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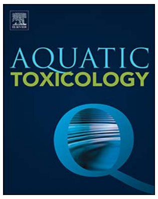

Research paper

The impact of acute PAH exposure on the toadfish glucocorticoid stress response

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ABSTRACT

The objective of the present study was to determine whether the polycyclic aromatic hydrocarbons (PAHs) associated with the Deepwater Horizon (*DWH*) oil spill impacted the stress response of teleost fish. The hypothesis was that intraperitoneal (IP) treatment with PAHs associated with the *DWH* oil spill or waterborne exposure to *DWH* oil high energy water-accommodated fraction (HEWAF) would result in the downregulation of the stress response of Gulf toadfish, *Opsanus beta,* a benthic marine teleost fish that resides in the Gulf of Mexico. *In vivo* plasma cortisol levels and adrenocorticotropic hormone (ACTH)-mediated cortisol secretion by *in vitro* isolated kidney tissue were measured. Toadfish at rest IP-treated with naphthalene had higher plasma cortisol compared to fluorene-treated and control fish; phenanthrene-treated fish tended to have higher plasma cortisol levels that fluorene-treated and controls. When subjected to an additional crowding stress, naphthalene and phenanthrene-treated fish were no longer able to mount a stress response compared to fluorene-treated and control fish, suggesting exhaustion of the stress response. Supporting this *in vivo* data, there tended to be less cortisol released by the kidney *in vitro* from naphthalene and phenanthrene-treated fish in response to ACTH compared to controls. In contrast, toadfish at rest exposed to 3% Slick A HEWAF did not have significantly different plasma cortisol levels compared to controls. But, exposed fish did have significantly less cortisol released by the kidney *in vitro* in response to ACTH. When toadfish were subjected to an additional stress, there were no significant differences in plasma cortisol or ACTH, suggesting the action of a secondary secretagogue to maintain plasma cortisol *in vivo*. Combined, these data suggest that in response to acute PAH exposure, there may be internalization or downregulation of the melanocortin 2 receptor (MC2R) that mediates the action of ACTH.

1. Introduction

On April 20th 2010 there was an explosion on the Deepwater Horizon (*DWH*) offshore drilling rig that ultimately resulted in the release of approximately 3 million barrels of oil into the Gulf of Mexico (DWH NRDA Trustees, 2016). The oil was released under high pressure at depth, which allowed for substantial contact time between the oil and seawater as the oil rose through the water column (Reddy et al., 2012). Chemical dispersants to break down the oil were also sprayed at the wellhead and water surface and, combined, these factors enhanced the dissolution of the toxic polycyclic aromatic hydrocarbons (PAHs) found in oil, resulting in significantly elevated PAH concentrations along the Gulf of Mexico coast (Allan et al., 2012; Hong et al., 2015). For marine vertebrates, exposure to a polluted environment such as this would typically result in an endocrine stress response that promotes survival and helps restore homeostasis. However, there is overwhelming evidence indicating that PAH exposure interferes with the vertebrate stress response.

Most of what we know about the impacts of PAHs on the fish glucocorticoid stress response, which is controlled by the hypothalamic-pituitary-interrenal (HPI) axis, is based on field studies from polluted sites found in temperate environments or laboratory studies on temperate species (*e.g.*, Thomas et al., 1980; Thomas and Rice, 1987; Hontela et al., 1992; Hontela et al., 1995; Hontela, 1998; Girard et al., 1998; Wilson et al., 1998; Aluru and Vijayan, 2004; Aluru and Vijayan, 2006; Kennedy and Farrell, 2005; Oliveira et al., 2007; Tintos et al., 2007; Tintos et al., 2008; Gesto et al., 2008). The majority of these studies have shown a decrease in plasma levels of the stress hormone, cortisol, in response to PAH exposure, through a variety of mechanisms. Examples include pituitary atrophy, as measured by a reduction in the size of pituitary corticotropes, that would lead to a reduction in adrenocorticotropic hormone (ACTH) release and cortisol secretion

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(Hontela et al., 1992), internalization/downregulation of the melanocortin 2 receptor (MC2R) that mediates the response to ACTH at the level of the interrenal tissue (Wilson et al., 1998; Hontela, 1998; Girard et al., 1998) and impacts on multiple sites along the steroidogenic pathway to inhibit glucocorticoid production (Aluru and Vijayan, 2006). Reductions in plasma cortisol are believed to be a consequence of, at least in part, the interaction of certain PAHs with the aryl hydrocarbon receptor (AhR) that leads to changes in the transcription of several genes, including cytochrome P4501A1 (CYP1A) (Hahn, 1998; Till et al., 1999; Billiard et al., 2002; Billiard et al., 2004). However, not all PAHs target the AhR. Furthermore, several studies on fish have shown a significant increase in plasma cortisol in response to PAH exposure that would suggest a stimulation of the HPI axis and not an inhibition of cortisol biosynthesis (Thomas et al., 1980; Thomas and Rice, 1987; Aluru and Vijayan, 2004; Kennedy and Farrell, 2005; Oliveira et al., 2007; Gesto et al., 2008; Tintos et al., 2008).

The impact of the PAHs associated with the *DWH* spill on the stress response of subtropical/tropical vertebrate organisms has not been directly studied. However, evidence suggests that impacts on the stress response are likely. An activation in AhR, as evidenced by the consistent induction of CYP1A, has been measured in residents of the Gulf of Mexico, mahi-mahi (*Coryphaena hippurus*), that have been exposed to *DWH* slick oil (ID: OFS-20100719) high energy water accommodated fraction (HEWAF) (Xu et al., 2016). Whether the activation of AhR and induction of CYP1A in response to *DWH* oil downregulates cortisol steroidogenesis is not known; however, bottlenose dolphins (*Tursiops truncates*) captured from Barataria Bay, Louisiana, an area that received substantial oiling over an extended period of time following the *DWH* spill, had significantly reduced circulating cortisol levels following capture stress compared to dolphins captured from an area with no oil contamination (Schwacke et al., 2014). These data suggest that activation of the AhR may be mediating a disruption in the function of the hypothalamic pituitary adrenal (HPA) axis, analogous to the fish HPI axis.

The objective of the present study was to determine whether PAHs associated with the *DWH* oil spill impacted the glucocorticoid stress response of teleost fish. The hypothesis of the present study was that intraperitoneal (IP) treatment with PAHs associated with the *DWH* oil spill, in particular the 2-ringed PAH, naphthalene, or the 3-ringed PAHs, fluorene and phenanthrene, or waterborne exposure to *DWH* oil will result in the downregulation of the stress response of Gulf toadfish, *Opsanus beta,* a benthic marine teleost fish that resides in the Gulf of Mexico. *In vivo* plasma cortisol levels in response to exposure as well as ACTH-mediated cortisol production and secretion by *in vitro* isolated interrenal tissue from control or exposed toadfish were measured.

2. Materials and methods

2.1. Experimental animals

Gulf toadfish (*Opsanus beta*) were captured by commercial shrimpers using roller trawls in Biscayne Bay, Florida (Florida Fish and Wildlife Conservation Commission Special Activity License #SAL-12-0729-SR). Biscayne Bay borders the City of Miami and is not a pristine environment (*e.g.* Litz et al., 2007); however, it was not impacted by the *DWH* oil spill. After capture, fish were immediately transferred to the laboratory where they were held for at least two weeks before experimentation. Upon arrival in the lab, fish were placed in fresh water for 15 min and then treated with a dose of malachite green (final concentration 0.05 mg L^{-1}) in formalin (15 mg L^{-1}) (AquaVet) to prevent infection by the ciliate, *Cryptocaryon irritans*. Fish were kept in 50 L glass aquaria at a stocking density of approximately 12 g fish·L^{-1} (*i.e.*, 6–20 fish per 50 L depending on their size). Fish were held in aquaria with flowing, aerated seawater at a temperature of 20–22 °C and were fed weekly with squid. All procedures were approved by the University of Miami Institutional Animal Care and Use Committee (IACUC).

2.2. Experimental treatment

Series i: Intraperitoneal (IP) treatment with individual PAHs

To determine the impact of PAH exposure on the ability of fish to elevate circulating cortisol in response to social stress, four groups of toadfish were anesthetized in MS222 (1 g L^{-1}; Finquel) and then injected intraperitoneally using a 18G needle attached to a 100 µL Hamilton syringe with either peanut oil alone (75.7 ± 8.8 g, $N = 25$; 2 µL peanut oil g fish^{-1}) or with the PAHs naphthalene (81.8 ± 12.7 g, $N = 18$), fluorene (99.2 ± 12.5 g, $N = 18$) or phenanthrene (90.1 ± 6.7 g, $N = 17$; 5 µg PAH·2 µL peanut oil^{-1} g fish^{-1}). Following implantation, fish were either left undisturbed in individual 2 L tubs ($N = 9$–10 per treatment) or placed together in crowded conditions (8 fish per 10 L water; ~80 g fish L^{-1}) for 72 h ($N = 8$ per treatment, except for control where $N = 16$). After 72 h, fish were removed from the water and blood samples were drawn immediately *via* caudal puncture using a 23G needle attached to a disposable syringe rinsed with heparinized saline (50 i.u. mL^{-1}; Sigma-Aldrich). Blood from each fish was sampled within a 5 min period; the time period was short enough so that plasma cortisol levels would be indicative of resting levels of fish held in a laboratory and not the result of sampling. Collected samples were centrifuged for 3 mins and the plasma decanted. Plasma from each sample was flash frozen in liquid nitrogen (N_2), and then stored at −80 °C until measured for circulating levels of cortisol. The fish were then over-anesthetized (3 g L^{-1}; MS-222) and the kidney removed from uncrowded fish for *in vitro* analysis of ACTH-stimulated cortisol secretion.

Series ii: Waterborne exposure to DWH *Slick A high energy water accommodated fraction* (*HEWAF*)

The oil (referred to herein as Slick A) used to prepare all high energy water accommodated fractions (HEWAFs) was collected during the *DWH* spill on July 29, 2010 from the hold of barge number CTC02404, which was receiving slick oil from various skimmer vessels (sample ID CTC02404-02), and was subsequently transferred under chain of custody to the University of Miami. Slick A HEWAF was prepared as previously described (Mager et al., 2014). Preparation of Slick A HEWAF occurred less than 24 h before exposure using the same seawater to which toadfish were acclimated. Dilutions of the stock Slick A HEWAF was performed in bulk and each solution was vigorously mixed on a stir plate for at least 5 min.

An initial pilot experiment was done to determine which Slick A HEWAF concentration would result in a change in plasma cortisol levels in resting toadfish within 24 h of exposure. A range of HEWAF dilutions (0.5, 1, 3, 10 and 30%) was prepared. Toadfish ($N = 78$, 33.3 ± 1.4 g) were placed in 1 L glass chambers filled with 0.750 L seawater and bubbled with air through glass Pasteur pipettes and left to acclimate for 36 − 48 h. The sides of chambers were shielded and chambers were covered with shielded glass panes to avoid evaporation and disturbance. At the beginning of the exposure, all seawater was removed from the glass chambers containing acclimated toadfish by small-diameter siphon and then immediately replaced with 0.750 L control seawater ($N = 20$) or 0.5% ($N = 12$), 1% ($N = 12$), 3% ($N = 14$), 10% ($N = 10$) and 30% ($N = 10$) Slick A HEWAF by decanting out of a glass graduated 1 L cylinder. After 24 h exposure, a blood sample was taken *via* caudal puncture with a 23G needle on a disposable syringe rinsed with heparinized saline. The blood sample was centrifuged (16, 000*g* for 5 min) and the plasma retained and frozen immediately in liquid N_2 for later analysis of cortisol. Initial and final measurements of water temperature, pH, dissolved oxygen (dO_2) and salinity were made and did not significantly differ between treatments (Supplementary Table 1).

Based on the findings of the pilot experiment (Supplementary Fig. 1), time-course experiments were then completed using 3% Slick A HEWAF. Toadfish ($N = 80$) were allowed to acclimate in 1 L glass chambers as described above. Seawater was removed from glass chambers by siphon and immediately replaced with either control seawater or 3% Slick A HEWAF. Groups of toadfish were removed from chambers after 1 h (control $N = 6$, HEWAF-exposed $N = 6$), 2 h (control $N = 7$, HEWAF-exposed $N = 7$), 4 h (control $N = 14$, HEWAF-exposed $N = 13$), 8 h (control $N = 8$, HEWAF-exposed $N = 8$), or 24 h (control $N = 6$, HEWAF-exposed $N = 5$) of exposure and a blood sample was taken immediately *via* caudal puncture as described above. The blood sample was centrifuged and the plasma retained and frozen immediately in liquid N_2 for later analysis of cortisol. The fish were then over-anesthetized (3 g L^{-1}; MS-222), the peritoneal cavity opened and the fish sexed by assessing the gonad visually (68–70% success rate), and the kidney removed for *in vitro* analysis of ACTH-stimulated cortisol secretion. Measurements of water temperature, pH, dO_2 and salinity were made initially and then throughout the exposure period and did not significantly differ between treatments (Supplementary Table 2). Control seawater and Slick A HEWAF samples (0.250 L) were collected for chemical analysis at each time point for measurement of Σ PAH concentration (Supplementary Fig. 2).

A final group of toadfish ($N = 32$) was exposed to the additional environmental challenge of air exposure for 15 min after exposure to either control or 3% Slick A HEWAF conditions for up to 8 h. Fish were acclimated in their glass chambers as described above, the water was changed and fish were exposed for 1, 2, 4 or 8 h to control or 3% Slick A HEWAF (control $N = 4$, HEWAF-exposed $N = 4$ for each time point). Fish were then removed from their individual chambers with a net and were left sitting air exposed for 15 min. Immediately after air exposure, a blood sample was taken *via* caudal puncture with a 23G needle on a 1 mL syringe rinsed with EDTA (15% EDTA; Sigma-Aldrich), centrifuged and the plasma retained and frozen immediately in liquid N_2 for later analysis of both cortisol and ACTH. Measurements of water temperature, pH, dO_2 and salinity were made initially and then throughout the exposure period and did not significantly differ between treatments (Supplementary Table 3).

2.3. Experimental preparations

2.3.1. In vitro isolated kidney preparation

Whole kidneys were collected from over-anesthetized uncrowded fish IP injected with individual PAHs from *Series i* and both control and 3% Slick A HEWAF-exposed fish from *Series ii*. Individual kidneys were dissected from fish and immediately placed in 1 mL of ice cold Liebovitz's L-15 media with L-glutamine (L-15; Cellgro by MediaTech, Inc.) and kept on ice as previously described (Medeiros and McDonald, 2012). The entire kidney (containing the interrenal cells that secrete cortisol as well as other cell types such as chromaffin and renal cells) was weighed and cut into 1 mm^3 pieces. The kidney pieces were then transferred to one of the wells in a 24-well sterile culture plate with 1 mL of fresh L-15 media, covered with tinfoil, and placed on an orbital plate rotator (Lab-Line) set at approximately 125 rpm. Tissue pieces were then pre-incubated at room temperature (approximately 25 °C) for 2 h in 1 mL L-15 media, with bath changes at 1 h and 1.5 h. After 2 h, a 35 μL sample of the pre-incubation media was taken to verify that the tissue was no longer spontaneously secreting cortisol. After the sample was taken, the tissue was washed with 1 mL L-15 media for fifteen minutes, the L-15 media removed and replaced with L-15 media containing ACTH so that ACTH concentrations were either 3.3×10^{-7} M or 3.3×10^{-6} M. With this *in vitro* preparation, any cortisol left in the interrenal tissue upon dissection diffuses out during the 2 h pre-incubation period and so only new cortisol that is produced in response to ACTH is secreted into the bath. A 35 μL sample was taken from every well at t = 0, 0.5, 1, 2 h and frozen immediately in liquid N_2 before being stored at −80 °C to be analyzed for cortisol.

2.4. Analytical techniques

Plasma cortisol was quantified using the MP Biomedical cortisol radio-immuno assay (RIA) kit, with the cortisol standards diluted by half so that protein concentrations were within the range measured in toadfish. Plasma ACTH was quantified using the MP Biomedical ACTH RIA kit. Temperature and dO_2 were measured using a ProODO hand held optical dO_2 probe and meter (YSI). pH was measured using a PHM201 m (Radiometer) and a combination glass electrode. Salinity was measured using a refractometer and total ammonia determined using the diacetyl-monoxime method (Ivancic and Degobbis, 1984). To measure Σ PAH concentration, amber sample jars (250 mL) were filled to capacity, labelled, stored at 4 °C and shipped overnight on ice to ALS Environmental (Kelso, WA) for analysis by gas chromatography/mass spectrometry − selective ion monitoring (GC/MS-SIM; based on EPA method 8270D). Initial PAH samples were collected from bulk dilutions and final (1, 2, 4, 8, 24 h) samples were composites of approximately equal volumes collected from replicates. Only initial samples were collected for control treatments. Reported Σ PAH values represent the sum of 50 select PAH analytes (Supplementary Table 4).

2.5. Statistics

Data are reported as means ± S.E.M. and N = number of fish. Statistical significance was set at $P < 0.05$ and analysis was completed using the statistical package found in Sigma Plot 11.0 (Systat Software, Inc.). In all cases, normality of the data was checked with a Shapiro Wilk test. If not normally distributed, the data were either log-, ln- or square root-transformed. For Fig. 1, the data were log-transformed and a two-way ANOVA with PAH and stressor type as the main factors followed by a Holm-Sidak multiple comparisons test was used. For Fig. 2, a one-way ANOVA with PAH as the main factor was used. For Fig. 3, the data were ln-transformed and a two-way ANOVA with exposure regime and time (Fig. 3A) or sex (Fig. 3B) as the main factors followed by a Holm-Sidak multiple comparisons test was used. For Figs. 4 and 5, the data were square root-transformed and a two-way ANOVA with exposure regime plus ACTH dose (Fig. 4), time (Fig. 5A) or sex (Fig. 5B) as the main factors followed by a Holm-Sidak multiple comparisons test was used. A Student's *t*-test was used to compare the differences between two means (Fig. 6). For Supplementary Fig. 1, the data were not normally distributed and did not become so upon transformation, so a one-way ANOVA based on ranks with PAH concentration as the main factor followed by a Dunn's multiple comparisons test was used to determine if the data were statistically significant.

3. Results

(i) Intraperitoneal (IP) treatment with individual PAHs

Plasma cortisol levels of toadfish at rest treated with naphthalene were 3-fold higher than control and fluorene-treated fish, with fish treated with phenanthrene showing a similar, although not statistically significant, elevation (Fig. 1A). Once subjected to the additional stress of crowding, plasma cortisol levels in control fish were on average 4-times higher in toadfish subjected to the additional crowding stress compared to toadfish at rest (Fig. 1A *cf.* Fig. 1B). Similarly, in the fluorene-treated fish plasma cortisol levels were on average 4.5-times higher in the crowded fish than toadfish at rest (Fig. 1A *cf.* Fig. 1B). In contrast to toadfish at rest, plasma cortisol levels of crowded toadfish treated with phenanthrene was significantly lower than crowded control and fluorene-treated fish, with fish treated with napthalene showing a similar, although not statistically significant, decrease (Fig. 1B), with values that were over 50% lower than those measured at rest (Fig. 1A *cf.* Fig. 1B). *In vitro* isolated kidneys showed measurable cortisol secretion in response to 3.3×10^{-7} M ACTH (Fig. 2). While not statistically significant, the differences in cortisol secretion from interrenal cells from control and PAH-treated fish reflect that of the *in vivo* plasma cortisol levels measured in fish in response to the additional crowding stress (Fig. 2 *cf.* Fig. 1B). In particular, the mean cortisol secreted by the interrenal preparations from fish treated with naphthalene and phenanthrene tended to be only half the mean levels of

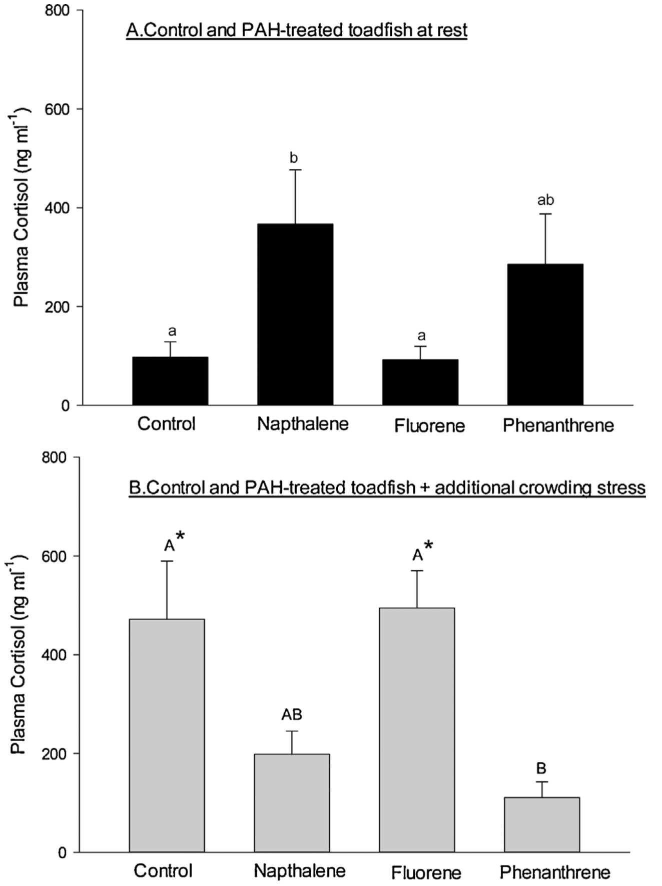

Fig. 1. (A) *In vivo* plasma cortisol concentrations in toadfish injected with 2 µL peanut oil·g fish^{-1} (vehicle control) or 5 µg PAH·2 µL peanut oil^{-1} g fish^{-1} at rest (uncrowded conditions, $N = 9–10$ for all treatments) or (B) subjected to an additional crowding stress ($N = 16$ for control, $N = 8$ for PAH-treated). Values are means + /− S.E.M; $P < 0.05$, different letters denote a statistically significant difference between PAH treatments within resting or crowded fish, asterisks denote a significant difference between resting and crowded fish within PAH treatment. The effect of crowding depended on which PAH was present, resulting in a statistically significant interaction between the level of crowding and PAH ($P < 0.001$).

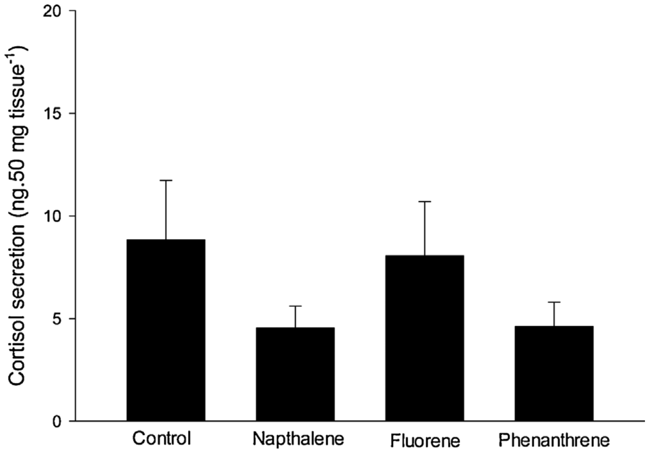

Fig. 2. *In vitro* ACTH-stimulated cortisol release from isolated toadfish kidneys removed from uncrowded fish injected with peanut oil (2 µL peanut oil·g fish^{-1}; vehicle control), naphthalene, fluorene or phenanthrene (5 µg PAH·2 µL peanut oil^{-1} g fish^{-1}; N = 9–10 for all treatments). Values are means + /− S.E.M.

interrenal tissue from the control and fluorene-treated fish (Fig. 2).

(ii) Waterborne exposure to DWH *Slick A HEWAF*

While not statistically significant, exposure to 3–10% Slick A HEWAF for 24 h tended to have the maximal inhibitory effect on plasma cortisol levels compared to those of control fish (Supplementary Fig. 1). Thus, the 3% Slick A HEWAF was used for further exposures as it would be closer to environmentally realistic PAH concentrations. Indeed, Σ PAH concentrations in the 3% Slick A HEWAF preparation decreased exponentially over the 24 h exposure period and measured concentrations ranged from 72.4 ± 2.3 ($N = 11$) µg L^{-1} to

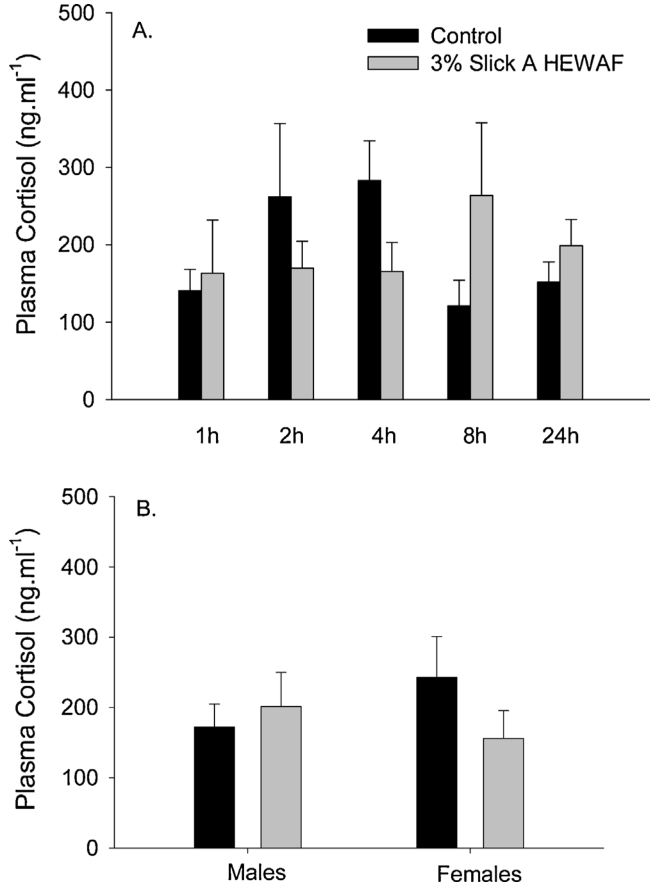

Fig. 3. (A) *In vivo* plasma cortisol concentrations in control or 3% Slick A HEWAF at t = 1 h (control $N = 6$; HEWAF $N = 6$), 2 h (control $N = 7$; HEWAF $N = 7$), 4 h (control $N = 14$; HEWAF $N = 13$), 8 h (control $N = 8$; HEWAF $N = 8$) and 24 h (control $N = 6$; HEWAF $N = 5$). (B) *In vivo* plasma cortisol concentrations from all time-points in response to control ($N = 17$ males, $N = 12$ females) or 3% Slick A HEWAF ($N = 17$ males, $N = 9$ females) in different sexes. Values are means + /− S.E.M.

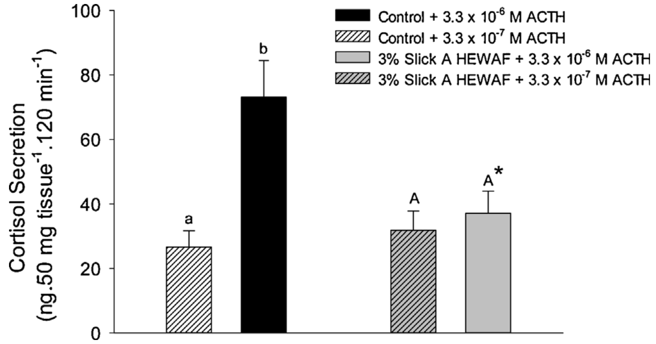

Fig. 4. *In vitro* ACTH-stimulated cortisol release from isolated toadfish kidneys from toadfish exposed to control seawater for up to 8 h and then stimulated with either a low (3.3×10^{-7} M; $N = 29$) or a high (3.3×10^{-6} M; $N = 18$) dose of ACTH or exposed to 3% Slick A HEWAF for up to 8 h and then stimulated with either a low ($N = 24$) or high ($N = 20$) dose of ACTH. Values are means + /− S.E.M; $P < 0.05$, different letters denote a statistically significant difference between exposure regime within an ACTH dose, asterisks denote a significant difference between ACTH dose within an exposure regime.

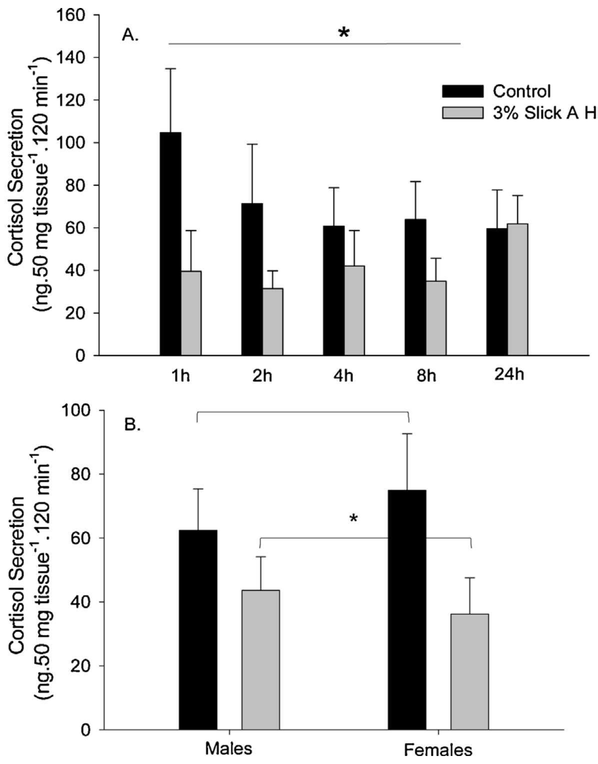

Fig. 5. (A) *In vitro* ACTH-stimulated cortisol release from isolated toadfish kidneys from toadfish exposed to control or 3% Slick A HEWAF for t = 1 h (control $N = 6$; HEWAF $N = 6$), 2 h (control $N = 7$; HEWAF $N = 5$), 4 h (control $N = 9$; HEWAF $N = 6$), 8 h (control $N = 7$; HEWAF $N = 7$) and 24 h (control $N = 6$; HEWAF $N = 5$). (B) *In vitro* ACTH-stimulated cortisol release from isolated toadfish kidneys from toadfish of different sexes exposed to control ($N = 18$ males, $N = 12$ females) and 3% Slick A HEWAF ($N = 15$ males, $N = 8$ females) at all time-points. A dose of 3.3×10^{-6} M ACTH was used. Values are means +/− S.E.M.* $P < 0.05$ significantly different between treatments.

10.2 ± 2.5 ($N = 8$) μg L^{-1} (Supplementary Fig. 2). Using the equation of the fitted line ($y = 14.6 + 56.2e^{-0.57x}$, $r^2 = 0.96$, $P = 0.007$), this resulted in a mean Σ PAH concentration over the 24 h exposure period of 19.8 ± 2.6 μg L^{-1} and 28.9 ± 6.2 μg L^{-1} over the first 8 h of exposure. In comparison, control seawater had a Σ PAH concentration of 0.05 ± 0.02 (N = 11) μg L^{-1}.

No significant differences in plasma cortisol were measured between control and 3% Slick A HEWAF-exposed fish at any time point *in vivo* (Fig. 3A). Furthermore, the impact of sex had no effect on plasma cortisol levels either under control conditions or after exposure to 3% Slick A HEWAF (Fig. 3B). However, *in vitro* kidney isolated from control fish showed a dose-dependent stimulation in cortisol secretion in response to ACTH, with 2.7-fold higher cortisol secretion in response to ACTH at 3.3×10^{-6} M compared to 3.3×10^{-7} M (Fig. 4). This dose response to ACTH was not measured in fish exposed to 3% Slick A HEWAF (Fig. 4). Furthermore, at the higher ACTH dose (3.3×10^{-6} M) there was a significant decrease in cortisol secretion between controls and 3% Slick A HEWAF-exposed fish (Figs. 4 and 5), with no measureable differences detected between controls and 3% Slick A HEWAF-expose fish in response to 3.3×10^{-7} M ACTH (Fig. 4). Breaking this down by exposure duration, there was an impairment of ACTH-stimulated cortisol secretion with the 3.3×10^{-6} M ACTH dose in fish that had been exposed to 3% Slick A HEWAF for at least 8 h compared to controls (Fig. 5A) with the sensitivity to ACTH appearing to return after 24 h of exposure (Fig. 5A) as PAH levels in the water dissipated (Supplementary Fig. 2). There was no impact of sex on the sensitivity of the *in vitro* kidney to ACTH-mediated stimulation of cortisol production (Fig. 5B), although the kidneys of both males and females exposed to 3% Slick A HEWAF secreted less cortisol in response to ACTH than controls. To determine whether the insensitivity to ACTH measured *in vitro* persisted *in vivo*, a subset of fish was exposed to the additional environmental challenge of air exposure to stimulate endogenous ACTH release after up to 8 h of either control or 3% Slick A HEWAF exposure. However, no significant difference in plasma cortisol (Fig. 6A) or plasma ACTH (Fig. 6B) secretion was measured between controls or 3% Slick A HEWAF-exposed fish.

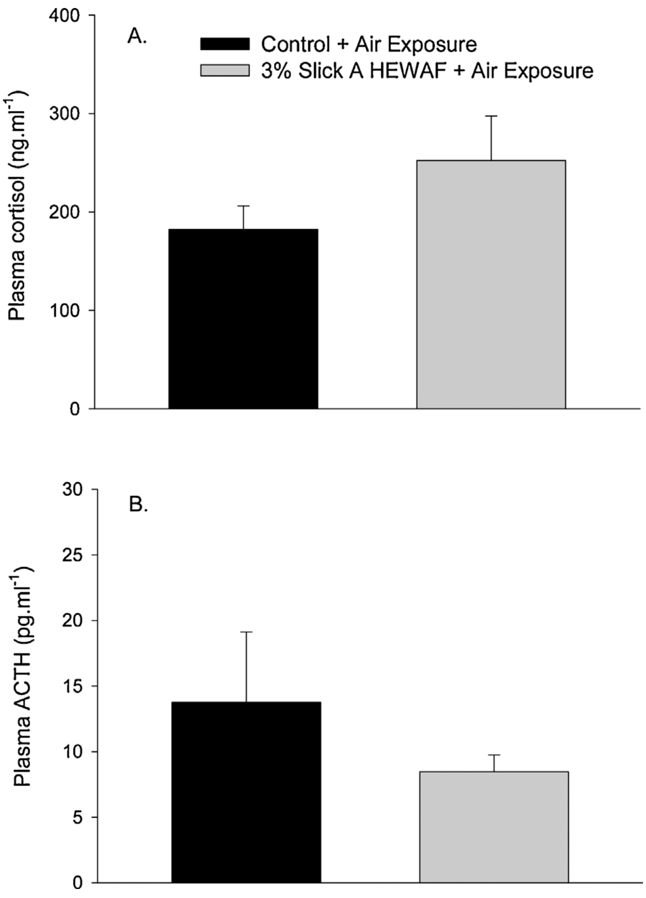

Fig. 6. (A) *In vivo* plasma cortisol concentrations in response to up to 8 h of control seawater ($N = 16$) or 3% Slick A HEWAF ($N = 16$) exposure with a subsequent 15 min air exposure. (B) *In vivo* plasma ACTH concentrations in response to up to 8 h of control ($N = 8$) or 3% Slick A HEWAF ($N = 7$) exposure with a subsequent 15 min air exposure. Values are means + / − S.E.M.

4. Discussion

(i) Intraperitoneal (IP) treatment with individual PAHs

The perception of stress is associated with an initial alarm stage followed by stages of resistance and exhaustion (Selye, 1973; reviewed by Mommsen et al., 1999; Barton, 2002). The alarm stage is characterized by a sharp increase in circulating cortisol levels, which in the present study occurred in resting toadfish treated with either naphthalene, and to a certain extent, phenanthrene, and suggests the perception of a stressor − whether it be *via* sensory stimulation (*e.g.*, olfaction or gustation) or pain perception is not known. Our findings are consistent with previous work that has shown that acute exposure to naphthalene, phenanthrene and PAH mixtures can result in an increase in plasma cortisol (Aluru and Vijayan, 2004; Kennedy and Farrell, 2005; Oliveira et al., 2007; Gesto et al., 2008). However, they contradict a study in freshwater rainbow trout (*Oncorhynchus mykiss*) that demonstrated a decrease in plasma cortisol in response to 2–10-fold higher levels of IP-injected naphthalene (Tintos et al., 2007). Interestingly, toadfish in the present study responded to fluorene differently than to naphthalene or phenanthrene, with a response that was not significantly different from controls. All three of these PAHs have been reported to be weak or inactive at the fish AhR (Billiard et al., 2002; Billiard et al., 2004; Barron et al., 2004). While there are many conflicting studies, recent work on Nile tilapia (*Oreochromis niloticus*) IP-treated with naphthalene or phenanthrene at the same dose as our study showed no induction of CYP1A (measured as EROD activity) either 24 or 72 h post-treatment (Pathiratne and Hemachandra, 2010). The different response to fluorene suggests that it may be either less potent or have a different mechanism of action than naphthalene and phenanthrene. For example, fluorene may have only a limited capacity to stimulate sensory organs and, thus, elicit a stress response.

If the environmental conditions are stressful enough, an animal can reach a stage of exhaustion, which is characterized by reduced cortisol levels and an inability to further mount a stress response (Selye, 1973). Physiologically, a state of exhaustion can be achieved by internalization/downregulation of the melanocortin 2 receptor (MC2R), which mediates the response of the interrenal tissue to ACTH, due to repeated stimulation (Baig et al., 2002; Li et al., 2013), or, in the more chronic cases, pituitary atrophy which would result in reduced ACTH release by the pituitary. Both of these responses have been measured in response to PAH exposure. For example, rainbow trout exposed to PAHs show a reduction in interrenal ACTH sensitivity when measured *in vitro* (Wilson et al., 1998; Hontela, 1998; Girard et al., 1998; Aluru and Vijayan, 2006), without a change in the enzymes involved in cortisol steroidogenesis (Aluru and Vijayan, 2004). On a more long-term basis, yellow perch (*Perca flavescens*) and northern pike (*Esox lucius*) that have lived their life in an environment contaminated with PAHs showed pituitary atrophy and a significant reduction in the ability to increase plasma cortisol levels in response to the stress of capture (Hontela et al., 1992). In the present study, naphthalene and phenanthrene-treated toadfish exposed to the additional crowding stress might have been pushed to the exhaustion stage, perhaps *via* internalization/downregulation of MC2R, explaining their lower cortisol levels compared to control and fluorene-treated fish that responded to crowding with an elevation in plasma cortisol typically measured in crowded toadfish (reviewed by Wood et al., 2003). Considering the brief exposure duration, pituitary atrophy is a less likely explanation for the observed HPI axis exhaustion. This is similar to findings in rainbow trout treated with the 4-ringed PAH-like compound, β-naphthoflavone (BNF), that had higher plasma cortisol levels compared to untreated fish, but when subjected to an additional stressor, cortisol levels in BNF-treated fish were lower than controls (Wilson et al., 1998; Aluru and Vijayan, 2004; Aluru and Vijayan, 2006; Gesto et al., 2008). It is possible that the reduced plasma cortisol measured in napthalene- and phenanthrene-treated fish exposed to crowding stress could be due to inhibition at the level of the steroidogenic pathway. Under normal circumstances, ACTH released from the pituitary stimulates steroidogenic acute regulatory (StAR) protein and 11β-hydroxylase which leads to an increase in cortisol production and secretion (Lehoux et al., 1998; Aluru and Vijayan, 2006). The activation of AhR and induction of CYP1A that occurs in response to PAH exposure has been shown to downregulate StAR and cytochrome P450 side chain cleavage (P450scc) mRNA expression with no change in 11β–hydroxylase (Aluru and Vijayan, 2006). However, a reduction in cortisol production due to downregulation of steroidogenesis was not evident in resting toadfish treated with naphthalene or phenanthrene, suggesting that exposure to these PAHs for 72 h likely did not affect cortisol biosynthesis directly.

Perhaps lending more clarity to the *in vivo* findings, *in vitro* cortisol release by kidney tissue isolated from naphthalene- and phenanthrene-treated fish at rest in response to 3.3×10^{-7} M ACTH tended to be lower compared to control and fluorene-treated fish, but the attenuation was not statistically significant. The *in vitro* preparation eliminates the potential role that pituitary atrophy may play in reducing the stress response, since the ACTH concentration in which the preparations are bathed is consistent between treatments. That the statistically significant difference between the treatment groups measured *in vivo* was not measured *in vitro* suggests that reduced ACTH release by the pituitary could play at least a partial role in the reduced stress response of naphthalene and phenanthrene-treated fish *in vivo*; plasma ACTH levels were not measured in these fish to ascertain whether their pituitary function was indeed limited. However, what may be more likely, especially considering the rather acute time frame of exposure, is an internalization/downregulation of MC2R due to repeated stimulation (Baig et al., 2002; Li et al., 2013) that may manifest itself as a decrease in binding maximum (B_{max}) and be only evident when the tissue is exposed to high ACTH concentrations. It could be that the ACTH concentrations (3.3×10^{-7} M) used to induce cortisol secretion *in vitro* may not have been high enough for significant differences between treatments to be measured (see below in *Series ii*).

(ii) Waterborne exposure to DWH *Slick A HEWAF*

Our investigation on the impacts of waterborne exposure of *DWH* Slick A HEWAF, a complex mixture of many different PAHs including naphthalene, phenanthrene and fluorene (Forth et al., 2017), led to findings that could be predicted by the *Series i* individual PAH work as well as lent some insight on *Series i* results. Waterborne exposure to 3% Slick A HEWAF did not result in significant differences in plasma cortisol concentration compared to control fish. This was despite the fact that Slick A HEWAF contains both naphthalene and phenanthrene that were shown to stimulate the HPI axis in *Series i* fish at rest. The differences between *Series i* and *Series ii* may have been due to the fact that the concentration of Σ PAHs in the waterborne exposure (20 μg L^{-1}) were significantly lower than what fish were exposed to in *Series i* (5 mg kg^{-1} *via* IP injection). Specifically, naphthalene, phenanthrene, and any other PAH that might have stimulated the HPI axis, made up a small proportion of the Σ PAH concentration (in our 3% HEWAF preparations, 0–0.3% of the Σ50 PAHs was naphthalene, 2.2% was phenanthrene and 0.6% was fluorene; Supplementary Table 5). The waterborne exposure duration was also substantially shorter compared to IP treatment of *Series i* (1–24 h *cf.* 72 h). Lastly, it is possible that the response to Slick A HEWAF is dictated by only a proportion of the PAHs found within the complex mixture. Supporting this idea, the activation of AhR, as evidenced by the consistent induction of CYP1A, has been measured in mahi-mahi that have been exposed to *DWH* slick oil (ID: OFS-20100719) HEWAF at Σ PAH concentrations that were slightly lower than the average Σ PAH concentration of the present study (~12 μg L^{-1}; Xu et al., 2016 *cf.* 20 μg L^{-1}). Therefore, the AhR-mediated response on the HPI axis, which past studies have shown is downregulation in steroidogenesis (Aluru and Vijayan, 2006), may be competing with hyperactivation of the stress axis followed by an internalization/downregulation of the MC2R, which may not be AhR mediated (Aluru and Vijayan, 2004).

Removing kidneys from fish that had been exposed to Slick A HEWAF and stimulating them with the same concentration of ACTH (3.3×10^{-7} M) used in the individual PAH study of *Series i* resulted in no difference in cortisol secretion compared to control fish. It was not until kidneys were exposed to 10-fold higher ACTH concentrations (3.3×10^{-6} M) that a significant reduction in cortisol secretion was measured. This range of ACTH concentrations is within the effective range established for this toadfish *in vitro* kidney preparation in a previous study (Medeiros and McDonald, 2012). That differences in cortisol secretion were only measured in kidney exposed to the higher ACTH concentration suggest that the MC2R in Slick A HEWAF-exposed fish may be internalized resulting in reduced cortisol secretion capacity, limiting the maximal ACTH response. If there was an impairment in cortisol steroidogenesis in response to Slick A HEWAF exposure, cortisol secretion should be reduced in Slick A HEWAF-exposed fish in response to either ACTH concentration. Interestingly, the sensitivity to ACTH appeared to return when water Σ PAH concentrations were approximately 10 μg L^{-1} (measured at 24 h), suggesting a potential recovery as Σ PAH concentrations went below 15 μg L^{-1} (as measured at 8 h) and a threshold for an acute response at Σ PAH concentrations > 15 μg L^{-1}.

Interestingly, plasma cortisol levels *in vivo* were not only maintained in these fish but, for both controls and Slick A HEWAF-exposed, were slightly higher that would be expected of toadfish held at rest in the laboratory (*c.f. Series i* control fish held at rest) − likely due to being held in glass chambers and/or the water change associated with the start of the experiment. This was despite an impairment in cortisol secretion. The apparent inconsistency between *in vitro* and *in vivo* results could be explained by *in vivo* toadfish at rest not being stressed enough, *i.e.*, toadfish at rest did not have high enough levels of endogenous ACTH for a difference in cortisol secretion to be detected, much like the response of *in vitro* interrenal tissue to lower ACTH concentrations. If that was the case, elevating the stress response *in vivo*, similar to our *Series i* fish that were exposed to the additional crowding stress, might result in reduced plasma cortisol levels in Slick A HEWAF-exposed fish *in vivo*. However, when control and Slick A HEWAF-exposed fish toadfish were exposed to the secondary stressor of a 15-min air exposure, both groups of fish had similar plasma ACTH, ruling out both compensation by secreting more ACTH and exhaustion at the level of the pituitary, and similar plasma cortisol levels, ruling out impaired steroidogenesis. Knowing that there is indeed problems with cortisol secretion at the level of the interrenal tissue does suggest that another type of compensation may be involved *in vivo*. For example, cortisol production and secretion is also stimulated by other secretagogues such as angiotensin II (Arnold-Reed and Balment, 1994), atrial natriuretic peptide (ANP; Arnold-Reed and Balment, 1991), and serotonin (5-HT; Medeiros and McDonald, 2012). These secretagogues could be upregulated in Slick A HEWAF-exposed fish to maintain the stress response *in vivo*.

5. Conclusions

In conclusion, our findings demonstrate that the glucocorticoid stress response of Gulf toadfish may be impaired in response to the oil released during the 2010 *DWH* oil spill. Furthermore, the present study suggests that there may be a downregulation in the MC2R, most likely an internalization/downregulation of the receptor, in response to relatively short-term exposure to naphthalene, phenanthrene and 3% Slick A HEWAF. Whether the findings of the present study translate to the long-term exposure conditions experienced by Barataria Bay dolphins or other aquatic organisms found in contaminated environments cannot be deduced. Furthermore, additional impacts on the stress response, for example a decrease in steroidogenesis or pituitary atrophy, in response to chronic exposures cannot be ruled out. Future work should investigate whether the steroidogenic pathway is impaired when organisms are exposed over the long-term and whether there is a reduction in MC2R mRNA expression or binding kinetics in response to PAH- or *DWH* oil exposure. Future work should also look into compensatory mechanisms that may be at play, *i.e.*, 5-HT or other potential secretagogues that could allow the HPI axis and cortisol secretion to continue unimpeded *in vivo* and the costs associated with that compensation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2017.08.014.

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