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.
(Hontela et al., 1992), internalization/downregulation of the melano-
cortin 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 stereoidogenic
pathway to inhibit glucocorticoid production (Aluru and Vijayan,
2006). Reductions in plasma cortisol are believed to be a consequ-
ence of, at least in part, the interaction of certain PAHs with the aryl hy-
drocarbon 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 ex-
posure 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 di-
rectly 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
truncatus) 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 activ-
ation 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 telost 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 resident 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 shrim-
pers 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 experi-
mentation. Upon arrival in the lab, fish were placed in fresh water
for 15 min and then treated with a dose of malachite green (final con-
centration 0.05 mg L$^{-1}$) in formalin (15 mg L$^{-1}$) (AquaVent) 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 in-
jected 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 phe-
nanthrene (90.1 ± 6.7 g, N = 17; 5 μg PAH2μL peanut oil$^{-1}$ g
fish$^{-1}$). Following implantation, fish were either left undisturbed in in-
dividual 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 im-
mediately via caudal puncture using a 23G needle attached to a dis-
posable 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 (N2), 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 ac-
commodated 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 cus-
tody to the University of Miami. Slick A HEWAF was prepared as pre-
viously 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 dis-
turbance. At the beginning of the exposure, all seawater was removed
from the glass chambers containing acclimated toadfish by small-dia-
meter 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) and then in-

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

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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 \( \Sigma \) 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 \( \mu \)L 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 \( \times \) \( 10^{-7} \) M or 3.3 \( \times \) \( 10^{-8} \) 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 \( \mu \)L 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 (Ivanic and Degobbis, 1984). To measure \( \Sigma \) 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 \( \Sigma \) 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-, In- 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 In-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. For Figs. 6 and 7, the data were 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 naphthalene 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 \( \times \) \( 10^{-7} \) 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...
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
ACTH at 3.3 × 10^−6 M resulted in a mean cortisol secretion of 0.05 ± 0.02 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 μg L^−1 (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 vitro (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 measurable differences detected between controls and 3% Slick A HEWAF-exposed 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.

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 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/down-regulation of MC2R, explaining their lower cortisol levels compared to controls, and any other PAH that might have stimulated the HPI axis, made up a small proportion of the ∑PAHs in mahi-mahi that have been exposed to DWH slick oil (ID: OOS-20100719) HEWAF at a PAH concentration of the present study (less than 15 μgL−1) used to induce cortisol secretion in vitro (as measured at 24 h). Interestingly, the AhR-mediated response on the HPI axis, which past studies have shown is downregulated 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).

(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 PAHs as well as lent some insight on Series ii 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 ∑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 ∑PAH concentration in our 3% HEWAF preparations, 0–0.3% of the 250 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: OOS-20100719) HEWAF at a PAH concentration that were slightly lower than the average ∑PAH concentration of the present study (~12 μgL−1; Xu et al., 2016 cf. 20 μg L−1). Therefore, the AhR-mediated response on the HPI axis, which past studies have shown to be downregulated 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 ∑PAH concentrations were approximately 10 μg L−1 (measured at 24 h), suggesting a potential recovery as ∑PAH concentrations went below 15 μg L−1 (as measured at 8 h) and a threshold for an acute response at ∑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 DW4H 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 3β 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 DW4H 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.

References


