitary. StAR = steroidogenic acute regulatory protein; P450scc = cytochrome P450 side chain cleavage.

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inter-renal cells. To test these 2 hypotheses, Gulf toadfish were continuously exposed to low, environmentally relevant concentrations of *Deepwater Horizon* oil for 1 wk. To determine how the stress response may recover from oil exposure, an additional group of fish was exposed for 1 wk and allowed to recover for 1 wk. Various endpoints were measured throughout the exposure and recovery groups to determine the mechanism of cortisol release impairment and the implications of recovery.

MATERIALS AND METHODS

Experimental animals

Gulf toadfish (*O. beta*) were collected by commercial shrimpers using trawl nets in Biscayne Bay, Florida, USA (Florida Fish and Wildlife Conservation Commission Special Activity License SAL-16-0729-SR) from June to July 2018 for series I and December 2018 to March 2019 for series II. After they were brought to the laboratory, fish were treated every 2.5 wk with freshwater and a dose of malachite green (0.1 mg L^{-1}) and formalin (30 mg L^{-1}) to treat for the parasite *Cryptocaryon irritans*. Treatment ended approximately 1 wk before experimental exposures. Procedures were approved by the University of Miami Institutional Animal Care and Use Committee.

Experimental series

Exposure set-up. The present study was done in conjunction with that of Alloy et al. (2021, this issue), and more details about the exposure system can be found there. Briefly, fish were placed in a flow-through system and exposed to control conditions (0.006 ± 0.001 [$n = 4$] μg L^{-1} ∑PAHs) or one of 3 concentrations (low, 0.009 ± 0.002 [$n = 4$]; medium, 0.059 ± 0.006 [$n = 4$]; and high, 2.818 ± 0.284 [$n = 4$] μg L^{-1} ∑PAHs) of high-energy water accommodated fraction (HEWAF) from *Deepwater Horizon* surface oil (OFS-20100719-JUNIPER-001; Alloy et al. 2021, this issue). The HEWAF preparation was similar to those of Alloy et al. (2015) and Reddam et al. (2017). A stock HEWAF concentration was created by mixing 4 g of oil/L of seawater in a Waring industrial blender on low power for 30 s and allowing it to separate in a glass separatory funnel for 1 h. This HEWAF protocol allowed us to extract as much PAH/unit oil mass as possible without chemical assistance. This process, even with the 1-h settling period, may lead to droplet oil (Singer et al. 2000; Redman and Parkerton 2015), which may not be as environmentally realistic as truly dissolved oil. This stock HEWAF was continuously pumped into a header tank with continual input of seawater to create the high oil concentration, which was delivered to the exposure tanks. The medium and low concentrations were then created through an in-line dilution series using the previous header tank and seawater input and similarly delivered to exposure tanks. Control tanks received only received seawater. Further details of the system and flow rates can be found in Alloy et al. (2021, this issue). Although the low concentration is similar to the control concentration, the serial dilution was expected to create a different PAH profile than the control seawater.

Temperatures ranged from 24 to 26 °C, pH was approximately 8.25, and salinity was approximately 33 ppt. There were 8 19-L replicate exposure tanks for each concentration with 10 fish each (average individual mass 29.5 ± 0.4 g). Fish were separated into 2 groups (4 tanks each); the first group of fish (exposure only) were exposed to control or oil conditions for up to 7 d, and blood and tissue samples were collected during the exposure at 4 h, 8 h, 1 d, 3 d, and 7 d. A separate group of fish (exposure + recovery) were exposed to the same control or oil conditions for 7 d as the first group; however, unlike the first group, they were left alone during this entire 7-d period, with no samples taken. After their 7-d exposure, these fish underwent a recovery period of clean seawater for another 7 d where PAH concentrations were control = 0.007 ± 0.002, low = 0.009 ± 0.002, medium = 0.009 ± 0.002, and high = 0.012 ± 0.005 ($n = 4$) μg L^{-1} PAHs. During this recovery period, blood and tissue samples were collected at 4 h, 8 h, 1 d, 3 d, and 7 d.

Sampling protocol. At each of the sampling time points for both exposure and recovery groups, 8 fish from each oil concentration (control, low, medium, and high) were removed from the tanks (2 from each of the 4 tanks/group). Samples (see next paragraph) were immediately taken from half of these fish ($n = 4$) and considered samples from "unstressed" fish. The other half ($n = 4$) were exposed to an additional environmental stressor of a simulated predator chase and considered samples from "acutely stressed" fish. Fish were placed in a container with control seawater and aeration and a helical agitator that was set at the lowest speed. The attachment was placed all the way down in the container for 30 s, which resulted in the fish actively swimming to avoid the attachment, and then the attachment was lifted slightly so that fish could rest on the bottom but still experience disturbance from the rotating attachment for 15 to 30 min. At $t = 15$, 20, 25, and 30 min, 1 of the 4 fish was removed from the mixer and sampled because only one fish could be sampled at a time. The entire experiment was repeated twice so that there was $n = 8$ for each concentration, time point, and stress treatment for both exposure and recovery groups.

For each fish, a blood sample was immediately taken via caudal puncture with a 1-mL syringe and a 23-gauge needle prerinsed with a 50-μM ethylenediaminetetraacetic acid solution. The blood was centrifuged for 3 to 4 min at 14 000 *g* at 4 °C, and plasma was immediately frozen in liquid nitrogen for later analysis of ACTH and cortisol. After blood sampling, fish were euthanized in MS-222 (tricaine methanesulfonate; 3 g L^{-1}; Western Chemicals) buffered with sodium bicarbonate, and fish mass and sex were recorded. The brain was dissected according to Medeiros et al. (2014), and the pituitary mass was recorded in a preweighed bullet tube as a measure for pituitary atrophy. The pre-optic area (which contains the hypothalamic region), pituitary, and kidney were removed and frozen in liquid nitrogen for later analysis of CYP1A, CRF, MC2R, StAR, P450scc, and 11βH. The entire kidney was removed and analyzed because a

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definitive study determining the exact location of inter-renal cells has not been done on Gulf toadfish.

Analysis

Analytical techniques. Water samples from each concentration were sent to ALS Environmental (Kelso, WA, USA) to quantify ∑PAHs concentrations using gas chromatography–mass spectrometry/selective ion monitoring (GC-MS(SIM)), and samples were also taken daily for analysis via hexane liquid–liquid extraction and peak fluorescence (described in Alloy et al. 2021, this issue). Plasma ACTH was quantified using a ^{125}I-ACTH radioimmunoassay (RIA) kit (MP Biomedicals), and plasma cortisol was quantified using the ^{125}I-cortisol RIA kit (MP Biomedicals).

Quantitative real-time PCR. Total RNA from each tissue (pre-optic area, pituitary, kidney) was isolated according to the protocol included with the Trizol reagent (Invitrogen), and then treated with DNAse I using the TURBO DNA-free kit (Invitrogen). The quantity and purity of the total RNA was measured using SpectraDrop micro-volume microplate quantification (Molecular Devices) and the 260/280 ratio. Samples from which we obtained <300 ng/μL total RNA that had a 260/280 ratio of <1.7 were not used in this analysis. Complementary (c)DNA was synthesized using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) and diluted 10-fold. Real-time quantitative polymerase chain reaction (qPCR) primers for StAR, P450scc, and 11*β*H were designed from partial cDNA sequences obtained using degenerate primers (Table 1). The reference gene used was elongation factor 1α (EF1α; primer sequences obtained from Grosell et al. 2009), and the genes of interest were toadfish CRF (primer sequences obtained from Medeiros et al. 2014), toadfish CYP1A (primer sequences described in Alloy et al. 2021, this issue), toadfish StAR (GenBank Accession # MW256752), toadfish P450scc (GenBank Accession # MW256753), and toadfish 11*β*H (GenBank Accession # MW256754; Table 1). Efforts to design MC2R primers were unsuccessful.

An Mx3005P Multiple Quantitative PCR System (Stratagene) was used to perform qPCR on the cDNA samples in duplicate using Power SYBR Green qPCR Master Mix (Applied Biosystems) as the reporter dye. The cycling parameters used were 95 °C for 10 min; 40 cycles (pre-optic area and pituitary) or 50 cycles (kidney) at 95 °C for 30 s, 60 °C for 33 s, and 72 °C for 30 s. No-template controls, no-reverse transcriptase controls, and a melt curve analysis were completed to confirm there was no contamination or primer dimer formation. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) was used for each gene of interest to determine fold changes from the control of each time point. Mean messenger (m)RNA expression values were rescaled to the control for each time point and normalized to the reference gene EF1α.

Potential changes in molecular endpoints were measured initially for only the most acute (4-h exposure and 4-h recovery) and the most chronic (7-d exposure and 7-d recovery) time points. If data were not conclusive with just these time points measured, further time points were analyzed. If differences were measured during exposure, the trajectory of recovery was further analyzed. To determine whether toadfish perceived oil as an environmental stressor, CRF mRNA expression in the pre-optic area in unstressed fish was quantified for the 2 earliest (4 and 8 h) and the most chronic exposure time point measured in the present study (7 d). To determine whether CYP1A was induced in the HPI axis during exposure and recovery, CYP1A

TABLE 1: Degenerate primer sequences used for partial cDNA cloning of target genes and quantitative polymerase chain reaction primers used for mRNA expression

Primer type	Name	Sequence (5′–3′)	Product (bp)
Degenerate	StAR-F	TGC CTG CAA CKT TYA AAC TGT G	601
	StAR-R	ACA TSC CRG CMA RGA AGC A	
	P450scc-F	GGA CAA GCT RTC CAT TGA AG	474
	P450scc-R	GCT ATY CTG CGW CCT AAA CAC	
	11*β*H-F	CTG GAA GCC AGY TGY CAT GT	441
	11*β*H-R	GGG TTR CGR CCC ARC TCR WAC AG	
qPCR	EF1α-F[a]	AGG TCA TCA TCC TGA ACC AC	143
	EF1α-R[a]	GTT GTC CTC AAG CTT CTT GC	
	CRF-F[b]	TTC ATC CGA CTG GGC AAC GG	147
	CRF-R[b]	ATC GGC GGG TCG TCG GAC CTC CTT	
	CYP1A-F[c]	GGA CAA CAT TCG TGA CAT CAC	160
	CYP1A-R[c]	CCA TGA CAG ACC GGT GG	
	StAR-F	TGC CTG CAA CGT TTA AAC TGT G	180
	StAR-R	CAA TCC GAG AAC CGA GGA GG	
	P450scc-F	GGA CAA GCT ATC CAT TGA AG	192
	P450scc-R	TTC AGC ATC TCC GTC ATG	
	11βH-F	AAG CCA GTT GTC ATG TGC TG	200
	11βH-R	GAA GAT GTG GTC CCA TGC AG	

[a]Grosell et al. 2009.
[b]Medeiros et al. 2014.
[c]Alloy et al. 2021, this issue.
F = forward primer; R = reverse primer; qPCR = quantitative polymerase chain reaction; StAR = steroidogenic acute regulatory protein; P450scc = cytochrome P450 side chain cleavage; CYP1A = cytochrome P4501A1; CRF = corticotrophin-releasing factor; 11βH = 11β-hydroxylase.

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mRNA expression was measured in a mix of unstressed and acutely stressed fish (stress treatment did not affect CYP1A mRNA expression; data not shown) in the hypothalamic pre-optic area (exposure: 4 h, 7 d; recovery: 4 h, 8 h, 1 d, 3 d, and 7 d), pituitary (exposure: 4 h, 3 d; recovery: 4 h, 8 h, 1 d, and 3 d), and kidney (exposure: 4 h, 7 d; recovery: 4 h, 8 h, 1 d, 3 d, and 7 d). Due to the small size of the pituitary, 2 pituitaries of the same exposure concentration, time point, and stress treatment were combined and analyzed together to extract enough RNA for analysis. This need was not realized until after pituitaries from the 7-d exposure and recovery were analyzed (and mRNA expression was inconsistent); thus, the most chronic exposure and recovery time point for the pituitaries is 3 d. To determine whether steroidogenic pathway proteins are responsible for stress response impairment, StAR, P450scc, and 11*β*H mRNA expression were quantified in the kidney of stressed fish at the most acute and chronic time points (exposure: 4 h, 7 d; recovery: 4 h, 7 d).

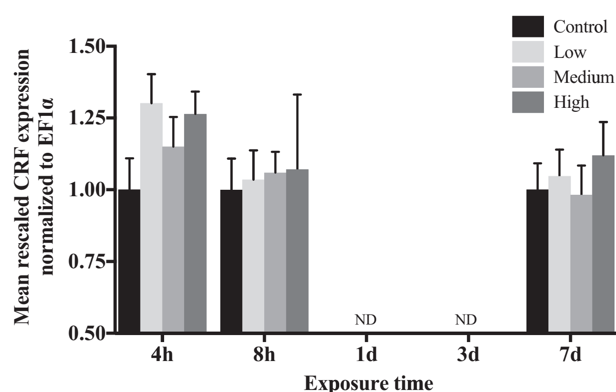

FIGURE 2: There were no significant differences in corticotrophin-releasing factor (CRF) mRNA expression in the pre-optic area during exposure at 4 h, 8 h, or 7 d in unstressed control compared with oil-exposed fish. Oil concentrations were as follows: low 0.009, medium 0.06, and high 2.8 μg L^{-1} sum polycyclic aromatic hydrocarbons (∑PAHs). Mean CRF mRNA expression was rescaled to the control for each time point and normalized to elongation factor 1α (EF1α). Values are mean ± standard error of the mean ($n = 7$–8 for each time point). ND = no data were analyzed at that time point.

Statistical analysis

All statistical tests were performed using GraphPad Prism software (Ver. 7.0a). For plasma ACTH concentration, plasma cortisol concentration, % pituitary mass of total body mass, and mRNA expression of CRF, CYP1A, StAR, P450scc, and 11*β*H, each time point and stress treatment were analyzed separately to determine differences between controls and oil concentrations. Data were tested for normality using the D'Agostino–Pearson test or the Shapiro–Wilk test if the sample size was too small. If data were not normally distributed, they were either log or square root transformed to perform a parametric one-way analysis of variance and Tukey's multiple comparison test with oil concentration as the main factor. If data were not normal after transformation, a nonparametric Kruskal–Wallis test with Dunn's multiple comparison was used to compare differences within each time point. Outliers were removed via the ROUT outlier test ($\alpha = 0.0001$) for ACTH, cortisol, and CYP1A and StAR mRNA expression. For time points in which CYP1A mRNA expression was only measured in control and high exposed fish, a Student's *t* test was used. Combined control values for ACTH and cortisol were also compared with a Student's *t* test. Statistically significant differences were considered if $p < 0.05$.

RESULTS

Is exposure to PAHs associated with Deepwater Horizon oil perceived by fish as an environmental stressor?

The goal of this set of analyses was to determine whether continuous PAH exposure is perceived as an environmental stressor that could ultimately cause overactivation and eventual fatigue of the HPI axis. The CRF mRNA expression for PAH-exposed unstressed fish at any concentration was not significantly different from controls at 4 h, 8 h, and 7 d in the exposure group (Figure 2). Furthermore, plasma ACTH concentrations in unstressed PAH-exposed fish were not significantly different from unstressed controls at any time points (Figure 3A and B). During the exposure period, there were no differences in plasma ACTH between time points in unstressed control fish (Figure 3A), but during recovery, plasma ACTH was elevated during recovery at 8 h compared with 4 h and 1 d (Figure 3B). There were no effects of oil exposure on % pituitary mass (Table 2). There were also no effects on plasma cortisol concentrations between unstressed control and unstressed PAH-exposed fish at any of the time points for the exposure and recovery groups (Figure 4A and B). During exposure, there were no differences in plasma cortisol between time points in control fish (Figure 4A), but during recovery, plasma cortisol was elevated at 1 d compared with 7 d (Figure 4B).

Is there AhR activation throughout the HPI axis and are there downstream effects?

The goal of this set of analyses was to determine whether exposure to PAHs activated the AhR and induced CYP1A mRNA expression throughout the HPI axis and caused impairment of cortisol release through changes in transcription of various genes. There were no differences in CYP1A mRNA expression between unstressed and acutely stressed fish (data not shown), so data were combined for some time points (Figure 5). In the pre-optic area, CYP1A mRNA expression in high-exposed fish was significantly and immediately elevated by 85-fold after 4 h of exposure compared with controls, with elevation persisting for 7 d (95-fold higher compared with control fish; Figure 5A). Similarly, CYP1A mRNA expression was significantly elevated in high-exposed fish in the recovery group by 108-fold 4 h following recovery and appeared to slowly decrease, returning to control levels by 3 d into the

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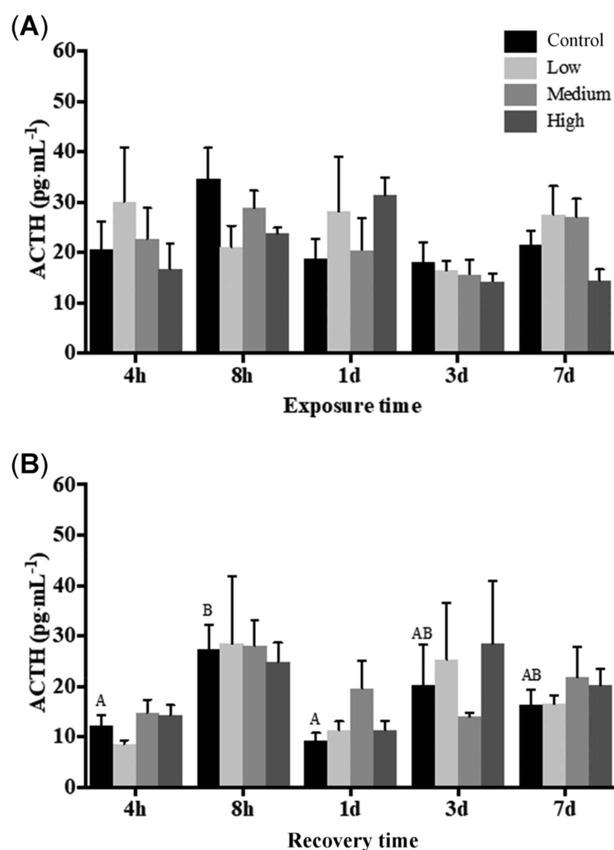

FIGURE 3: There were no significant differences in plasma adrenocorticotropic hormone (ACTH) concentrations between unstressed control and oil-exposed fish at 4, 8, 1, 3, and 7 d during (**A**) exposure or (**B**) recovery. Values are mean ± standard error of the mean (n = 5–8 for each time point except recovery group 3 d when n = 4). Different capital letters denote significant difference between time points in control fish ($p < 0.05$).

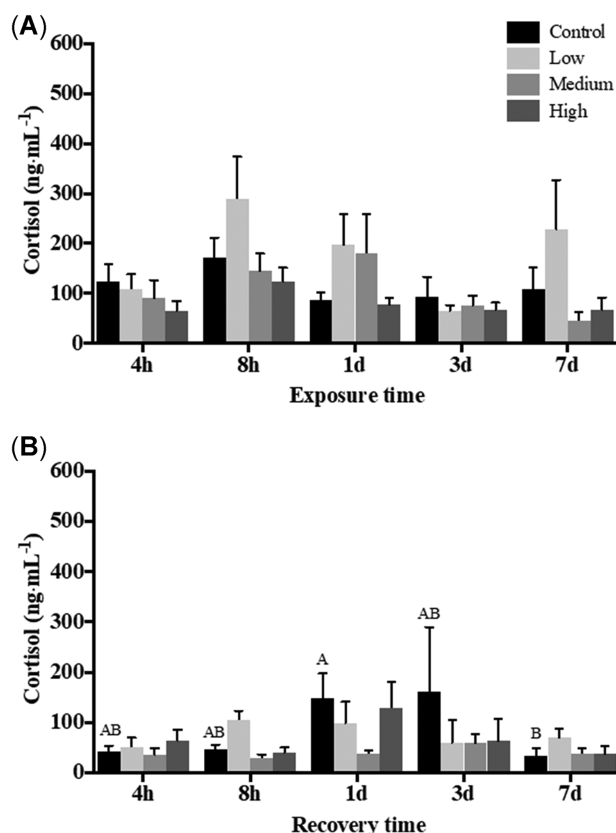

FIGURE 4: There were no significant differences in plasma cortisol concentrations between unstressed control and oil-exposed fish at 4 h, 8 h, 1 d, 3 d, and 7 d during (**A**) exposure or (**B**) recovery. Values are mean ± standard error of the mean (n = 5–8 for each time point except recovery group 3 d when n = 4). Different capital letters denote significant difference between time points in control fish ($p < 0.05$).

recovery (Figure 5B). The CYP1A mRNA expression in the pre-optic area in medium oil-exposed fish was also greater, by 2- to 4-fold, than controls in the exposure group (Figure 5A). In the recovery group, medium oil-exposed fish had 2- to 8-fold higher CYP1A compared with controls 4 h into recovery but it was no longer significantly elevated after 1 d of recovery (Figure 5B). There was no significant effect on CYP1A mRNA expression in low oil-exposed fish in the pre-optic area in the exposure or recovery groups (Figure 5A and B).

In the pituitary, CYP1A mRNA expression was significantly elevated in the high-exposed fish: by 10-fold after 4 h of exposure compared with controls, with the elevation persisting for at least 3 d (217-fold higher compared with controls; Figure 5C). The CYP1A mRNA expression in the pituitary was significantly elevated in high-exposed fish compared with controls in the recovery group by 5-fold 4 h following recovery and by 34-fold following 8 h of recovery (Figure 5D). After 1 d of recovery, pituitary CYP1A mRNA expression was no longer

TABLE 2: There was no significant difference in % pituitary mass of total body mass in control or oil-exposed fish at 4 h, 8 h, 1 d, 3 d, or 7 d in unstressed and stressed fish combined for both exposure and recovery groups[a]

		4 h	8 h	1 d	3 d	7 d
Exposure	Control	$1.59 \pm 0.13 \times 10^{-3}$ (16)	$1.34 \pm 0.08 \times 10^{-3}$ (14)	$1.46 \pm 0.12 \times 10^{-3}$ (16)	$1.42 \pm 0.14 \times 10^{-3}$ (16)	$1.37 \pm 0.12 \times 10^{-3}$ (16)
	Low	$1.30 \pm 0.13 \times 10^{-3}$ (15)	$1.40 \pm 0.12 \times 10^{-3}$ (16)	$1.37 \pm 0.10 \times 10^{-3}$ (16)	$1.50 \pm 0.13 \times 10^{-3}$ (16)	$1.15 \pm 0.08 \times 10^{-3}$ (16)
	Medium	$1.44 \pm 0.07 \times 10^{-3}$ (16)	$1.41 \pm 0.08 \times 10^{-3}$ (16)	$1.67 \pm 0.15 \times 10^{-3}$ (15)	$1.59 \pm 0.12 \times 10^{-3}$ (15)	$1.23 \pm 0.06 \times 10^{-3}$ (16)
	High	$1.59 \pm 0.12 \times 10^{-3}$ (16)	$1.56 \pm 0.14 \times 10^{-3}$ (16)	$1.19 \pm 0.11 \times 10^{-3}$ (16)	$1.43 \pm 0.10 \times 10^{-3}$ (16)	$1.39 \pm 0.10 \times 10^{-3}$ (16)
Recovery	Control	$1.16 \pm 0.20 \times 10^{-3}$ (15)	$0.99 \pm 0.20 \times 10^{-3}$ (16)	$1.16 \pm 0.12 \times 10^{-3}$ (16)	$1.70 \pm 0.16 \times 10^{-3}$ (8)	$1.31 \pm 0.08 \times 10^{-3}$ (15)
	Low	$1.25 \pm 0.13 \times 10^{-3}$ (15)	$1.33 \pm 0.14 \times 10^{-3}$ (16)	$1.09 \pm 0.13 \times 10^{-3}$ (16)	$1.45 \pm 0.10 \times 10^{-3}$ (8)	$1.42 \pm 0.13 \times 10^{-3}$ (9)
	Medium	$1.16 \pm 0.11 \times 10^{-3}$ (16)	$0.98 \pm 0.10 \times 10^{-3}$ (16)	$1.07 \pm 0.09 \times 10^{-3}$ (16)	$1.76 \pm 0.19 \times 10^{-3}$ (8)	$1.38 \pm 0.10 \times 10^{-3}$ (15)
	High	$1.11 \pm 0.08 \times 10^{-3}$ (16)	$1.37 \pm 0.08 \times 10^{-3}$ (16)	$1.23 \pm 0.09 \times 10^{-3}$ (16)	$1.85 \pm 0.14 \times 10^{-3}$ (8)	$1.87 \pm 0.20 \times 10^{-3}$ (15)

[a]Mean ± standard error of the mean (no).

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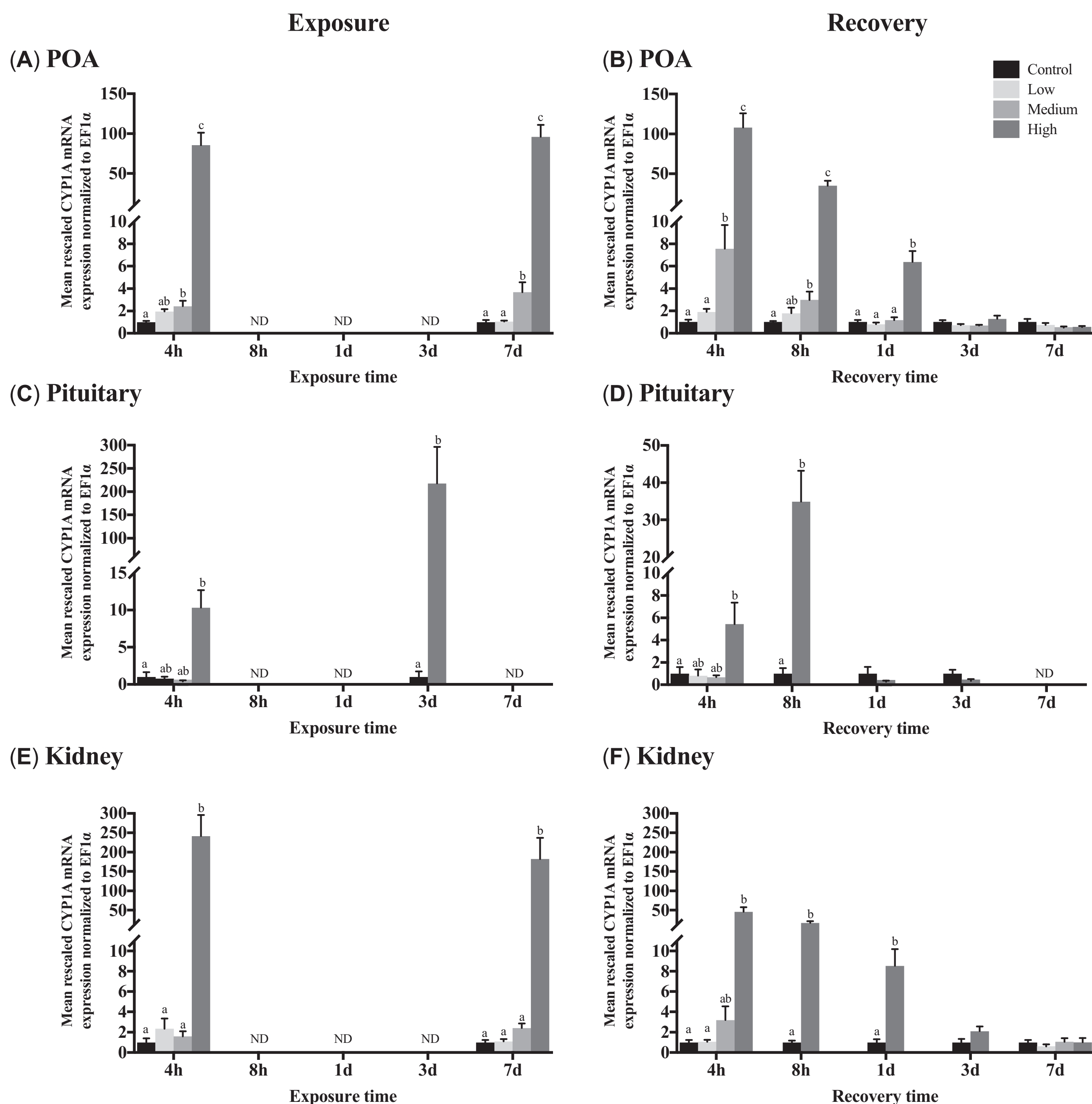

FIGURE 5: A significant elevation in cytochrome P4501A1 (CYP1A) mRNA expression was measured in the (**A** and **B**) pre-optic area (POA; $n = 5–16$), (**C** and **D**) pituitary ($n = 4–12$), and (**E** and **F**) kidney ($n = 4–8$) in oil-exposed fish compared with controls in exposure (**A**, **C**, and **E**) and recovery (**B**, **D**, and **F**) groups. Mean mRNA expression of CYP1A was rescaled to the control for each time point and normalized to elongation factor 1α (EF1α). Values are mean ± standard error of the mean, and different letters denote significant differences within each time point ($p < 0.05$). ND = no data were analyzed at that time point.

significantly different from controls (Figure 5D). There was no significant effect on CYP1A mRNA expression in low or medium oil-exposed fish in the pituitary (Figure 5C and D).

In the kidney, high-exposed fish CYP1A mRNA expression was significantly and immediately elevated by 182-fold compared with controls within 4 h of exposure and elevation persisted (241-fold increase compared with controls) after 7 d of exposure (Figure 5E). After 4 h of recovery, high-exposed fish still demonstrated a significant 46-fold elevation compared with controls that gradually reduced over time (Figure 5F). After 3 d of recovery, kidney CYP1A was no longer significantly different from controls (Figure 5F). Similar to the pituitary, there was no significant effect on CYP1A mRNA expression in low or medium oil-exposed fish in the kidney (Figure 5E and F).

After exposure to an acute simulated predator chase, plasma ACTH concentrations were not significantly different between acutely stressed control and PAH-exposed fish in exposure and recovery groups (Figure 6A and B). During the exposure period, there were no differences in plasma ACTH between time points in acutely stressed control fish (Figure 6A),

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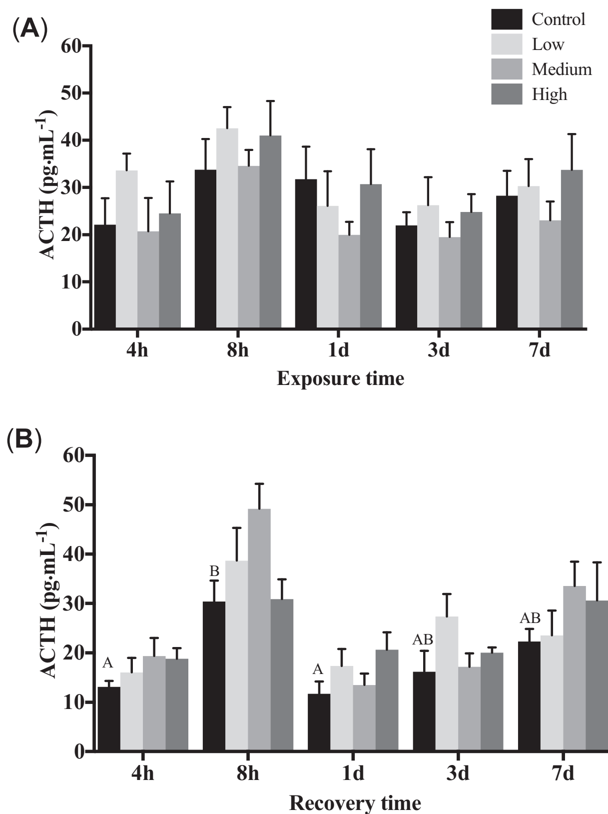

FIGURE 6: There were no significant differences in plasma adrenocorticotropic hormone (ACTH) concentrations between acutely stressed control and oil-exposed fish at 4 h, 8 h, 1 d, 3 d, and 7 d during (**A**) exposure or (**B**) recovery. Values are mean ± standard error of the mean ($n = 5$–8 for each time point except recovery group 3 d when $n = 4$). Different capital letters denote significant difference between time points in control fish ($p < 0.05$).

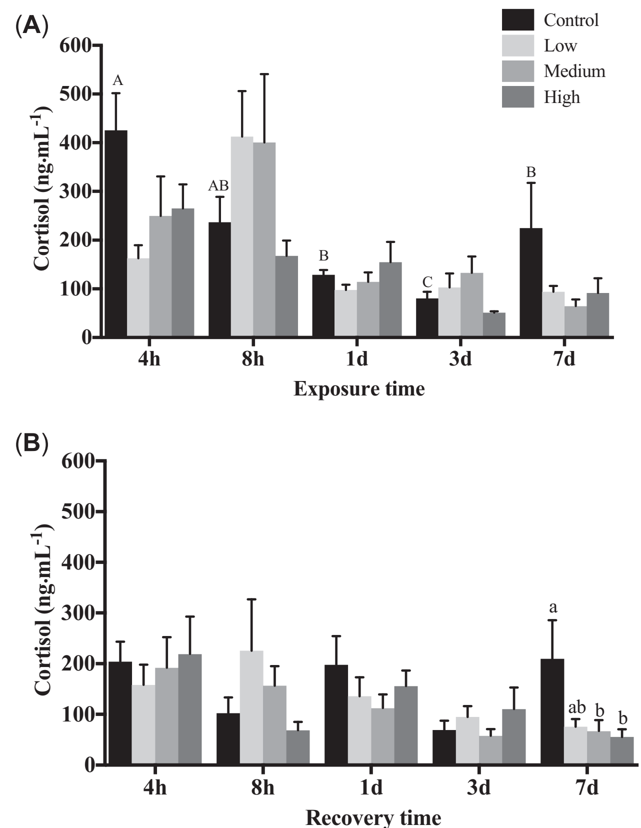

FIGURE 7: There were no significant differences measured in plasma cortisol concentrations between acutely stressed control and oil-exposed fish during (**A**) exposure, but a significant decrease in plasma cortisol was measured during (**B**) recovery after 7 d. Values are mean ± standard error of the mean ($n = 5$–8 for each time point except recovery group 3 d when $n = 4$). Different lowercase letters denote significant difference within a time point ($p < 0.05$). Different capital letters denote significant difference between time points in control fish ($p < 0.05$).

but during recovery, acutely stressed control fish had elevated ACTH at 8 h compared with 4 h and 1 d (Figure 6B).

Plasma cortisol was not significantly different between acutely stressed control and PAH-exposed fish during the exposure period at any of the time points (Figure 7A). During the recovery period, there were also no differences in plasma cortisol between acutely stressed control and PAH-exposed fish from 4 h to 3 d (Figure 7B). However, after 7 d of recovery from a 7-d PAH exposure, plasma cortisol in response to an acute simulated predator chase was significantly reduced in medium (67.2 ± 21.6 ng mL^{-1}) and high (55.3 ± 15.1 ng mL^{-1}) oil-exposed fish compared with controls (210.0 ± 75.7 ng mL^{-1}) with no effect at the low oil concentration (75.9 ± 14.9 ng mL^{-1}; Figure 7B).

Notably, the magnitude of the cortisol response to the acute simulated predator chase of control fish in the exposure group significantly decreased over time, with the response after 3 d being only 19% of that measured at 4 h (Figure 7A). At 7 d, the magnitude of the response in control fish began to return to levels measured at 1 d but was still only 53% of the 4-h response (Figure 7A). Looking at control fish of the recovery group, there were no significant differences between time points (Figure 7B).

There was no corresponding effect of oil exposure on StAR, P450scc, and 11βH mRNA expression in the kidney in acutely stressed fish in exposure or recovery groups (Table 3).

DISCUSSION

Is exposure to PAHs associated with Deepwater Horizon *oil perceived by fish as an environmental stressor?*

Results from a previous study on the effects of PAHs and *Deepwater Horizon* oil on Gulf toadfish (Reddam et al. 2017) as well as other studies (Hontela et al. 1992; Wilson et al. 1998; Gesto et al. 2008) suggested that exhaustion or overstimulation of the stress response could be responsible for impaired cortisol release (Mommsen et al. 1999). However, in the present study, exposure to environmentally realistic PAH concentrations (<2.8 μg L^{-1} ∑PAHs) that are lower than in many studies to date (reviewed by Pasparakis et al. 2019), especially for stress (Reddam et al. 2017), had no effect on CRF mRNA expression, circulating ACTH concentrations, and pituitary mass, an indicator of potential pituitary atrophy; thus overstimulation of the

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TABLE 3: StAR, P450scc, and 11β-H mRNA expression in the kidney at 4 h and 7 d of exposure and recovery in acutely stressed fish[a]

		Exposure group		Recovery Group	
Gene	Concentration	4 h (no.)	7 d (no.)	4 h (no.)	7 d (no.)
StAR	Control	1.00 ± 0.98 (6)	1.00 ± 0.39 (8)	1.00 ± 0.34 (7)[a]	1.00 ± 0.63 (8)
	Low	1.20 ± 1.19 (7)	0.31 ± 0.09 (8)	17.2 ± 7.86 (8)[ab]	1.37 ± 1.21 (4)
	Medium	4.77 ± 4.55 (6)	0.24 ± 0.11 (8)	23.3 ± 10.2 (8)[b]	0.40 ± 0.09 (6)
	High	33.3 ± 21.8 (8)	0.96 ± 0.49 (8)	12.0 ± 6.72 (7)[ab]	0.07 ± 0.03 (6)
P450scc	Control	1.00 ± 0.74 (6)	1.00 ± 0.24 (8)	1.00 ± 0.38 (7)	1.00 ± 0.46 (8)
	Low	0.51 ± 0.23 (7)	0.34 ± 0.04 (8)	0.74 ± 0.17 (8)	1.33 ± 0.85 (4)
	Medium	0.76 ± 0.46 (7)	0.63 ± 0.14 (8)	1.25 ± 0.39 (8)	0.73 ± 0.15 (6)
	High	2.28 ± 1.44 (8)	1.15 ± 0.34 (8)	1.38 ± 0.47 (8)	0.32 ± 0.10 (7)
11β-H	Control	1.00 ± 0.65 (6)	1.00 ± 0.35 (8)	1.00 ± 0.55 (7)	1.00 ± 0.44 (8)
	Low	3.20 ± 2.06 (7)	0.64 ± 0.11 (8)	0.39 ± 0.12 (8)	1.98 ± 0.98 (4)
	Medium	2.08 ± 0.83 (7)	0.93 ± 0.17 (8)	0.54 ± 0.25 (8)	1.31 ± 0.24 (6)
	High	1.38 ± 0.47 (8)	1.26 ± 0.29 (8)	0.37 ± 0.16 (8)	0.68 ± 0.13 (7)

[a]Mean mRNA expression of each gene was rescaled to the control for each time point and normalized to elongation factor 1α (EF1α). Values are mean ± standard error of the mean (no.). Different letters denote significant difference within a time point ($p < 0.05$).
StAR = steroidogenic acute regulatory protein; P450scc = cytochrome P450 side chain cleavage; 11β-H = 11β-hydroxylase.

stress axis does not appear to be the cause of impairment. However, it is likely that 1 wk of PAH exposure may not have been long enough to observe pituitary atrophy because a lifelong environmental PAH exposure has been shown to cause atrophy in northern pike and yellow perch and a consequent reduction in cortisol release in response to capture stress (Hontela et al. 1992). The need to understand the impacts of long-term exposures is environmentally relevant because the oil well was uncapped for several months during the *Deepwater Horizon* spill, which would result in persistent elevations in PAHs.

It is possible that CRF could have been elevated within the first 4 h of oil exposure, which would suggest immediate perception of stress. This elevation may have been missed because CRF mRNA expression can be up-regulated within approximately 8 min of perceived stress in toadfish (Medeiros et al. 2014). However, even if a transient elevation was missed, it did not translate to downstream effects within the HPI axis. It is unknown whether toadfish perceive PAHs through olfaction, gustation, or pain perception (Reddam et al. 2017); however, our results suggest that if they do perceive it at these low concentrations, they do not respond to it negatively. Some species of fish have been found to avoid oil and PAHs at varying concentrations possibly through olfaction (Lari et al. 2015; Claireaux et al. 2017; Lari and Pyle 2017; Schlenker et al. 2019). However, the lowest avoidance threshold has been found in European seabass, which avoid the water-soluble fraction of oil at 8.54 $\mu g\, L^{-1}$ PAHs (Claireaux et al. 2017), so it is unknown whether toadfish were able to detect our exposure concentrations of ≤2.8 $\mu g\, L^{-1}$ ∑PAHs.

Consistent with no changes in CRF and ACTH, there were also no changes in circulating cortisol in unstressed fish. This is similar to Reddam et al. (2017), who found that toadfish had no changes in plasma cortisol in vivo up to 24 h after water exposure to 3% HEWAF (10–72 $\mu g\, L^{-1}$). However, exposure concentration and route of exposure may be important. For example, Reddam et al. (2017) also found that 72 h after intraperitoneal injections of individual PAHs, toadfish experienced a significant increase in plasma cortisol to naphthalene and a nonsignificant increase to phenanthrene, but not fluorene. Therefore, our results suggest that PAH concentrations <3 $\mu g\, L^{-1}$ in *Deepwater Horizon* oil do not affect circulating cortisol levels in toadfish at rest. There were no effects on CRF, ACTH, pituitary mass, and cortisol so steroidogenic pathway effects were not measured in unstressed fish. Therefore, our first hypothesis, that toadfish perceive exposure to PAHs associated with *Deepwater Horizon* oil as an environmental stressor, is not supported.

Is there AhR activation throughout the HPI axis and are there downstream effects?

Elevations in CYP1A mRNA expression in the pre-optic area, pituitary, and kidney in both exposure and recovery groups suggest that the AhR was immediately activated throughout the HPI axis in response to very low concentrations of PAHs between 0.06 and 2.4 $\mu g\, L^{-1}$ PAHs. The CYP1A mRNA expression was elevated in all 3 stress axis tissues in fish exposed to the highest concentration of PAHs, with CYP1A returning to baseline faster in the pituitary compared with the pre-optic area and kidney. The kidney had the highest fold change from the controls out of any of the 3 tissues, which is similar to Atlantic salmon injected with β-naphthoflavone (BNF; an AhR agonist) in which the kidney had the greatest fold increase compared with the brain and other tissues analyzed (Rees et al. 2003). The medium PAH concentration only induced a significant elevation in CYP1A mRNA expression in the pre-optic area, suggesting that the pre-optic area may be more sensitive to AhR activation than both the pituitary and the kidney, which could have implications for other physiological processes (i.e., reproduction, metabolism) that may be controlled by that region of the brain.

To our knowledge, our study is the first to investigate CYP1A induction in the HPI axis after exposure to low concentrations of PAHs associated with *Deepwater Horizon* oil and also to investigate a timeline for recovery. The PAHs and AhR agonists have been previously found to increase CYP1A mRNA expression in the hypothalamus, pituitaries, and kidneys in fish

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(Andersson et al. 1993; Aluru and Vijayan 2004, 2006; Chung-Davidson et al. 2004; Kim et al. 2008); however, previous studies have investigated impacts associated with higher waterborne concentrations or intraperitoneal injections, in particular, of BNF, an AhR agonist not associated with *Deepwater Horizon* oil. For example, river pufferfish experienced an increase in CYP1A mRNA expression in the brain and kidney after 6 to 12 h of 1 μM (~272 μg L^{-1}) BNF waterborne exposure, and mRNA expression remained elevated throughout the entire 96-h exposure (Kim et al. 2008). In the present study, toadfish similarly exhibited immediate elevation in pre-optic area, pituitary, and kidney CYP1A after exposure to *Deepwater Horizon* oil; however, we found that CYP1A mRNA expression decreased after 3 d when waterborne PAH concentrations returned to background. Intraperitoneal injections in the range of mg kg^{-1} BNF (Aluru and Vijayan 2004, 2006; Chung-Davidson et al. 2004) likely resulted in circulating concentrations that were orders of magnitude higher than what toadfish in the present study experienced from a waterborne exposure. This may explain why we were able to measure recovery in our fish once they were removed from the waterborne exposure compared with these other studies that used intraperitoneal injection treatment or did not investigate recovery after water exposure. In addition, exposure route affects CYP1A mRNA expression differentially in tissues, because zebrafish with similar body burdens of benzo[*a*]pyrene had higher CYP1A mRNA expression after intraperitoneal injection compared with waterborne exposure in the liver and the heart (Gerger and Weber 2015). However, to our knowledge this has not been investigated in HPI tissues.

In the present study, the small size of the pituitary resulted in difficulties in RNA extraction and inconsistent fold changes from the controls between time points; however, because CYP1A has been found to be induced in other teleost pituitaries, our results likely suggest that AhR was also activated in toadfish pituitaries. For example, exposure to low concentrations of the AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (6 pg L^{-1}) increased CYP1A immunoreactivity in gilthead seabream pituitary and hypothalamus tissues (Ortiz-Delgado et al. 2002).

Despite AhR activation in the hypothalamus and pituitary, there was no effect of PAH exposure on ACTH release after exposure to the acute environmental stressor of a simulated predator chase. Similar to these results, toadfish did not experience any changes in plasma ACTH after 8 h of 3% HEWAF (geomean of total PAH concentrations 28.9 ± 6.2 μg L^{-1}) and 15-min air exposure as an additional environmental stressor compared with controls (Reddam et al. 2017). The insensitivity of ACTH to PAH exposure suggests that PAH exposure does not impact CRF release from the hypothalamus in response to an acute stressor. This was not directly measured in fish of the present study because the elevation in CRF in response to the acute simulated predator chase was likely immediate (within 5 min) and transient and would have been missed with our acute stress regime. The insensitivity of ACTH to PAH exposure also suggests that PAH exposure does not affect the pituitary's ability to release ACTH. It also suggests there is no compensatory up-regulation in ACTH secretion or hyperactivity of the pituitary due to potential downstream effects on inter-renal cortisol secretion, a result that is further supported by a lack of pituitary atrophy.

Similar to ACTH, cortisol release in response to the acute simulated predator chase in PAH-exposed fish was not impaired during the 7-d exposure period. Only after 7 d of recovery did toadfish previously exposed to medium and high PAH concentrations experience a significant impairment in the ability to release cortisol in response to an acute simulated predator chase. These data suggest that the ability of toadfish to secrete cortisol in response to acute stress is not immediately affected by exposure to environmentally realistic concentrations of PAHs. Instead, impacts are slow to manifest and are not experienced by the fish until they are well into recovery—a phenomenon that has not been previously described in fish.

Although this may be the case, it should be noted that acclimation stress, disturbance stress, and/or changing social interactions within each tank may have confounded the toadfish's ability to mount an acute stress response (Sorensen et al. 2013), because even control fish experienced a decrease in the magnitude of the cortisol response to the acute stressor over time. Indeed, PAHs have been shown to have differential effects on stress axis function that depended on whether fish were exposed simultaneously to an acute or chronic stressor (Gesto et al. 2008; Reddam et al. 2017). Thus, a different experimental design is needed to better test our second hypothesis, that impairment of cortisol release may be due to a PAH activation of the AhR throughout the HPI axis that causes changes in the transcription of various genes. However, that a reduction in plasma cortisol levels was measured after 7-d recovery is consistent with the bottlenose dolphin studies in which the blood samples were collected over 1 yr after the oil spill, and problems in mounting a stress response were evident (Schwacke et al. 2014). Furthermore, dolphins still experienced an impairment in the stress response up to 4 yr following the oil spill, and the impairment appeared to be a delay in the response to secrete cortisol rather than suppression (Smith et al. 2017). This is believed to be due to adrenal compromise in dolphins because necropsies revealed adrenal cortical atrophy (Venn-Watson et al. 2015). Interestingly, the alteration in cortisol secretion is not evident at rest but is a factor when the organism attempts to mount a response to an acute environmental stressor (Schwacke et al. 2014; Reddam et al. 2017; Smith et al. 2017). Although fish and dolphins have different routes of oil exposure, the impact on cortisol release appears to be consistent. Therefore, AhR activation, CYP1A induction, and the subsequent reduction in cortisol release do provide some support for the second hypothesis. However, after 7 d of recovery, plasma cortisol is still impaired even when CYP1A is no longer induced, suggesting a disconnect between CYP1A induction and HPI axis impairment.

Because the decrease in cortisol release is not due to alterations in the hypothalamus or the pituitary, we investigated inter-renal proteins involved in steroidogenesis that could be

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the cellular mechanism responsible for the impairment. For example, in stressed rainbow trout, BNF ingestion inhibits the cortisol response through down-regulation of StAR and P450scc but not 11βH (Aluru and Vijayan 2006). However, in the present study, we did not measure a decrease in the mRNA expression of steroidogenic proteins after 7-d exposure or recovery that corresponded with the suggested impairment after 7-d exposure and the clear impairment of cortisol secretion after 7 d of recovery. Furthermore, there was a tremendous amount of variation in these measurements. It is also possible that we did not observe consistent effects due to differences in stress coping styles (Koolhaas et al. 1999; Overli et al. 2007), which has not yet been investigated in toadfish. Because there was considerable variation in the gene expression of steroidogenic proteins, we hypothesize that coping styles may result in reactive toadfish experiencing a stimulation in proteins involved in steroidogenesis compared with proactive toadfish (Koolhaas et al. 1999). In addition, there could be effects on protein function that we are not capturing by measuring mRNA expression alone.

Two other mechanisms that were not addressed in the present study are alterations in the MC2R, which binds to ACTH and begins the process of steroidogenesis within the inter-renal cells, and/or alterations with cholesterol biosynthesis, which is the precursor for steroidogenic hormones. In response to PAHs, the MC2R displays reduced sensitivity or internalization (Baig et al. 2002; Li et al. 2013), which would result in a delay (Smith et al. 2017) or impairment of cortisol production and secretion. Furthermore, Reddam et al. (2017) hypothesized that the impairment in cortisol secretion in toadfish after a 24-h exposure to approximately 20 μg L^{-1} PAHs was at the level of the MC2R. Although our attempt to determine changes in MC2R gene expression in the present study was unsuccessful, future studies will investigate its function using an in vitro kidney preparation (Reddam et al. 2017) because evidence leads to this receptor playing a role in impairment. With respect to cholesterol, oil exposure has been shown to enhance teleost cholesterol biosynthetic pathways (Sørhus et al. 2017; Xu et al. 2017) or to reduce cholesterol concentrations in mahi-mahi larval homogenates exposed to 8.3 μg L^{-1} PAHs from *Deepwater Horizon* oil (McGruer et al. 2019). In mammals, cholesterol used for steroidogenesis can be synthesized in the adrenal gland or obtained from circulating lipoproteins (Hu et al. 2010; Bochem et al. 2013), but to our knowledge, the source of cholesterol for fish steroidogenesis has never been directly investigated. Assuming similar sources, liver and plasma total cholesterol concentrations were found to be unchanged after 7 d of PAH exposure and following 7 d of recovery in the same toadfish used in the present study (Alloy et al. 2021, this issue). Kidney cholesterol concentrations were not measured in the present study but perhaps lower cholesterol at 7-d recovery may be responsible for reduced cortisol synthesis.

Overall, our study determined that low, environmentally relevant concentrations of *Deepwater Horizon* oil are not chronically perceived as an environmental stressor in toadfish. Instead, the vertebrate stress response may be impaired through AhR activation. Oil concentrations as low as 0.06 and 2.4 μg L^{-1} PAHs activate the AhR throughout the HPI axis in toadfish, and this activation, measured as CYP1A induction, in the brain and kidney is reduced after a few days of recovery in clean seawater. When toadfish recover for 7 d in clean seawater after an uninterrupted 7-d PAH exposure, they exhibit impaired cortisol release that cannot be explained by changes in StAR, P450scc, or 11βH at the level of mRNA expression. Future research should investigate potential impacts of *Deepwater Horizon* oil exposure on the function of the MC2R and cholesterol availability within the kidney. There appears to be a disconnect between the cortisol secretion impairment and the activation of the AhR, and future research should investigate why organisms would experience a continued impairment after oil exposure and AhR inactivation.

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