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OXIDATIVE STRESS RESPONSES OF GULF KILLIFISH EXPOSED TO HYDROCARBONS FROM THE DEEPWATER HORIZON OIL SPILL: POTENTIAL IMPLICATIONS FOR AQUATIC FOOD RESOURCES

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Abstract: Ecosystem effects of polycyclic aromatic hydrocarbons (PAHs) remain under investigation following the Gulf of Mexico Deepwater Horizon oil spill. *Fundulus grandis*, an established indicator of aquatic ecosystem health, was investigated because this species shares genes and biochemical pathways with higher trophic–level fish and plays an important role in the gulf food chain. Oxidative stress responses including hepatic cytochrome P4501A (CYP1A) and serum antioxidant capacity were evaluated in fish exposed to PAHs. Fish were exposed to water-accommodated fractions (WAFs) of crude oil (7.0 ± 0.10 mg/L C6-C28) after which solutions were diluted below the level of detection over 8 h using 15 ppt aerated artificial seawater. Before euthanasia, fish remained in aquaria for 12 h, 24 h, or 48 h. Three replicate experiments were conducted at each time point using unexposed fish as experimental controls. Significant differences ($p < 0.05$) in CYP1A induction were observed in exposed versus control fish at 24 h. Expression of CYP1A increased by 25%, 66%, and 23% in exposed fish at 12 h, 24 h, and 48 h, respectively. Significant increases were observed in antioxidant capacity of nonenzymatic antioxidants in exposed versus control fish at each time point. Given the activity of CYP1A, radicals formed during PAH detoxification likely resulted in increased oxidant load requiring elevated antioxidant defenses. Research is needed to determine the duration of oxidative stress responses considering the potential for lipid oxidation in exposed fish or species feeding on exposed fish. *Environ Toxicol Chem* 2014;33:370–374. © 2013 SETAC

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INTRODUCTION

Impact assessment of the 2010 Deepwater Horizon oil spill in the Gulf of Mexico (USA) remains on-going. As a result of the explosion at the Macondo well, millions of gallons of crude oil were released into the gulf and distributed along the shores of Louisiana, Mississippi, Alabama, and Florida (USA). Although no traces of visible oil remain, sublethal effects of exposure to polycyclic aromatic hydrocarbons (PAHs) have been reported in numerous species ranging throughout the water column, including aquatic food resources such as red snapper and king mackerel, among others [1,2]. For this reason, research continues not only to assess the fate and effects of the spill on aquatic resources throughout the food chain but also to explore fish biomarker responses induced by PAHs to assist in early detection of exposure in the case of future spills.

Aquatic organisms detoxify PAHs and other xenobiotics by phase I metabolism. During this process, which includes the activity of cytochrome P4501A (CYP1A), reactive oxygen-derived radical species (ROS) are produced as byproducts of xenobiotic metabolism. Thus, exposed organisms encounter toxicity-induced impact from the hydrocarbons themselves and from the reactive byproducts generated during detoxification. If the rate of ROS generation surpasses the rate of decomposition, these highly reactive radicals can impact the integrity of lipids, proteins, and nucleic acids, resulting in oxidative stress induction, alterations in antioxidant defenses, and lipid oxidation of unsaturated fatty acids including beneficial omega-3 fatty acids [3,4]. Given the potential for homeostatic disruption, it is imperative to identify biomarker responses to PAH exposure that would allow for early detection and monitoring of fisheries among species that share genes and biochemical pathways with higher trophic level fish as well as in species that play an important role in the aquatic food chain. Hence, fish species meeting both of these criteria represent the ideal model for evaluating the present and future impact of PAH exposure on aquatic resources.

Within the Gulf of Mexico and the Atlantic Ocean, *Fundulus* species are emerging as a model indicator of the health of aquatic ecosystems [5,6]. In addition, according to the US National Institutes of Health, biomarker studies in fish models including *Fundulus* have been deemed critical to understanding the impact of PAH exposure in humans because of similarities in physiological responses to exposure [7]. Within the food chain, *Fundulus grandis*, also known as gulf killifish, serve as feeder fish for trout, redfish, flounder, and other species commonly consumed by humans along the Gulf of Mexico and around the United States.

Based on the unique characteristics of this species, research investigating gulf killifish has shed light on the biological effects of PAH release in the Gulf of Mexico. For example, since the Deepwater Horizon oil spill, the period of acute impact has passed; however, 1 yr from the initial landfall of oil, the effects on killifish harvested from oiled sites along the Gulf Coast indicate significant stress responses including upregulation of CYP1A in gill, liver, intestine, and kidney compared with fish collected from nonoiled reference or control sites [5]. In addition, in experimental laboratory exposures, gulf killifish embryos exposed to field-collected PAH-containing sediments also exhibited significant increases in CYP1A expression along

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with developmental abnormalities compared with embryos exposed to sediments collected from nonoiled reference or control sites. As with any field study in the marine environment, it can be and has been argued that the induction of stress responses is not solely related to PAH exposure. Thus, the research should be extended to include controlled PAH exposures of gulf killifish to understand the impact of exposure on this model species. For this reason, the present study evaluated the acute effects of PAH on gulf killifish exposed to crude oil from the Deepwater Horizon oil spill for the purpose of assessing antioxidant capacity and CYP1A induction as oxidative stress responses to PAH exposure in a prominent species within the Gulf of Mexico aquatic food chain.

MATERIALS AND METHODS

Sample animals

Adult *F. grandis* (10–12 cm total length and 12–20 g total body wt) were obtained from a private company (Aquatic Innovations, LLC., Auburn, AL, USA) where fish were raised in inland saline ponds. During acclimation and prior to PAH exposure, fish were housed in recirculating 15-ppt saltwater systems maintained at 24 °C to 26 °C, aerated with airstones and regenerative blowers (Sweetwater; Pentair). During the holding period, water quality—including ammonia, pH, nitrite, and salinity—was monitored daily and fish were fed daily at a rate of approximately 2% body weight with a commercially available fish food (AquaMax Fingerling Starter 300; Purina Aquatics). All procedures involving these animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University (Auburn, AL, USA) in accordance with the guidelines of the IACUC of the US National Institutes of Health.

Chemicals and equipment

Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and fluorescein were obtained from Aldrich, and 2,2-azobis (2-amidino-propane) dihydrochloride (AAPH) was purchased from VWR. Nonweathered Macando oil 252 (MC252), the type of oil released from the Deepwater Horizon oil spill platform, was obtained through the British Petroleum Gulf Coast Restoration Initiative.

Preparation of water-accommodated fractions of crude oil

Artificial seawater (15 ppt) was prepared using commercially available sea salts and municipal water dechlorinated by passage through activated charcoal. Experimental oil exposures were performed using a water-accommodated fraction (WAF) of crude oil prepared using a standardized low-energy mixing method of Singer et al. [8] in which oil was layered onto 22 L of artificial seawater contained in 23-L polycarbonate carboys (Nalgene Clearboy Round Carboy; Thermo Scientific). The crude oil–artificial seawater combination was mixed for 18 h with magnetic stirrers at an approximate rate of 1.664 *g* to produce a swirl with no detectable vortex.

Exposure procedures

Spiked-exposure toxicity testing was conducted using standardized testing procedures modified to accommodate larger organisms [8]. Exposure chambers were filled with the prepared WAF, and 15 *F. grandis* were placed in each chamber after which the tops were sealed to minimize volatilization. Flushing of WAFs from exposure chambers was started immediately after chambers were sealed and aerated artificial seawater (15 ppt) was flushed into the chambers at a rate of 200 mL/min over 8 h. The length of flushing was based on previous experiments showing that all WAFs are washed from the system within this time period [9]. Water samples were collected from the chambers at the time of exposure (time 0) as well as at 4 h and 8 h after full-strength WAF exposure. Analysis of total hydrocarbon concentration in exposure chambers was measured by ACZ Laboratories according to the US Environmental Protection Agency method M8015D, which measures nonhalogenated organics by gas chromatography coupled with a flame ionization detector at a limit of detection of 0.05 mg/L [10]. After the initial exposure and subsequent flushing conducted over 8 h, chambers were unsealed, and air stones were placed within them for the remaining test period (12 h, 24 h, or 48 h) prior to fish euthanasia and sample collection. Three replicate experiments were conducted for each time point (12 h, 24 h, or 48 h). Experimental controls for each replicate included unexposed *F. grandis* ($n = 15$) maintained in a chamber without WAFs. To limit variability between exposed and unexposed fish, chamber flushing for control fish was conducted as described above for fish exposed to WAFs.

Serum and tissue collection

For blood collection, fish were anesthetized in a 200-ppm solution of MS222 (Tricaine S) buffered to neutral with sodium bicarbonate. Whole blood was collected via venipuncture of the caudal vertebral vein in the caudal peduncle, placed into 0.5-mL polypropylene tubes (Eppendorf), and allowed to clot for 30 min at 4 °C. Following centrifugation, serum was collected and stored at –80 °C until analysis. Following blood collection, fish were returned to buffered MS222, where they remained until they expired from anesthetic overdose. The peritoneal cavity was immediately opened and the liver was removed. A 2-mm portion of hepatic tissue was collected with a sterile 2-mm dermal punch (Integra Miltex) and placed into tubes containing 0.5 mL guanidinium buffer (6 M guanidine–HCl, 10 mM urea, 20% [v/v] Triton X-100, 10 mM Tris–HCl, pH 4.4). Samples were homogenized, and total nucleic acids were extracted according to the method of DeGraves et al. [11] using glass-fiber matrix binding with the High-Pure PCR Template Preparation Kit (Roche Molecular Biochemicals) and elution in 200 µL elution buffer (10 mM Tris-HCl, 1 mM ethylenediamine tetraacetic acid [EDTA], pH 8.0).

Hepatic CYP1A mRNA transcription per CYP1A gene

Because nucleic acid sequences of *F. grandis* were not available, the upstream sequence of the *F. grandis* CYP1A gene including portions of exons 1 and 2 and intron 1 were determined using the *F. heterocolitus* mitochondrial DNA sequence as a template [12]. DNA was removed by DNase digestion from the extracted total nucleic acids, and total RNA was re-extracted. Primers designed from *F. heteroclitus* CYP1A sequences were used to amplify an upstream portion of the *F. grandis* CYP1A mRNA from both genders in single-step reverse transcriptase polymerase chain reaction (PCR). Sequencing of female and male mRNAs revealed that they were identical, and the boundary between exon 1 and exon 2 was determined by alignment with the *F. heteroclitus* CYP1A gene sequence containing exon1, intron 1, and a portion of exon 2. Determination of the 309–base pair *F. grandis* CYP1A intron 1 sequence was accomplished by DNA sequencing of amplification products from genomic DNA. These sequences were used for design of real-time PCR using fluorescence resonance energy transfer (FRET) probes for fluorescence readout of the amplification (Figure 1).

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AAAACATTTTTTTTTCTGTTGTTGGACACGCATCTCTGGAATTAGAGTGTTCGTCTTCTTTTTATCATTC
AGCTAAAGGTaaggtgattacctgcatgataaaagttatttctaagaaaggattgagatgattatttaag
acgtagatttactgttaatcagtcatatgtacatactatatgcaatcttttaaactgaaagttatgaatt
atgacctagacatacacactattgtgtatattcttaaagtgtgctaagatcaccaaagtgcagcaagtct
agattaaagttctcacttgggacctcgctgtgtaatcactatgttgcacaacacttctttcttctctctg
tcaacactgatctaattccctcctatttaatttacaggtTGAGCAGAGAACAGAGAAAAGTTGTCATCAT
GGCATTAATGATACTGCCATTCATTGGAGCACTCTCAGTGTCTGAGGGTTTGATAGCCTT
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Figure 1. Intron 1 and portions of exons 1 and 2 of the *Fundulus grandis* CYP1A gene. Exons are indicated by capital letters, and intron 1 by lower-case letters. Positions of primers are underlined, the single 5′Lightcycler Red 640-labeled fluorescence resonance energy transfer (FRET) acceptor probe on the terminal portion of exon 1 is indicated by bold underlined letters, and the 3′FAM-6-labeled FRET donor probes on intron 1 (genomic DNA polymerase chain reaction [PCR]) and exon 2 (mRNA PCR) are indicated by bold letters.

All oligonucleotides were designed using the Vector NTI software (Invitrogen). The fluorescein probes were 3′ labeled with carboxyfluorescein (6-FAM) and used unpurified as a FRET energy donor probe excited by 488 nm light. The LC Red 640 probe was 5′-labeled, 3′-phosphorylated, purified by high-performance liquid chromatography, and used as the acceptor probe (Integrated DNA Technologies). Primers and probes used for determination of CYP1A gene copies included the following: Fgrand_CYP1A_UP, TTTTCTGTTGTTGGACACGCATCTCTGG; Fgrand_CYP1A_DNA_DN, GCTGCACTTTGGTGATCTTAGCACACTT; Fgrand_CYP1A_LCR640, LCR640-ACCTTTAGCTGAATGATAAAAAGAAGACGAACAC-Phosphate; Fgrand_CYP1A_DNA_6-FAM, CTTAGAAATAACTTTTATCATGCAGGTAATCACCT-6FAM. Primers and probes used in the single-step reverse transcription PCR for determination of CYP1A mRNA copies included the following: Fgrand_CYP1A_UP, Fgrand_CYP1A_mRNA_DN, ACTGAGAGTGCTCCAATGAATGGCAGT; Fgrand_CYP1A_LCR640; Fgrand_CYP1A_mRNA_6FA M, TGATGACAACTTTTCTCTGTTCTCTGCTC-6FAM.

Nucleotide fragments representing the *F. grandis* gene and mRNA amplification fragments were synthesized and inserted into a pIDTSMART cloning Vector (Integrated DNA Technologies). The plasmid was linearized with *Hind*III (Promega) followed by inactivation of the restriction enzyme at 65 °C for 20 min. The DNA was quantified by PicoGreen DNA fluorescence assay (Invitrogen) for preparation of quantitative standards on a background of purified pGEM plasmid DNA (Promega) in Tris-EDTA buffer.

The copy numbers of *F. grandis* CYP1A genes and mRNAs were determined by FRET PCR and one-step reverse transcription FRET PCR, respectively, performed in a LightCycler 1.5 real-time platform with Software version 3.5 (Roche Molecular Biochemicals) as previously described [11,13]. Ten microliters of 1:1000 diluted total liver nucleic acids were used as input in both PCRs for a total volume of 20 μL. Reaction chemistry and thermal cycling protocols of DeGraves et al. [11] and Wang et al. [13] were followed. Data were analyzed as 640 nm:530 nm (F2/F1) fluorescence ratios. To verify specificity of the amplification, the melting curve for probe annealing to the PCR products was determined by monitoring the fluorescence from 50 °C to 80 °C with a temperature transition rate of 0.2 °C/s.

Determination of serum antioxidant capacity

The synergistic antioxidant capacity of serum excluding enzymatic antioxidants was measured according to the oxygen radical absorbance capacity ($ORAC_{FL}$) assay using the FLUOstar Optima plate reader (BMG Labtech) equipped with an incubator and 2 injection pumps according to a previously validated method by Prior et al. [14] for the analysis of antioxidant capacity in biological samples. The peroxyl radical generator was AAPH, and Trolox, a water-soluble analogue of vitamin E, served as the standard. Calibration curves of the standard ranging from 3.25 μM to 25 μM Trolox were prepared fresh for each assay run. The decrease in fluorescence was determined by measuring excitation at 535 nm and emission at 595 nm to exhibit a fluorescence decay curve based on the antioxidant capacity of the sample. The final $ORAC_{FL}$ values were calculated as the area under the fluorescence decay curve (AUC) according to the following equation: $(AUC_{sample} - AUC_{buffer})/(AUC_{Trolox} - AUC_{buffer}) \times$ dilution factor of sample $\times$ initial Trolox concentration (in mM). Antioxidant capacity results are expressed as mmol Trolox equivalents (TE)/mL.

Statistical analysis

Data were subjected to independent *t* tests to assess significant differences ($p < 0.05$) in oxidative stress biomarkers between exposed and control fish for each postexposure holding period. Statistical analysis was performed using Statistical Analysis Software, version 9.2 (SAS Institute). Percentage increases in oxidative stress biomarkers were calculated based on the biomarker response of control fish and exposed fish at each time point.

RESULTS AND DISCUSSION

Hydrocarbon analysis of the WAFs in exposure tanks revealed a mean concentration of 7.0 ± 0.10 mg/L at time 0 and 0.5 ± 0.22 mg/L at 4 h after full-strength WAF exposure. The mean concentration was below the limit of detection at 8 h after full-strength WAF exposure. Following experimental exposure, significant differences ($p < 0.05$) in hepatic CYP1A expression were observed in exposed versus control fish at 24 h. At this time interval, fish exposed to WAF of crude oil exhibited a 66% increase in CYP1A expression compared with unexposed fish (Figure 2). The 3-fold increase in CYP1A transcripts supports the hypothesis that hydrocarbon exposure transiently induces the expression of this hepatic cytochrome, which is responsible for assisting in the biotransformation of xenobiotic compounds. Although significant differences ($p < 0.05$) in CYP1A expression were not observed in exposed versus control fish at 12 h or 48 h, fish exposed to WAFs of crude oil exhibited a 23% increase in CYP1A expression at both time periods. These results are in agreement with numerous other studies reporting the use of CYP1A as a biomarker response to xenobiotic exposure in fish throughout the water column including rainbow trout [15] and Artic charr [16] along with field-collected killifish [5,17]. The level and sustained impact of CYP1A induction in each of these studies was influenced by the concentration of xenobiotic in the

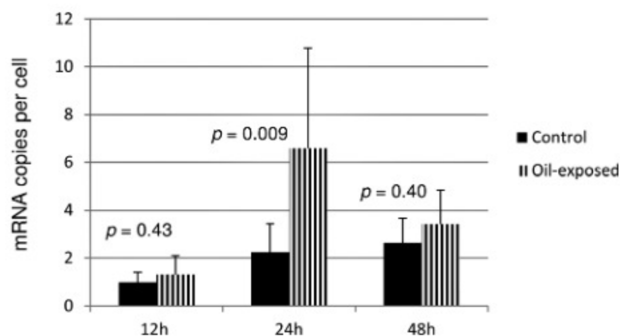

Figure 2. Expression of CYP1A in liver tissue of *Fundulus grandis* exposed to water-accommodated fractions of crude oil. Results reflect the mean and standard deviation of 3 experimental trials performed at each time point with all samples assayed in triplicate.

exposure medium as well as the length of exposure. In addition, chemical dispersants were used in the aftermath of the Deepwater Horizon oil spill; however, dispersants have been shown to influence PAH sensitivity in some aquatic organisms, especially during critical life stages of development [15,18]. Taken collectively, oxidative stress responses in fish within the gulf ecosystem may have been more pronounced than depicted in the present study as a result of higher PAH concentrations, longer exposures to PAH, and the use of chemical dispersants, namely Corexit EC9500A® and Corexit EC9527A®.

Given the detoxification action of CYP1A, ROS and xenobiotic free radicals formed during PAH metabolism may result in oxidant overload unless an organism is capable of mounting a significant antioxidant response involving both nonenzymatic as well as enzymatic antioxidants. In the present study, significant differences ($p < 0.05$) in the serum capacity of nonenzymatic antioxidants were observed in exposed versus control fish at 12 h, 24 h, and 48 h after exposure. At each time point, fish exposed to WAFs of crude oil exhibited a $\geq$25% increase in antioxidant capacity compared with unexposed fish (Figure 3). When CYP1A expression is evaluated in concert with antioxidant capacity, the results suggest that the abundance of ROS and xenobiotic free radicals generated after PAH detoxification requires antioxidant activity for a period beyond peak induction of CYPIA to accommodate the oxidant overload. While numerous studies have reported the increase in activity of antioxidant enzymes in various fish species following xenobiotic exposure [19–23], few studies have evaluated the influence of PAH exposure on nonenzymatic, chain-breaking antioxidants including serum proteins. Nevertheless, it is well documented that proteins can inhibit oxidation, perform antioxidant functions in response to stress, and influence antioxidant capacity of assays such as the ORAC assay [14,24,25]. Thus, in the present study, proteinated rather than deproteinated serum was assayed to account for the synergistic activity of all nonenzymatic antioxidants within serum. In addition, rather than test single antioxidant enzymes, the $ORAC_{FL}$ assay was selected based on its validated use in assessing the synergistic capacity of nonenzymatic antioxidants within biological samples isolated from numerous organisms [14,26–28]. Among studies evaluating nonenzymatic antioxidant capacity as an exposure biomarker, a significant increase ($p < 0.05$) in nonenzymatic antioxidant capacity was reported concomitantly with enzymatic antioxidant induction in eel as well as catfish following exposure to xenobiotic-containing water [19,20]. Taken collectively, the findings of the present study are in agreement with other studies suggesting the beneficial use of both nonenzymatic and enzymatic antioxidant responses as biomarkers of exposure to aqueous pollutants. Interestingly, among studies evaluating antioxidant biomarker responses in conjunction with CYP1A induction, the antioxidant responses positively correlate with the induction of CYP1A, after which there is a steady decline in enzymatic and nonenzymatic antioxidant defenses. Such findings suggest that the detoxification process may assist in exacerbating the antioxidant defenses of an organism. Nevertheless, such a response pattern would be expected to differ based on the xenobiotic compound, the concentration and length of exposure, and the species exposed. To thoroughly investigate this hypothesis, the period of holding post exposure would need to be extended well beyond the period of CYP1A induction.

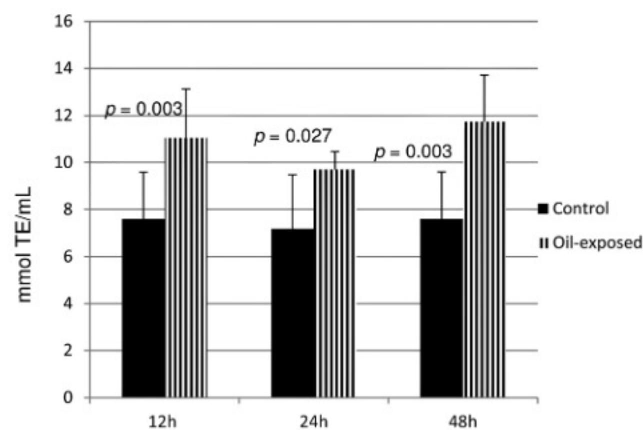

Figure 3. Serum antioxidant capacity of *Fundulus grandis* exposed to water-accommodated fractions of crude oil. Results reflect the mean and standard deviation of 3 experimental trials performed at each time point with all samples assayed in triplicate. Trolox equivalents (TE) were calculated using Trolox, a water-soluble analogue of vitamin E.

The implications of the present study are far-reaching, and thus the authors caution against regarding the relevance of these effects to gulf killifish only. First, this species represents an established model for evaluating the impact of toxicant exposure because it shares genes and biochemical pathways with higher trophic level fish and is an important species within the gulf aquatic food chain. Second, if PAH exposure and the resulting ROS generated from endogenous defense systems overwhelm antioxidant defenses, lipids, proteins, and DNA would become substrates for oxidation. For example, studies have demonstrated the concurrent effect of diminished antioxidant defenses and lipid oxidation in several species including catfish and tilapia exposed to xenobiotics including PAHs [21,29–31]. Based on the abundance of polyunsaturated fatty acids in fish, lipid oxidation or altered fatty acid composition would be expected to affect the nutritional value of the exposed fish, as was observed in haddock and cod exposed to PAHs [32]; furthermore, lipid oxidation or altered fatty acid composition would be expected to also affect unexposed fish as a result of bioaccumulation occurring when higher trophic level fish consume feeder fish with altered fatty acid composition. Thus, given the response similarities of gulf killifish and higher trophic level fish coupled with the importance of this species within the aquatic food chain, lipid oxidation testing may need to be included in biomonitoring of PAH-exposed ecosystems from which aquatic food resources are harvested.

Additional studies using various concentrations of crude oil with longer exposures and postexposure holding periods should be conducted to evaluate the potential for chronic or adaptive effects of PAH exposure in this model species and to

comprehensively understand the potential impact on fish within the aquatic food chain. Furthermore, antioxidant biomarker testing should be expanded to evaluate antioxidant capacity against other free radical species involved in oxidative stress and damage. In the present study, serum was exposed to only 1 radical species in the $ORAC_{FL}$ assay as a result of the limited amount of serum available from this small species; however, based on the biological relevance of the peroxyl radical in vivo, the antioxidant capacity of the serum measured against the peroxyl radical provides significant insight into the redox dynamics of *F. grandis* post exposure. Overall, the results of the present study assist in moving the science of environmental assessment forward by confirming the tandem use of these 2 biomarkers in this model species for comprehensively monitoring and protecting aquatic food resources following the release of hydrocarbons in aquatic ecosystems.

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