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Effects of the Deepwater Horizon Oil Spill on Deep Sea Fishes

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EFFECTS OF THE DEEPWATER HORIZON OIL SPILL ON DEEP SEA FISHES

by

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CERTIFICATE OF APPROVAL

The thesis “Effects of the Deepwater Horizon Oil Spill on Deep Sea Fishes” by Arianne Ella Leary

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Dedication

I would like to dedicate this work to my son, Elijah Ethen Leary. You have been my motivation and inspiration to pursue my passion for science and research. I love you to the moon and back, more than all the stars in the sky, to bottom of the ocean and from your nose to your toes. I hope that you will pursue your passions and dreams whatever they may be.

Acknowledgements

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CHAPTER 1:

MULTIBIOMARKER ASSESMENT OF POLYCYCLIC AROMATIC HYDROCARBON EXPOSURE IN DEEP SEA SHARKS AFTER THE DEEPWATER HORIZON OIL SPILL

Abstract

The Deepwater Horizon Oil Spill (DWH) released about 4.4 million barrels of crude oil into the Gulf of Mexico (GOM), making it one of the largest oil spills in U.S. history. Additionally, the depth of the spill (i.e., 1500 meters) created a unique research opportunity because most oil spills occur at the surface and affect coastal rather than deepwater habitats. Polycyclic aromatic hydrocarbons (PAHs) are the most toxic components of oil, and are often the focus of oil exposure studies. PAHs are quickly metabolized by vertebrates; therefore, indicators of biological responses to PAH exposure (PAH "biomarkers") such as the levels of PAH detoxification enzymes and the resulting metabolites are commonly used to examine oil exposure. This study measured multiple PAH biomarkers including hepatic activity of the PAH detoxification enzymes cytochrome P4501a1 (CYP1A) and glutathione-S-transferase (GST), as well as biliary PAH metabolites in deep sea sharks and bony fishes from areas affected by the Deepwater Horizon Oil Spill. Samples were collected from 2011-2013 from seven species of sharks, with special focus on the four most abundant deep sea species: *Centrophorus niakang, Centrophorus* cf *granulosus, Squalus cubensis* and *Squalus* cf *mitsikurii*. Overall enzyme activity was low in these sharks, yet it was higher in oiled sites compared to reference locations.

Additionally some species showed declining CYP1A activity since the time of the oil spill, suggesting exposure to CYP-inducing compounds during the beginning of the survey period. Last, PAHs of a petrogenic nature were more abundant in oiled sites compared to reference locations. Overall, this project provides the much need biomarker data for sharks as well as insight on exposure and metabolism of PAHs in deep sea sharks after the DWH.

Introduction

The Deepwater Horizon Oil Spill (DWH), which occurred on April 20, 2010 and persisted until July 15, is one of the largest oil spills in U.S. history, having released approximately 4.4 million barrels into the Gulf of Mexico (GOM) (Crone and Tolstoy, 2010). In addition, this was a unique incident because it occurred at a depth of 1500 m as opposed to other oil spills throughout history, which were predominantly surface spills (Crone and Tolstoy, 2010). In fact, three weeks after the DWH occurred, polycyclic aromatic hydrocarbon (PAH) concentrations were higher at a depth of 1360 m compared to surface concentrations (Diercks et al., 2010). The large amounts of liquid petroleum released and the depth at which it was concentrated poses significant health risks to resident organisms, especially deep water species. For example, studies on past oil spills have reported oil-associated responses, such as increased levels of detoxification enzymes (Jewett et al., 2002), increased occurrence of DNA adducts (Harvey et al., 1999), and impairment of estrogen-dependent pathways (Monteiro et al., 2000). Such effects are presumably due to exposure to polycyclic aromatic hydrocarbons (PAHs), the most toxic constituents of crude oil (Shanle and Xu, 2010, Velando et al., 2010, Whitehead et al.,

2012, Lee and Anderson, 2005, Martínez-Gómez et al., 2009), making these compounds a focus of research on the ecological effects of oil spills on marine wildlife.

Given concerns about PAHs, many projects evaluating oil contamination examine exposure to these compounds as a means to assess oil exposure and potential health effects in marine organisms (Jung et al., 2012, Tim-Tim et al., 2009, Lee and Anderson, 2005, Jewett et al., 2002, Monterio et al., 2000). Effects of PAH exposure rather than uptake are commonly examined due to the high metabolism of PAHs in most vertebrates. After PAHs are taken up by vertebrates, many of these compounds are metabolized by phase I and/or II detoxification enzymes, such as Cytochrome P4501A1 (CYP1A) and Glutathione-S-transferase (GST) in order to produce more water-soluble and excretable metabolites, which are released from the body via bile or urine. An increase in uptake of PAHs has been shown to increase both the levels of these detoxification enzymes as well as biliary PAH metabolites in many vertebrates, providing useful measures for assessing PAH exposure (Ferrira et al., 2006, Gorbi and Regoli. 2004). The intermediate PAH metabolites resulting from the actions of these enzymes are in some cases more toxic than the parent compound if not excreted because some can bind directly to DNA, forming DNA adducts or increasing free radical formation, resulting in oxidative stress. (Insausti et al., 2009). These intermediate metabolites are therefore often the cause of potential health effects rather than the less reactive parent compounds, so measuring biomarkers for the exposure and metabolism provides insight regarding potential health effects.

Because of the tendency for PAH detoxification enzymes and biliary PAH metabolites to increase in animals following oil exposure, both have been commonly used to assess oil exposure after oil spills. Previous oil spill studies have evaluated CYP1A activity in a variety of species, including invertebrate, fishes and birds. Studies on 7 different oil spills (Exxon Valdez, Prestige, Hebei Sprit, Columbia River, Erika, Coral Bunker and Sea Empress) reported elevated CYP1A levels in exposed wildlife ranging from as little as 5 days after the oil spill up to 7 years (Springman et al. 2008, Martinez-Gomez et al. 2009, Velando et al. 2010, Jewett et al., 2002). Although not as commonly used, GST activity has also been measured to evaluate health effects from oil exposure. GST levels, in addition to CYP1A activity, have been correlated with degree of oil exposure in fish populations, exhibiting increased activity 15 days to 3 years after an oil spill (Shailaja and D'Silva, 2003, Pathiratne and Hemachandra, 2010, Balk et al., 2011). GST had the highest induction, compared to other examined biomarkers, in a lab study evaluating direct exposure to crude oil in *Carassius auratus* (Wang et al., 2008). In this study, GST activity was higher in these species with increasing doses of PAHs, particularly petrogenic compounds.

Another common biomarker used to assess influences of exposure to oil-related pollutants is the concentration of PAH metabolites in bile. Fluorescent aromatic compounds (FACs) reflect the levels of PAH metabolites in bile, reflecting levels of exposure to these compounds. Biliary FACs levels were shown to increase in various species of bivalves and fishes in response to oil exposure following previous oil spills, such as Exxon Valdez, Hebei Spirit, Colombia River and Coral Bunker as soon as 20 days and up to 7 years after the oil spill (Jewett

et al., 2002, Krahn et al., 1986). Additionally, many petrogenic PAH-like metabolites were detected in higher quantities than other PAH metabolites, which are not typically abundant in crude oil (Moreira et al., 2004). FAC levels have also been shown to decrease from time of exposure as well as distance from the location of the oil spill (Balk et al., 2011).

As upper predators in aquatic environments, sharks often accumulate elevated concentrations of pollutants, such as PAHs, due to their higher trophic position (Marsili, 2000, Hassen et al., 2000). However, despite their ecological importance, few studies have examined the effects of oil spills or even typical sources of petroleum contamination on these fish. Nonetheless, there is good evidence to suggest commonly used biomarkers could be employed to assess the effects of oil exposure on sharks and their relatives. Hahn et al. (1998) has shown that CYP1A is inducible in *Mustelus canis*, demonstrating an increase in detoxification enzymes during exposure to the CYP1A inducer 5,6 benzoflavone. In addition, earlier field studies have suggested that CYP1A activity, as measured using the ethoxyresorufin-O-deethylase (EROD) assay, and biliary FACs were suitable biomarkers to monitor petroleum exposure in the Chilean catshark, *Schroederichthys chilensis,* from coastal areas in the South Pacific Ocean (Fuentes-Rios et al, 2005). Additionally, Sole et al. (2005) determined species-specific differences for GST and EROD levels for *S. chilensis* among other species in the Mediterranean (Sole et al., 2005). Both of these studies provided evidence that these enzyme activities can be used to determine pollutant exposure in elasmobranchs, but suggested lowered inducibility in these fishes than that observed in teleosts.

Therefore, the purpose of this research was to evaluate the potential health effects of oil exposure resulting from the DWH on deep sea shark species residing in the northeast Gulf of Mexico (GOM), while providing important data on PAH biomarkers for sharks. Areas of the GOM that were affected by the DWH are habitat for many squaliform sharks including: *Centrophorus niakang, Centrophorus* cf *granulosus, Squalus cubensis* and *Squalus* cf *mitsikurii.* Due to their abundance these species were focused on in this study, yet a total of ten sharks species were evaluated. The hypotheses that were tested in this study were that PAH biomarkers would be elevated in sharks residing in oiled areas, and that biomarker levels would decrease with time since the oil spill.

Methodology

Sample Collections

Research cruises were conducted from 2011-2014 in the Gulf of Mexico, throughout the continental slope from Louisiana to West Florida, including the DeSoto Canyon and areas adjacent to the DWH wellhead (Figure 1). Sample sites included oiled and unoiled sites spanning a variety of depths (200-2000m) and distances (25-250km) from the Deepwater Horizon wellhead. Some animals were also collected from less oiled or non-oiled reference sites (>400m from DWH) in the GOM on the West Florida slope. Animals were collected using bottom long-lines approximately 550m in length consisting of 50 hooks (20:10/0, 10:12/0, 10:11/0, 5:14/0 and 5:18/0) and 2 fish traps (1 cylindrical trap: 35cm x 60cm with 1cm mesh

and 1 chevron trap: 60cm x 60cm x 38cm with 2.5cm mesh). Following capture, animals were euthanized for collection of liver and bile samples. Liver was typically taken from lower right lobe, then flash frozen in liquid nitrogen and stored at -80˚C until used for measurements of PAH detoxification enzymes. Bile was collected with sterile 0.5 ml tuberculin syringes with 21-gauge needles and stored in black microcentrifuge vials and frozen at -20˚C until used for analysis of biliary PAH metabolites.

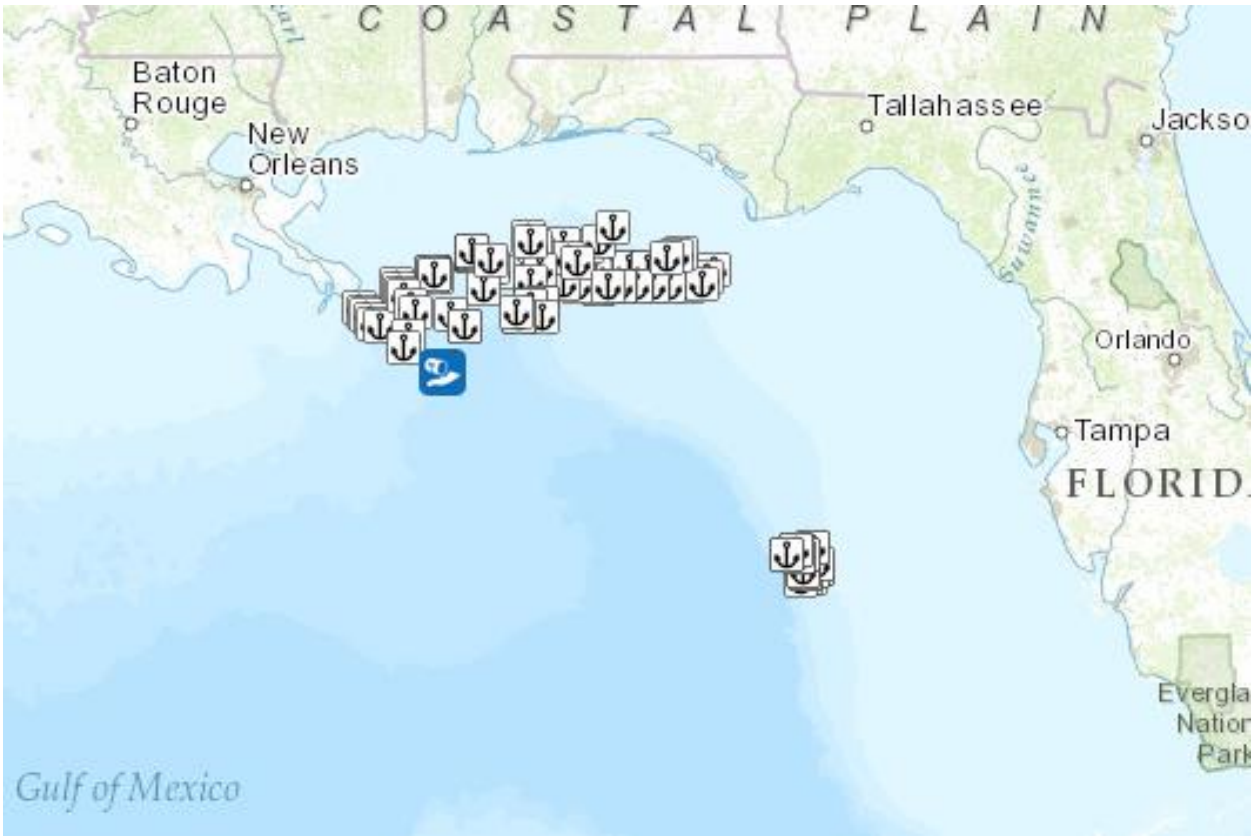

Figure 1.1 Map of Sampling Stations

Stations fished in oiled sites in Northeast Gulf of Mexico. Anchors indicate stations and oil spill indicates origin of Deepwater Horizon Oil Spill.

Liver Enzyme Activity

Hepatic CYP1A activity was measured using a modified version of the ethoxyresorufin-O-deethylase (EROD) assay described in Sepúlveda et al. (2004). Liver was homogenized (1: 5 w/v ratio) was homogenized in EROD homogenate buffer (10 mM TRIS (pH 7.4), 250 mM sucrose, 1 mM EDTA, 0.2 mM dithiothrietol, and 0.1 mM phenylmethylsulfonyl fluoride) using a bead homogenizer. Homogenates were centrifuged at 8,000*g* for 10 minutes at 4^{o}C. Supernatant was centrifuged a second time at 12,000*g* for 20 minutes at 4^{o}C and the resultant S9 fraction (the cytosolic fraction) was used to measure enzymatic activity of CYP1A. The EROD assay was performed using black 96-well microtiter plates, in which each well included 5 µL of homogenate (S9) was mixed with 193 µL of assay buffer (0.1 m NaPO4, pH 7.8) and 2 µL of substrate (100 mM ethoxyresorufin in methanol). The reaction was started by adding 5 µL of 5 mM NADPH per well, and fluorescence change at 530/25 ex, 590/35 em was measured every minute for 10 minutes. Each sample was assayed in triplicate alongside positive (5µl CYP1A standard) and negative (5µL EROD homogenate buffer) controls. GST activity was determined in the same homogenates using the commercially available Glutathione-S-Transferase Assay Kit (Cayman Chemicals, Ann Arbor, Michigan) following the manufacturer's instructions with minor modifications. Briefly, homogenates were diluted 1/5 in GST sample buffer and EROD homogenization buffer was added to negative and positive controls at volumes equivalent to those in diluted samples. Samples and both positive and negative controls were assayed in duplicate. Both CYP1A and GST activity were normalized using protein concentrations determined via the Bradford assay (Bradford, 1976).

Bile Metabolites

Relative levels of biliary FACs were measured using fixed wavelength fluorescence following a modified protocol from a previously published study (Insausti et al., 2009). Briefly, bile was diluted in 1:1000 using 48% ethanol, then clarified by centrifugation for 5 minutes at 8,000*g* at 4^{o}C, using supernatant for analysis. Samples were run in triplicate on black 96-well microtiter plates. The following PAHs (excitation/emission wavelengths in nm) were examined: naphthalene (290/335), pyrene (340/380), benzo(a)pyrene (380/430), chrysene (273/382) and phenanthrene (256/380) (Pathiratne and Hemachandra, 2010). Stocks for PAH standards were purchased from AccuStandard. Eight-point standard curves were made for each PAH and fluoresce was used to determine concentration equivalents (PHN 2-500ng/ml, CHR 2-500ng/ml, NPH 8-1000ng/ml, PYR 0.08-10ng/ml, BAP 0.039ng/ml).

Statistical Analysis

Overall means for each biomarker were compared between species using Kruskal-Wallis one-way ANOVA followed by Dunn's post-hoc test. Data from individual species were grouped by site and compared to determine if sharks from oiled sites were exhibiting signs of heightened PAH exposure. Temporal differences in PAH biomarker levels in *Centrophorus* spp. and *Squalus* spp. collected from oiled sites were examined by grouping individuals of each species collected from a single research cruise denoted by months since the occurrence from the DWH. The nonparametric Kruskal-Wallis test followed by Dunn's post-test were used to analyze data

because they did not fulfill the criteria (i.e., normal distribution and homogenous variances) for parametric analysis. GraphPad prism was used to perform analysis.

Results

Overall Findings

At least one biomarker was analyzed for 657 individuals representing of 11 different species, *Centrophorus* cf *granulosus* (279*), Centrophorus niaukang* (39), *Squalus* cf *mitsukurii* (171)*, Squalus cubensis* (115)*, Mustelus canis* (40)*, Carcharhinus altimus* (4), *Carcharhinus falciformis* (3), *Carcharhinus plumbeus* (1)*, Carcharhinus signatus* (3)*, Somniosus microcephalus* (1)*, Sphyrna lewini* (1). Overall CYP1A activity was low ranging from 0-4.5pmol*min^{-1}*mg^{-1}protein (Table 1). Although overall significant differences were observed between species (Kruskal-Wallis, $p<0.0001$), multiple comparison test were unable to isolate groups that differed significantly for CYP1A activity (Dunn's post-test, $p>0.05$). *S. mitsukurii* had a 15% of individuals with detectable CYP1A in oiled sites compared to only 3% in reference sites, although this was not determined statically significant (Table 1). *S. cubensis* had nearly a 25% positive detection in oiled sites, but this species was not caught in reference locations for comparisons (Table 1). The *Squalus* spp. had more positive CYP1A detections compared to the *Centrophorus* spp.; both groups had robust sample sizes (Figure 2A). GST activity in sharks was nearly always detected in sharks with an overall range of 0-213.9 nmol*min^{-1}*mg^{-1} protein among all species (Table 1). *Centrophorus* cf *granulosus* had the highest mean among species (58.63±2.948), which was

significantly different from *S.*cf *mitsukurii* (Dunn's post-test $p<0.0001$) (Figure 2B). Additionally, *S. cubensis* GST levels were significantly higher than *S.* cf *mitsukurii* (Dunn's post-test $p<0.0001$) (Figure 2B). Although it was not statistically significant, *Centrophorus* spp. from oiled sites had higher mean GST activity compared to *C. niaukang* in reference sites (Figure 2B).

Table 1.1 Liver Enzyme Activity in Sharks

Cytochrome P4501A1 (CYP1A) pmol*min^{-1}*mg^{-1} protein and glutathione-S-transferase (GST) activity nmol*min^{-1}*mg^{-1} protein activity ±SEM, range in italics and N in parentheses for all sharks. Centrophorus cf granulosus (CGRA), Centrophorus niaukang (CNIA), Squalus cf mitsukurii (SMIT), Squalus cubensis (SCUB), Mustelus canis (MCAN) Carcharhinus altimus (CALT), Carcharhinus falciformis (CFAL), Carcharhinus plumbeus (CPLU), Carcharhinus signatus (CSIG), Sphyrna lewinii (SLEW), Sominousus microcephalus (SMIC). Overall positive detections over sample size for CPY1A are provided. Sharks collected in reference locations are denoted by REF.

Biomarker	CNIA	REF CNIA	CGRA	MCAN	SMIT	REF SMIT	SCUB	CALT	CFAL	CPLU	CSIG	SLEW	SMIC
CYP	0.0±0.0 *0.0-0.0* (7)	0.0±0.0 *0.0-0.0* (12)	0.01257±0.006561 *0.0-0.9800* (167)	0.2343±0.1726 *0.0-4.410* (28)	0.1498±0.05211 *0.0-3.510* (88)	0.02241±0.02241 *0.0-0.6500* (29)	0.07048±0.02177 *0.0-0.9800* (63)	0.0±0.0 *0.0-0.0* (3)	1.783±0.3590 *1.080-2.260* (3)	0.0±0.0 *0.0-0.0* (1)	0.0±0.0 *0.0-0.0* (3)	2.010±0.0 *2.010-2.010* (1)	1.080±0.0 *1.080-1.080* (1)
+ CYP/N	0/7	0/12	3/167	2/28	13/88	1/29	14/63	0/3	2/3	0/1	0/3	1/1	1/1
GST	49.39±13.00 *15.80-122.1* (7)	26.84±5.684 *2.970-79.52* (16)	58.63±2.948 *0.0-213.9* (186)	43.55±4.008 *15.05-113.8* (34)	29.73±2.046 *3.480-102.1* (96)	25.46±2.864 *5.460-53.38* (21)	48.23±3.030 *2.310-157.3* (97)	98.72±44.69 *26.03-180.1* (3)	56.79±3.450 *53.34-60.24* (2)	100.2±0.0 *100.2-100.2* (1)	56.23±6.335 *43.57-62.99* (3)	54.46±0.0 *54.46-54.46* (1)	37.77±0.0 *37.77-37.77* (1)

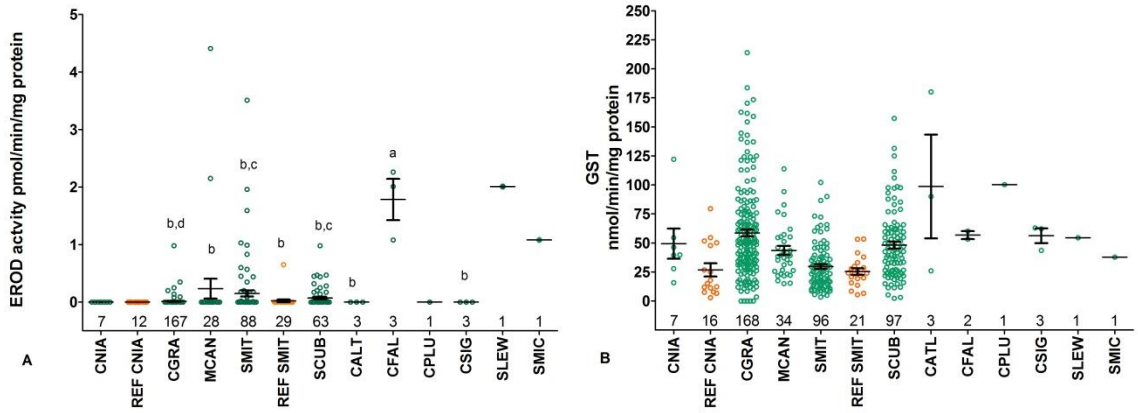

Figure 1.2 Liver Enzymes for Sharks

A) Cytochrome P4501a1 activity (EROD activity pmol*min-1*mg*-1protein) and B) Glutathione-S-transferase activity (nmol/min/mg protein) in individuals of each species -Centrophorus cf granulosus (CGRA), Centrophorus niaukang (CNIA), Squalus cf mitsukurii (SMIT), Squalus cubensis (SCUB), Mustelus canis (MCAN) Carcharhinus altimus (CALT), Carcharhinus falciformis (CFAL), Carcharhinus plumbeus (CPLU), Carcharhinus signatus (CSIG), Sphyrna lewinii (SLEW), Sominosus microcephalus (SMIC)- Grouped by oiled sites and reference (REF) Means are displayed as central line with error bars representing SEM. Sample Size is shown. Pairwise significant differences are denoted by different letters.

Most species had comparable FAC means with large variances, yet some species such as *C. altimus*, *C. falciformis* and *C. signatus* had higher means for most FACs. However, sample sizes were extremely low for these species (Table 2). *S.* cf *mitsukurii* from oiled sites had slightly higher FAC levels than reference site individuals (Figure 3). Overall, significant differences were observed for all FACs by species, PHN ($p=0.0017$), CHR ($p<0.0001$), NPH ($p=0.0009$), PYR ($p=0.0014$), BAP ($p=0.0021$) yet no pairwise differences were determined (Dunn's post-test, $p>0.05$) (Figure 3).

Table 1.2 Bile Metabolites in Sharks

Metabolite concentrations in shark's bile for five different polycyclic aromatic hydrocarbon- Chrysene-like metabolites (CHR), Phenanthrene-like metabolites (PHN), Naphthalene-like metabolites (NPH), Pyrene-like metabolites (PYR) and Benzo(a)pyrene-like metabolite (BAP) means ±SEM, range in italics and N in parentheses for all sharks. *Centrophorus* cf *granulosus* (CGRA), *Centrophorus niaukang* (CNIA), *Squalus* cf *mitsukurii* (SMIT), *Squalus cubensis* (SCUB), *Mustelus canis* (MCAN) *Carcharhinus altimus* (CALT), *Carcharhinus falciformis* (CFAL), *Carcharhinus plumbeus* (CPLU), *Carcharhinus signatus* (CSIG), *Sphyrna lewinii* (SLEW), *Sominousus microcephalus* (SMIC). Sharks collected in reference locations are

PAH	CNIA (16)	REF CNIA (22)	CGRA (256)	MCAN (32)	REF MCAN (1)	SMIT (122)	REF SMIT (30)	SCUB (83)	CATL (4)	CFAL (3)	CPLU (1)	CSIG (2)	SLEW (1)	SMIC (1)
PHN	34745±9318 0.0-145267	62975±17935 0.0-387431	53010±3710 0-546029	59537±8802 0.0-201976	133101±0.0 133101-133101	54930±5407 0.0-375658	28299±4324 0.0-80528	53168±4518 408.4-204662	515817±143185 315612-929010	124773±44561 53632-206832	15559±0.0 15559-15559	88280±53881 34399-142161	40977±0.0 40977-40977	50634±0.0 50634-50634
CHR	63825±14309 5358-194745	92161±22342 10154-486949	69656±3462 3390-314569	108629±13940 5838-329195	222681±0.0 222681-222681	73002±6679 0.0-438991	45269±5755 6160-110272	90463±8775 4923-494732	509240±973.8 508266-510213	190460±69847 83521-321767	9299±0.0 9299-9299	146897±83667 63230-230563	39452±0.0 39452-39452	20148±0.0 20148-20148
NPH	40489±11190 0.0-167464	56351±10218 0.0-168168	47492±2933 0.0-292706	74538±10009 0.0-210420	137387±0.0 137387-137387	47187±4782 0.0-307965	31456±4811 0.0-86093	62061±6225 0.0-257062	N/A	132500±46541 63317-221021	2584±0.0 2584-2584	108128±44486 63642-152613	33421±0.0 33421-33421	5746±0.0 5746-5746
PYR	522.0±284.0 0.0-3613	482.1±286.7 0.0-5977	803.0±85.55 0.0-10746	440.4±107.1 0.0-1766	1392±0.0 1392-1392	847.4±123.6 0.0-7272	389.0±103.3 0.0-2201	1359±184.7 0.0-8810	2579±818.1 649.2-4589	509.8±449.8 20.98-1408	0.0±0.0 0.0-0.0	0.0±0.0 0.0-0.0	0.0±0.0 0.0-0.0	0.0±0.0 0.0-0.0
BAP	3347±952.9 0.0-12167	2450±608.0 159.1-9083	1442±117.4 0.0-12917	720.7±131.9 0.0-2760	1109±0.0 1109-1109	1136±135.1 0.0-9685	731.5±125.6 0.0-2635	1228±121.1 0.0-5783	3796±1423 1923-8020	1062±213.9 663.2-1396	19.73±0.0 19.73-19.73	676.4±325.6 350.8-1002	129.9±0.0 129.9-129.9	282.2±0.0 282.2-282.2

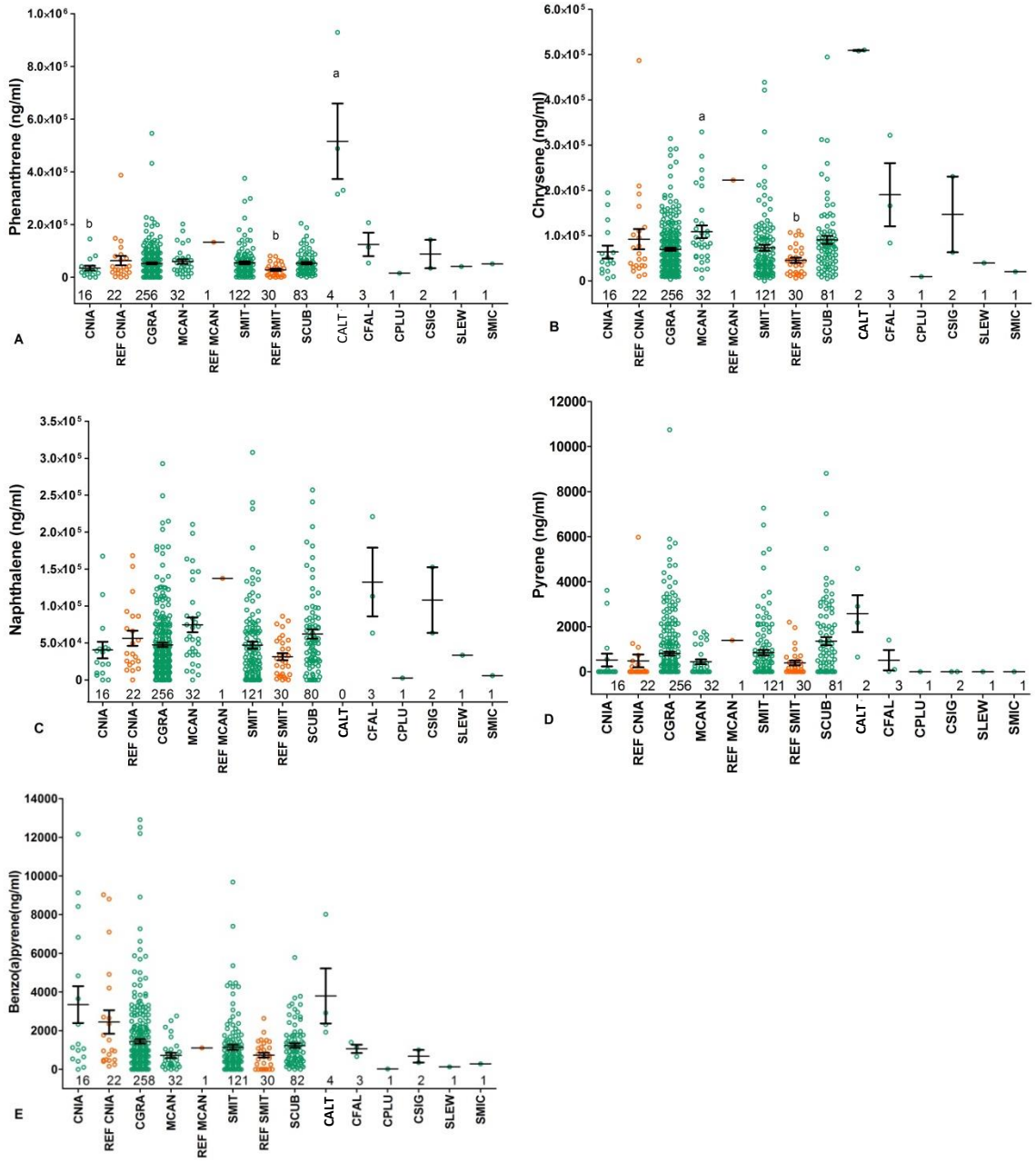

Figure 1.3 Bile Metabolites in Sharks. FACs (ng/ml) for individuals of each species: Centrophorus cf granulosus (CGRA), Centrophorus niaukang (CNIA), Squalus cf mitsukurii (SMIT), Squalus cubensis (SCUB), Mustelus canis (MCAN) Carcharhinus altimus (CALT), Carcharhinus falciformis (CFAL), Carcharhinus plumbeus (CPLU), Carcharhinus signatus (CSIG), Sphyrna lewinii (SLEW), Sominousus microcephalus (SMIC). Data from oiled sites are shown in blue, reference locations are shown in orange. Means are displayed as central line with error bars representing SEM. Sample size is shown. A) Chrysene-like metabolites, B) Phenanthrene-like metabolites C) Naphthalene-like metabolites, D) Pyrene-like metabolites, E) Benzo(a)pyrene-like metabolite. Pairwise significant differences are denoted by different letters (Kruskal-Wallis followed by Dunn's post-test).

Species with larger sample sizes were used to examine temporal and spatial trends among biomarkers, namely *Centrophorus* spp. and *Squalus* spp. *Centrophorus* spp., were grouped together for analysis. Additionally, only *Squalus* spp. were analyzed for trends in CYP1A activity due to minimal detection in *Centrophorus* spp.

Temporal Trends

Centrophorus spp. from oiled sites were analyzed for temporal trends by time of capture in months since the DWH. These data were also compared to reference sites. GST activity was significantly higher in several months (16, 24, 30, 39 and 42) for individuals from oiled sites compared to those observed from reference sites (Dunn's post-test $p<0.05$). Months 12 and 27 were similar to those in reference site animals, yet sample sizes were smaller than any other time period. Enzyme levels appeared to peak in months 30 and 39 and decrease in month 42; levels observed in months 30 and 39 were significantly greater than those in month 16 and 42 and references (Figure 4).

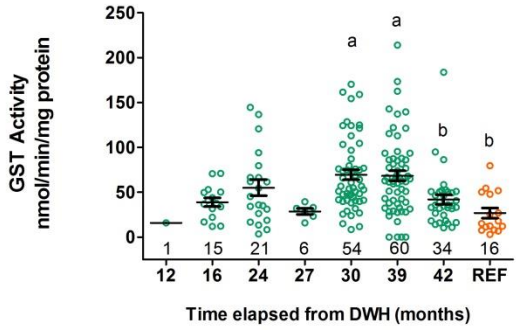

Figure 1.4 Glutathione-S-transferase in Centrophorus Spp.

GST activity (nmol/min/mg protein) for individual *Centrophorus* spp. for oiled sites by time since DWH (months) and individuals caught in reference locations (REF). Means are displayed as central line with error bars representing SEM. Sample sizes are shown. Pairwise significant differences are denoted by different letters (Kruskal-Wallis followed by Dunn's post-test).

FAC levels in *Centrophorus* spp. varied slightly throughout sampling periods (Figure 5). Levels of CHR, PHN and NPH metabolites were significantly higher in months 30 and 39 compared to months 16, 49 and reference sites (Kruskal Wallis and Dunn's post-test, $p<0.0001$) (Figure 5A-C).

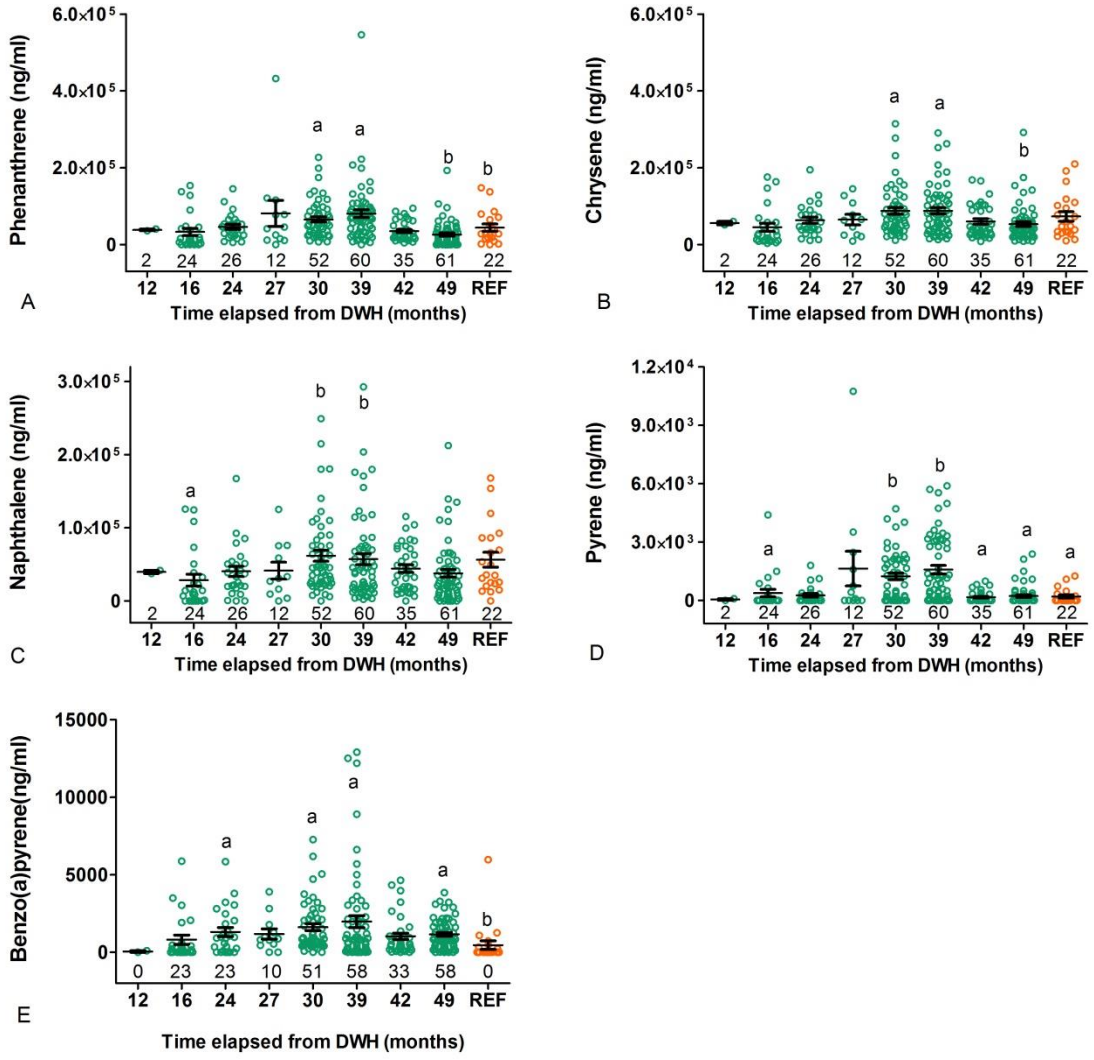

Figure 1.5 Bile Metabolites in Centrophorus spp.

FAC levels (ng/ml) for individual *Centrophorus* spp. for oiled sites by time since DWH (months) and individuals caught in reference locations (REF). Means are displayed as central line with error bars representing SEM. Sample sizes are shown. A) Chrysene-like metabolites, B) Phenanthrene-like metabolites, C) Naphthalene-like metabolites, D) Pyrene-like metabolites, E) Benzo(a)pyrene-like metabolites. Pairwise significant differences are denoted by different letters (Kruskal-Wallis followed by Dunn's post-test).

S. cubensis from oiled sites were analyzed for temporal trends by time of capture. Months 16 and 30 had higher CYP1A activity, yet only month 27 was statistically significant (Dunn's post-test, $p<0.05$) (Figure 6A). GST activity appears to peak in month 30, which was significantly greater than levels observed in month 12, then declined through month 42 (Dunn's post-test, $p<0.05$) (Figure 6A).

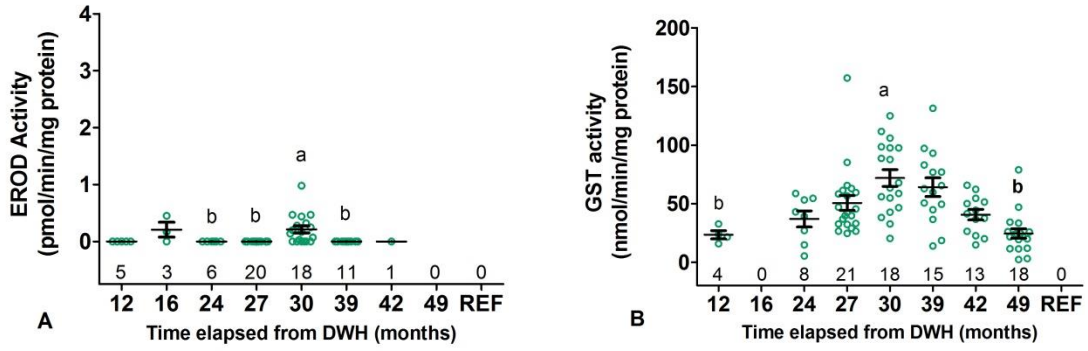

Figure 1.6 Liver Enzymes for Squalus cubensis

A) Cytochrome P4501a1 EROD activity pmol*min-1*mg*-1protein and B) Glutathione-S-transferase (GST) nmol*min^{-1}*mg^{-1} protein activity for *S. cubensis* from oiled sites grouped by time since DWH (months). No individuals were caught in reference (REF) sites. Means are displayed as central line with error bars representing SEM. Sample size is shown. Pairwise significant differences are denoted by different letters (Kruskal-Wallis followed by Dunn's post-test).

FAC levels for *S. cubensis* varied depending on time of capture. Levels observed in months 27 and 30 were statistically greater than months 16, 42 and 49 for PHN ($p<0.0001$), CHR ($p<0.0001$) and NPH ($p<0.0001$) (Figures 7A-C). PYR-like metabolites were significantly greater in month 30 than in months 39, 42 and 49 (Dunn's post-test, $p<0.0001$) (Figure 7D). BAP-like metabolites were greater in month 24 and 27 compared to month 42 (Dunn's post-test, $p<0.0001$) (Figure 7E).

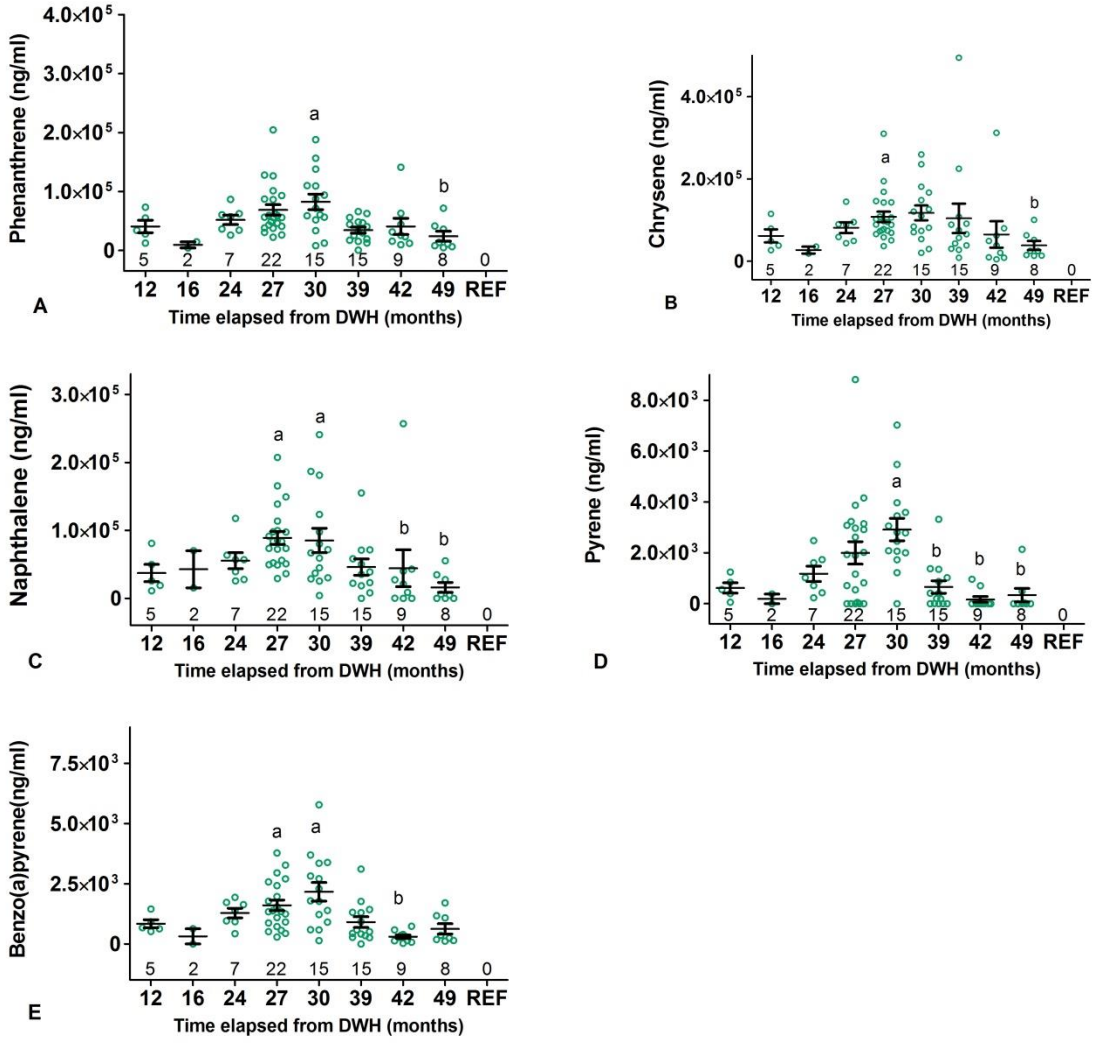

Figure 1.7 Bile Metabolites for Squalus cubensis

FAC levels (ng/ml) for individual *S. cubensis* from oiled sites by time since DWH (months). No individuals were caught in (REF) reference locations. Means are displayed as central line with error bars representing SEM. Sample sizes are shown. A) Chrysene-like metabolites, B) Phenanthrene-like metabolites, C) Naphthalene-like metabolites, D) Pyrene-like metabolites, E) Benzo(a)pyrene-like metabolites. Pairwise significant differences are denoted by different letters

S. cf *mitsukurii* were grouped by time in oiled locations, as well as compared to reference locations. Both enzyme levels showed higher activity in month 30 (Figure 8). CYP1A activity in month 12, 16, 27 and 30 was significantly higher than sharks collected in other sampling periods and reference locations (Dunn's post-test, $p<0.0001$) (Figure 8A). GST activity increased slightly from month 12 through month 30 and then began to marginally decrease through month 49 (Figure 8B).

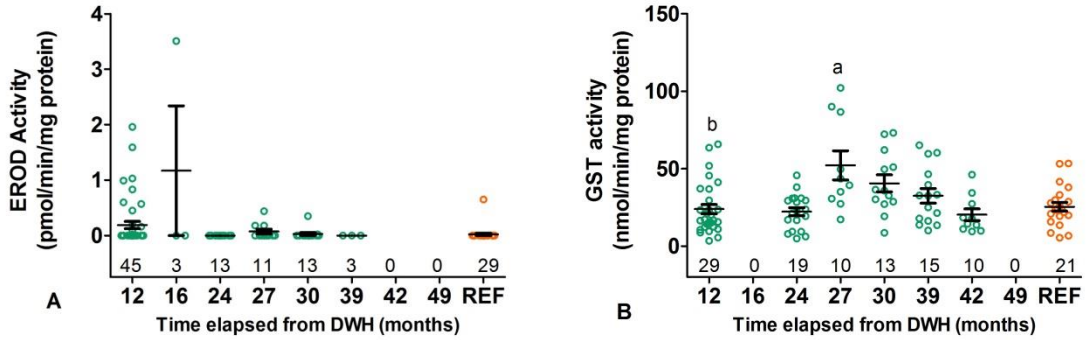

Figure 1.8 Liver Enzymes for Squalus cf mitsukurii

A) Cytochrome P4501a1 EROD activity pmol*min-1*mg*-1protein and B) Glutathione-S-transferase (GST) nmol*$min^{-1}mg^{-1}$ protein activity for *S.* cf *mitsukurii* grouped by for oiled sites by time since DWH (months). Means are displayed as central line with error bars representing SEM. Sample sizes are shown. Pairwise significant differences are denoted by different letters (Kruskal-Wallis followed by Dunn's post-test).

Analysis also demonstrated elevated FAC levels in *S.* cf *mitsukurii* in months 30, 27 and 39. These months were significantly higher than means in reference sites (Dunn's post-test, $p<0.05$). Biomarker levels appeared to increase slightly from each sampling period until month 30 then slightly decreased in subsequent collection periods (Figure 9). Months 27 and 30 were statistically higher than month 49 for PHN ($p=0.0008$), CHR ($p=0.0018$) and NPH ($p=0.0001$) (Figure 9A-C). These months were also significantly higher for PYR ($p<0.0001$) and BAP ($p<0.0001$), but only compared to month 42 (Figure 9D and E).

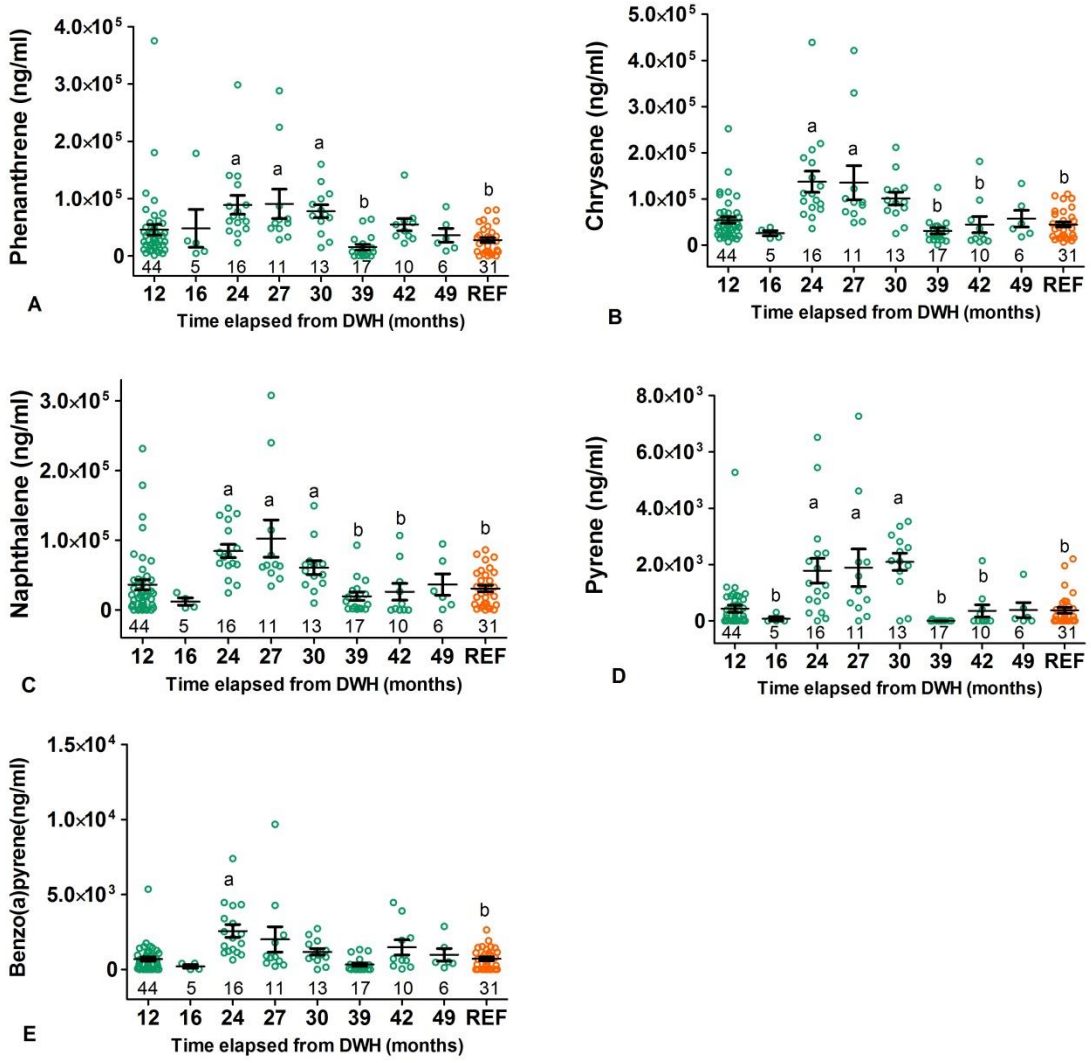

Figure 1.9 Bile Metabolites in Squalus cf mitsukurii

FAC levels (ng/ml) for individual *S.* cf *mitsukurii* for oiled sites by time since DWH (months). Mean displayed as central lines with error bars representing SEM. Sample sizes are shown. A) Chrysene-like metabolites, B) Phenanthrene-like metabolites, C) Naphthalene-like metabolites, D) Pyrene-like metabolites, E) Benzo(a)pyrene-like metabolites. Pairwise significant differences are denoted by different letters (Kruskal-Wallis followed by Dunn's post-hoc test).

Discussion

This study has provided a wealth of new information on PAH biomarkers in sharks, particularly deep sea sharks, which have not been well studied especially in context of ecotoxicology. Biomarker levels differ in comparison to previous studies and suggest the possibility of interspecies differences. Results indicate temporal and spatial differences among oiled and reference sites. These varying levels suggest PAH exposure from sources that could include DWH oil.

Although these biomarkers are seldom evaluated in sharks, the results from previous studies reported similar ranges or slightly higher than this study. The averages for CYP1A activity in the present study were less than 1 pmol*min-1*mg*-1protein compared to ~5pmol*min-1*mg*-1protein in the Chilean catshark, a much shallower dwelling species, collected in sites known to be oil-polluted (Fuentes-Rios et al. 2005). Previous studies within this lab have shown similar means of CYP1A activity for coastal shark species in the months following the DWH (Walker, 2011). GST levels (mean~50nmol/min/mg protein) were similar, but slightly lower than some other studies on elasmobranch species in polluted sites; e.g., 40-50 nmol/min/mg protein (Rudneva et al., 2010) and 80 nmol/min/mg protein (Velez-Alavez et al., 2013). Lower enzyme activity has been reported for benthic animals compared to epipelagic (Rudneva et al., 2014) elasmobranchs compared to teleosts (Rudneva et al. 2010), as well as deep sea species compared to shallower dwelling counterparts (Treberg et al., 2003). The

synergistic effect of these factors in the deep sea sharks provides rationale for overall lower enzyme levels and emphasize the necessity for further investigation.

Although there are a few studies that evaluated PAHs in sharks, comparisons were not possible due to differing methodologies used among studies. Sharks have been noted to accumulate 2-4 ringed PAHs in liver, muscle and gill (Hassan et al., 2000). While the current study did not evaluate PAHs in the same manner, these 2-4 ringed PAHs (NPH, PHN, CHR and PYR) showed more significant differences between sampling periods and sites than BAP (a 5 ringed PAH). Fuentes-Rios et al. (2005) evaluated PAH metabolites in the *Schroederichthys chilensi* using different methods, yet concluded that biliary FACs in all sampling sites indicated that petrogenic PAHs were ubiquitous in the whole embayment system being studied. This may also be true for the NE Gulf of Mexico due to similar values in the oiled sites. The deep sea sharks in the current study had slightly lower PAH metabolite levels compared to previously reported bony fishes (Insuasti et al. 2009). Lack of data immediately following the DWH is detrimental in fully evaluating the effects on these organisms.

Although CYP1A activity in this study was low, differences among species was observed. The most notable difference was the near non-detection in *Centrophorus* spp. compared to *Squalus* spp. and other species. Pelagic sharks, such as *C. falciformis and S. lewini* had slightly higher CYP1A activity (>1pmol*min-1*mg*-1protein) compared to the deep dwelling species, but were within ranges that Walker (2011) reported for coastal species. This may be due to

coastal species encountering CYP-inducing pollutants more frequently than deep sea species due to anthropogenic activity on the coast. *S. microcephalus,* a deep dwelling shark typically found in arctic waters, had CYP1A activity higher than the other deep water species in this study, as well. *S. microcephalus* has been noted to have higher levels of pollutants compared to other species in Canadian waters, such as PCBs and DDTs which are inducers of CYP1A (Fisk et al., 2005). Yet higher prevalence of CYP1A activity in individuals captured in DWH sites compared to reference sites requires further examination, which will be discussed later. GST activity differed among species as well. *Centrophorus* spp. from oiled sites and *S. cubensis* had higher GST means compare to other species. Yet most FACs were similar among species. Some large pelagic sharks, namely *C. signatus* and *C. altimus* had some higher levels of PHN, CHR and NPH, but sample sizes were low.

Temporal trends for CYP1A activity were only evaluated in *S.* cf *mitsukurii* due to the lack of CYP1A activity in the *Centrophorus* spp. and *S. cubensis* only inhabiting NE GOM in areas that were likely impacted by the DWH. Although CYP1A in one of the most robust biomarkers used in pollutant studies, many of the samples within this study had below detectable levels. Yet, *S.* cf *mitsukurii* collected in oiled sites had about 20% positive detections compared to 3% from the reference sites Although CYP1A may not be the most suitable biomarker due to low activity in shark species, it does indicate higher pollutant exposure in oiled sites compared to reference sites. Temporal trends for *S.* cf *mitsukurii* indicate more positive detections in earlier sampling periods (month 12, 16 and 27). Although this trend was not statically significant, its

biological importance may suggest that this increase in CYP1A activity is related to the timing of the DWH. *M. canis* CYP1A activity also had more positive detections in the earlier sampling period, supporting this same hypothesis.

Due to the low detection rate of CYP1A in sharks, GST provided more insight to oil exposure for sharks in the NE Gulf, due to its naturally higher abundance in vertebrates. *C.* cf *granulosus* and *C. niaukang* from oiled sites had higher GST activity compared to *C. niaukang* from reference sites. GST activity for most sharks, excluding *M. canis* and *C. niaukang,* also exhibited temporal differences with increases from early sampling periods to months 27, 30 and 39 followed by decreases in months 42 and 49. This may indicate increased exposure to pollutants, such as oil in these months. This could be due to oil-pollutants incorporation in the food web, considering these sharks are upper-mid trophic levels and exposure is likely through diet.

Biliary FAC concentrations in sharks provided some evidence for PAH metabolism. For example, temporal trends for petrogenic PAHs (CHR, PHN and NPH) in *S.* cf *mitsukurii* showed increased levels in months 24, 27 and 30, decreasing in month 39 and increasing again in month 49. These increased levels were also significantly higher than levels reported for reference locations. In other sharks many PAH metabolites, especially petrogenic PHN, CHR and NPH, followed similar trends. Both *S. cubensis* and *C.* cf *granulosus* had higher PHN, CHR and NPH levels for months 27, 30 and 39 compared to earlier and later months. This indicates higher

metabolism of petrogenic PAHs during these periods, which was also higher than the metabolism indicated by FACs in reference locations from this study. This supports the hypothesis that NE Gulf sharks experienced higher metabolism of oil-related pollutants compared to references locations. However, this perceived delay with increases in exposure and metabolism after the DWH was also supported by GST data. Many factors contribute to FAC levels of any given animal at a given time, such as feeding status, sex, reproductive cycle and recent exposure (Ferreira et al., 2006, Gorbi and Regoli 2004, Nicholas et al. 1999). For instance, the European eel was noted to have higher concentrations of metabolites during periods of slower metabolism, when feeding was irregular (Ariese et al., 1993). Additionally Ferreira et al (2006) determined through a depuration study that metabolites levels took 4 months to significantly decrease in the striped mullet, *Mugil cephalus*. Gorbi and Regoli (2004) observed one week accumulation and excretion patterns in the American eel. This information is lacking for sharks and needs to be examined in future studies.

Although both CYP1A activity and PAH metabolites are useful biomarkers in evaluating exposure, they are affected differently by natural factors and have been noted to have different time responses in other marine organisms (Trisciani et al., 2011; Kamman et al., 2008; Gorbi and Regoli, 2004). This may explain why CYP1A activity in the current study does not reflect the same trends as PAH metabolite levels. The same patterns observed for GST activity and petrogenic PAH metabolites suggest that these two biomarkers may have similar responses in sharks. Phase II enzymes typically modify intermediate metabolites that are products from

Phase I enzymes, which may not be water soluble enough for excretion. Therefore, more by-products of Phase II detoxification would result in excretion, which seems to be supported in this study. PAH exposure is primarily introduced to an organism via diet or gills. As the sampling of this study started a year the DWH, the likely exposure route is diet. These compounds would have to be incorporated into the food web for exposure of higher trophic levels, such as sharks. The sampling periods initially after the DWH is absent in this study, which may have shown higher values than those reported in this study. The perceived delay may indicate that PAHs have been introduced into the food web and over time moved into higher trophic levels.

This study has not only provided insight to potential effects from the DWH on deep sea sharks, but has also provided much needed biomarker information for shark species. The variety of factors that influence these biomarkers demonstrate the need for specie-specific and baseline data for toxicology studies after a catastrophe. Future studies should focus on providing data in a similar manner so studies are comparable, as well as collecting data on reproductive cycles and feeding status of individuals for consideration in analysis. Determining accumulation and excretion time for species is also needed. Additional evaluation of enzyme activity in gills compared to liver could be used to determine route of exposure.

CHAPTER 2:

POLYCYCLIC AROMATIC HYDROCARBON BIOMARKERS IN DEEP SEA TELEOSTS AFTER THE DEEPWATER HORIZON OIL SPILL SUGGEST EXPOSURE AND METABOLISM TO OIL POLLUTANTS

Abstract

The Deepwater Horizon Oil Spill (DWH) released nearly 5 million barrels of liquid petroleum into the Gulf of Mexico, making it one of the largest oil spills in U.S. history. At a depth of about 1,500 meters, this spill created a unique yet challenging research opportunity. It is vital to determine the effects on Gulf wildlife from oil-related pollutants, particularly the polycyclic aromatic hydrocarbons (PAHs), which are the most toxic components of oil. Due to the rapid metabolism of these compounds, a variety of PAH biomarkers such as PAH detoxification enzymes and products of PAH metabolism were used to evaluate health effects from the oil spill in deep sea fishes collected from 2011-2014. In particular, this study focused on three bony fish species, *Lopholatilus chamaeleonticeps, Urophycis cirrata* and *Urophycis floridana,* which are abundant in the NE Gulf of Mexico. Animals were evaluated for inducible PAH-metabolizing enzymes, specifically cytochrome P4501a1 (Cyp1A) and glutathione-S-transferase (GST) in the liver, as well as PAH metabolites in the bile. Enzymatic assays for both CYP1A and GST were used to assess exposure. PAH metabolite concentrations in bile were determined using fixed wavelength fluorescence to assess PAH metabolism. Species differences were observed within this study, specifically the biomarker levels were highest in *L. chamaeleonticeps*. CYP1A activity was more prevalent in the earlier sampling period, suggesting

exposure to CYP-inducing pollutants, such as PAHs from the DWH. GST activity proved to be variable without any specific trends. PAH metabolite levels, specifically petrogenic PAHs, mirrored the trends observed for CYP1A with a further spatial trend. *U. cirrata* collected closer to the origin of the DWH had higher PAH concentrations compared to those at further distances and reference sites. Some of these levels were higher than previously reported for anthropogenic polluted sites. Results indicate exposure and metabolism of petrogenic PAHs with some trends supporting relationships to the DWH. This study suggests that *L. chamaeleonticeps* experienced higher levels of exposure and metabolism compared to other species within this study. This may be explained by their burrowing habits, which could increase exposure to lipophilic pollutants that associate with sediment, such as PAHs. The current study supports exposure and metabolism of PAHs in several abundant NE Gulf deep sea species, yet additional studies are required to determine origin of PAHs, due to the abundance of natural oil seeps in this region.

Introduction

The Deepwater Horizon Oil Spill (DWH) which occurred on April 20, 2010 and persisted until July 15 of the same year, is one of the largest oil spills in U.S. history, having released approximately 4.4 million barrels of crude oil into the Gulf of Mexico (GOM) (Crone and Tolstoy, 2010). In addition, this was a unique incident because it occurred at a depth of 1,500 meters as opposed to other oil spills throughout history which were predominantly surface spills (Crone and Tolstoy, 2010). In fact, three weeks after the DWH occurred, concentrations of toxic oil

constituents were higher at a depth of 1,360 meters compared to surface concentrations (Diercks et al., 2010). The large amounts of liquid petroleum released and the depth at which it was concentrated poses significant health risks to resident organisms, especially deep water species. For example, previous studies on past oil spills have reported increased levels of detoxification enzymes and the metabolites from these processes (Lee and Anderson, 2005) as well as other oil-associated health effects, such as increases in the occurrence of DNA adducts (Harvey et al., 1999) and impairment of estrogen dependent pathways (Monteiro et al., 2000). Such effects are believed to be due to exposure to polycyclic aromatic hydrocarbons (PAHs), which are generally considered to be the most toxic constituents of crude oil (Shanle and Xu, 2010, Velando et al., 2010, Martínez-Gómez et al., 2009, Lee and Anderson, 2005), making these compounds a focus of research on the ecological effects of oil spills on marine wildlife.

The depth of this oil spill has the potential to affect demersal organisms. Certain deep water fish species such as the golden tilefish, *Lopholatilus chamaeleonticeps*, and hakes, *Urophycis cirrata* and *Urophycis floridana,* are likely targets for study due to their behaviors and abundance in the northeast Gulf of Mexico. The life history characteristics of *L. chamaeleonticeps*, such as slow growth, long life, complex breeding system and habitat specificity makes it vulnerable to experiencing adverse impacts from DWH. This non-migratory, demersal fish's habitat occurs at depths of 200-400m in the GOM and other regions. *L. chamaeleonticeps* are a burrowing fish (Grimes and Turner, 1999), which also puts it as risk for exposure to PAHs settled in sediment. Hakes are ecologically important species within the NE

Gulf of Mexico. Both *U. cirrata* and *U. floridana* are dominant demersal fishes in depths from 200-400 and 300-700 meters, respectively. These species are likely to encounter oil exposure from DWH due to their demersal habits and depth profiles. Additionally, hake species were evaluated after the Prestige Oil Spill as a demersal fish species in previous studies and were shown to exhibit indicators of stress after oil exposure (Raingeard et al., 2009, Marigómez et al., 2006). The preferred habitat and behaviors of *L. chamaeleonticeps* places it at substantial risk for PAH exposure and the commonality and distribution of the hakes make these species ideal for study specimens of PAH exposure and effects from the DWH.

Given their importance, many projects evaluating oil contamination examine biological effects of PAHs as a means for assessing oil exposure in marine organisms (Jung et al. 2012, Jung et al. 2011, Velando et al. 2010, Martinez-Gomez et al. 2009, Tim-Tim et al. 2009, Sole et al. 2008, Springman et al. 2008, Moreira-Santos et al. 2004, Jewett et al. 2002, Peters et al. 1999, Krahn et al. 1986b). Effects of PAH exposure rather than uptake are commonly examined due to the high metabolism of PAHs in most vertebrates. When PAHs enter an organism, they are generally metabolized by phase I and/or II detoxification enzymes, such as Cytochrome P4501A1 (CYP1A) and Glutathione-S-transferase (GST), respectively, particularly in the liver. The favored route of these metabolites is excretion via bile or kidney, and an increase in uptake of PAHs has shown to increase the presence of these metabolites in the bile (Ferrira et al., 2006, Gorbi and Regoli, 2004). However, enzymatic detoxification can also result in the production of highly reactive intermediate metabolites, which can be more toxic than the parent compound if

not excreted because some can bind directly to DNA, forming adducts, or increase radical formation, resulting in oxidative stress (Insausti et al., 2009).

Because of the tendency for PAH detoxification enzymes and biliary PAH metabolites to increase in animals following oil exposure, both are commonly used to assess oil exposure after an oil spill. Previous oil spill studies have evaluated CYP1A activity in a variety of species, ranging from birds, mussels and fishes. Studies on 7 different oil spills (Exxon Valdez, Prestige, Hebei Sprit, Columbia River, Erika, Coral Bunker and Sea Empress) reported elevated CYP1A levels ranging from as little as 25 days after the oil spill up to 7 years afterwards (Velando et al. 2010, Martinez-Gomez et al. 2009, Springman et al. 2008, Jewett et al., 2002). Although not as commonly used, GST activity has also been useful for measuring health impacts from oil exposure. GST levels, in addition to CYP1A activity, have been correlated with degree of oil exposure in fish populations in the North Sea (Balk et al., 2011). Several studies have reported significantly higher GST activity from 15 days to 3 years after oil spill (Martinez-Gomez et al. 2009, Moreira-Santos et al. 2004). Investigations on oil effects using the presence of biliary fluorescent aromatic compounds (FACs), which reflect the levels of PAH metabolites in bile, also indicate levels higher in contaminated areas compared to reference areas (Jung et al., 2012, Jung et al., 2011, Jewett et al., 2002, Krahn et al., 1986b). Additionally FAC levels have been shown to decrease from time of exposure as well as distance from source (Balk et al., 2011). Studies have reported significantly higher FAC concentrations for several oil spills, i.e., Exxon Valdez, Hebei Spirit, Colombia River and Coral Bunker, as soon as 20 days after the oil spill and

up to 7 years post-spill in a variety of aquatic species (Jewett et al., 2002, Krahn et al., 1986b). Additionally several petrogenic PAH-like metabolites were detected in higher quantities than pyrogenic PAH metabolites, which are not typically high in abundance in petroleum (Moreira et al., 2004).

The purpose of this study was to evaluate *L. chamaeleonticeps, U. cirrata* and *U. floridana* for probable health effects from oil exposure and metabolism as a result of the DWH. More specifically, this study examined if PAH biomarkers in theses fishes varied spatially or temporally in relation to the origin and time of this oil spill. Additional comparisons to reference animals for hake collected within this study were used to determine overall implications of oil exposure from the DWH. It was hypothesized that the biomarker levels would be more predominant near the time and origin of the DWH and decrease with time and distance.

Methodology

Sample Collections

Research cruises were conducted from 2011-2014 in the Gulf of Mexico near the Deepwater Horizon oil rig, and on both east and west sides of the Desoto Canyon (Figure 1). Sample sites included oiled and unoiled sites spanning a variety of depths (200-2000m) and distances (25-250km) from the Deepwater Horizon wellhead. Some animals were also collected from less oiled or non-oiled reference sites in the GOM on the West Florida Shelf in

2011-2012. Animals were collected using bottom long-lines approximately 550m in length consisting of 50 hooks (20:10/0, 10:12/0, 10:11/0, 5:14/0 and 5:18/0) and 2 fish traps (1 cylindrical trap: 35cm x 60cm with 1cm mesh and 1 Chevron: 60cm x 60cm x 38cm with 2.5cm mesh). Following capture, animals were euthanized for collection of liver using dissection tools and bile samples using sterile 0.5 ml tuberculin syringes with 21-gauge needles. Liver was flash frozen in liquid nitrogen and stored at -80˚C until used for measurements of PAH detoxification enzymes. Bile was stored in black microcentrifuge vials and frozen at -20˚C until used for analysis of biliary PAH metabolites.

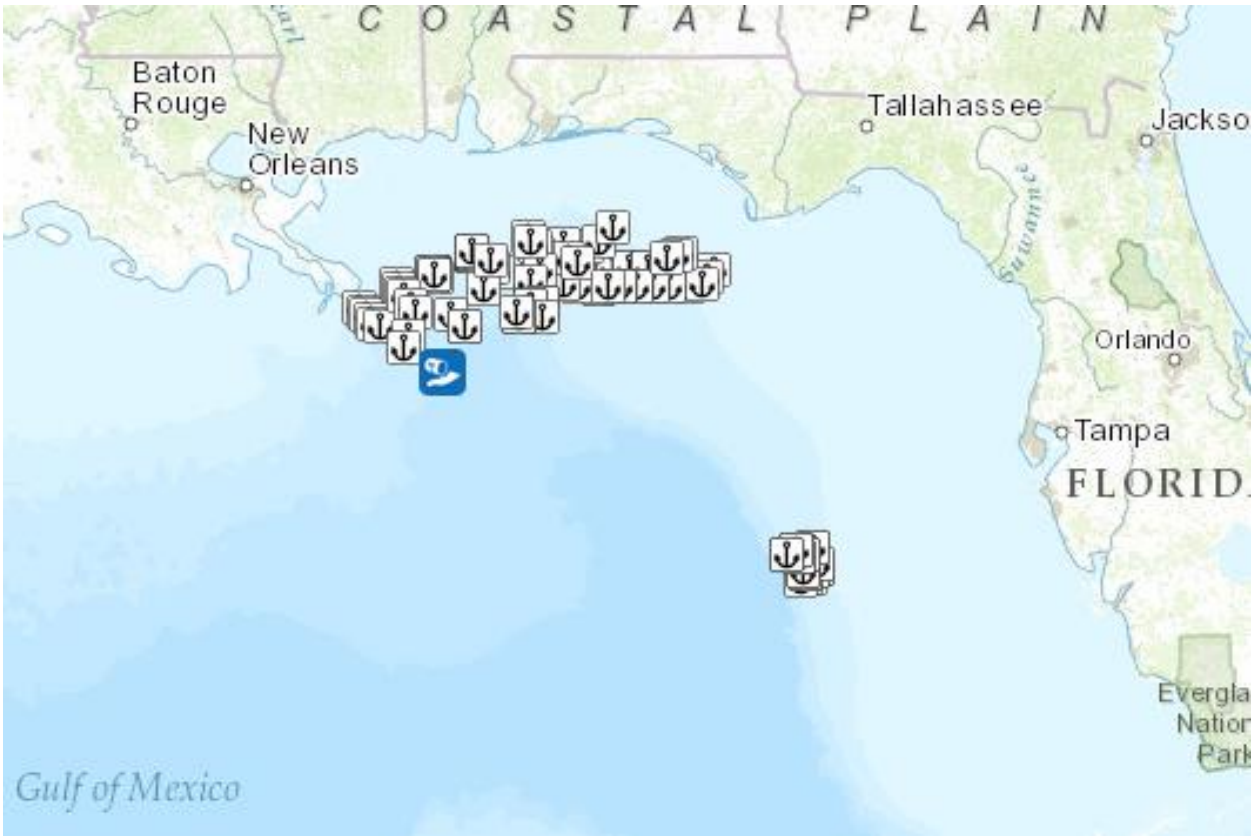

Figure 2.1 Map of Sampling Stations

Stations fished in the northeast Gulf of Mexico represented by anchors and origin of Deepwater Horizon is displayed as oil spill.

Liver Enzyme Activity

Hepatic CYP1A activity was measured in S9 fractions (or the cytosolic fraction) using a modified version of the ethoxyresorufin-O-deethylase assay described in Sepúlveda et al, (2004). Liver was homogenized (1: 5 w/v ratio) was homogenized in EROD homogenate buffer (10 mM TRIS (pH 7.4), 250 mM sucrose, 1 mM EDTA, 0.2 mM dithiothrietol, and 0.1 mM phenylmethylsulfonyl fluoride) using a bead homogenizer. Homogenates were centrifuged at 8,000*g* for 10 minutes at 4^{o}C. Supernatant was centrifuged a second time at 12,000*g* for 20 minutes at 4^{o}C and the resultant S9 fraction (the cytosolic fraction) was used to measure enzymatic activity of CYP1A. The EROD assay was performed using black 96-well microtiter plates, in which each well included 5 µL of homogenate (S9) was mixed with 193 µL of assay buffer (0.1 m NaPO4, pH 7.8) and 2 µL of substrate (100 mM ethoxyresorufin in methanol). The reaction was started by adding 5 µL of 5 mM NADPH per well, and fluorescence change at 530/25 ex, 590/35 em was measured every minute for 10 minutes. Each sample was assayed in triplicate alongside positive (5µl CYP1A standard) and negative (5µL EROD homogenate buffer) controls. GST activity was determined in the same homogenates using a commercially available Glutathione-S-Transferase Assay Kit (Cayman Chemicals, Ann Arbor, Michigan) following the manufacturer's instructions with minor modifications. Briefly, homogenates were diluted 1/5 in GST buffer, and EROD homogenization buffer was added to positive and negative controls at a quantity equivalent to that present in sample wells. Samples and controls were run in duplicate in clear 96-well microtiter plates. Both CYP1A and GST activity were normalized using protein concentration, which was determined via the Bradford assay (Bradford, 1976).

Bile FACs

Relative levels of biliary FACs were measured using fixed wavelength fluorescence following a modified protocol from a previously published study (Insausti et al., 2009). Briefly, bile was diluted 1/1000 in 48% ethanol and clarified by centrifugation for 5 minutes at 8,000*g* at 4^0C, using supernatant for analysis. The following PAHs (excitation/emission wavelengths in nm) were examined: naphthalene NPH (290/335), pyrene PYR (340/380), BAP benzo(a)pyrene (380/430), chrysene CHR (273/382) and phenanthrene PHN (256/380) (Pathiratne and Hemachandra, 2010). Eight-point standard curves were constructed for each PAH to use fluorescence to determine concentration equivalents (PHN 2-500ng/ml, CHR 2-500ng/ml, NPH 8-1000ng/ml, PYR 0.08-10ng/ml, BAP 0.039ng/ml). PAH standards were purchased from AccuStandard.

Statistical Analysis

Descriptive statistics were calculated for all biomarkers on a species-specific basis. Differences in biomarker levels among species and temporally within species were analyzed using nonparametric Kruskal-Wallis ANOVA followed by Dunn's Post hoc analysis because data did not fulfill criteria for parametric analysis. Because of a broad spatial range of bile samples from *U. cirrata,* nonparameteric Spearman correlation analysis was also performed using this dataset to determine if distance from the site of the oil spill was associated with variations in biliary FAC concentrations. Statistical tests were performed using Prism GraphPad.

Results

Overall Findings

A total of 675 individuals from 3 species (*L. chamaeleonticeps*: n =167, *U. cirrata:*, n = 351, *U. floridana*: n = 157) and 2 locations (sites in the Northeast Gulf of Mexico which are considered oiled: n = 629 and sites off of the West Florida shelf which are considered reference: n =46, *U. cirrata* and *U. floridana* only) were analyzed for at least one biomarker. Some biomarker analyses were not conducted on all species due to various reasons, e.g., insufficient samples for conducting analysis, loss of sample during processing.

Species Differences

Overall activity levels of detoxification enzymes were low, particularly CYP1A activity, which ranged from 0-28.5 nmol*$min^{-1}mg^{-1}$ protein with many non-detects. (Table 1). Although differences were not significant, positive detection rates for CYP1A were about 50% for all species in oiled sites as well as *U. floridana* from reference sites (Table 1). Levels for GST activity ranged from 0-86.23 pmol/min/mg protein (Table 1).

Table 2.1 Liver Enzymes in Bony Fishes
Hepatic liver enzyme levels Biomarker levels for each species (*L. chamaeleonticeps*- LCHA, *U. cirrata*- UCIR, *U. floridana*-UFLO) for oiled sites and reference sites (indicated by REF). Mean ± SEM, range (in italics) and N (in parentheses). Due to low detection rate of CYP1A, the number of positive detections/N is also provided.

Biomarker	LCHA	UCIR	REF UCIR	UFLO	REF UFLO
CYP1A	1.504±0.3762 *0.0-28.50* (102)	0.5571±0.09079 *0.0-5.029* (107)	0.02727±0.01830 *0.0-0.1500* (11)	1.142±0.2640 *0.0-12.24* (52)	0.9244±0.4262 *0.0-9.150* (22)
+ CYP1A/total	25/102	47/107	2/11	27/52	11/22
GST	11.34±0.9643 *0.0-36.92* (82)	15.33±5.043 *0.5900-86.23* (20)	2.380±0.0 *2.380-2.380* (1)	4.706±1.043 *1.100-9.310* (7)	22.51±6.700 *15.81-29.21* (2)

CYP1A activity was slightly higher in *L. chamaeleonticeps* and *U. floridana*, yet not statistically significant (Figure 2A). Differences for GST also proved not to be significant. GST activity was higher in in oiled sites compared to reference sites for *U. cirrata*. The contrary was observed for *U. floridana*. *U. floridana* had a small sample size, yet GST activity was higher in reference sites compared to oiled sites (Figure 2B).

PAH metabolite levels had pronounced SEM and large ranges for each species (Table 2).

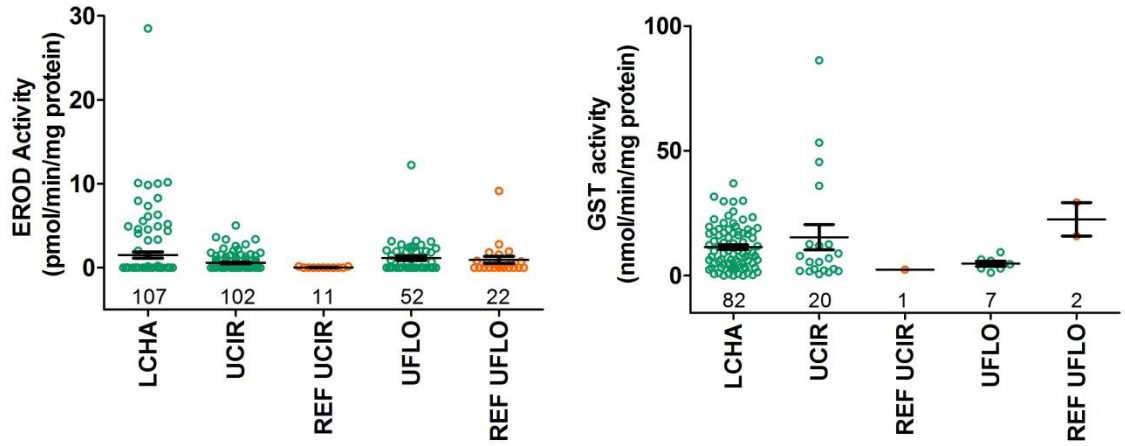

Figure 2.2 Liver Enzymes in Bony Fishes

A) Cytochrome P4501a1 (EROD activity nmol*min^{-1}mg^{-1} protein) and B) Glutathione-S-Transferase (pmol*min^{-1}mg^{-1} protein) for individuals grouped by species *(L. chamaeleonticeps*- LCHA, *U. cirrata*-UCIR, *U. floridana*-UFLO). Means are displayed as central line with error bars representing SEM. Sample sizes are shown. Pairwise significant differences are denoted by letters

Table 2.2 Bile Metabolites in Bony Fishes
PAH metabolite levels. A) Phenanthrene (ng/ml), B) Chrysene (ng/ml), C) Naphthalene (ng/ml), D) Pyrene (ng/ml), E) Benzo(a)pyrene (ng/ml) in individuals of each species (*L. chamaeleonticeps*- LCHA, *U. cirrata*- UCIR, *U. floridana*-UFLO) for oiled sites and reference sites (indicated by REF). Mean ± SEM, range (in italics) and N (in parentheses).

Biomarker	LCHA	UCIR	REF UCIR	UFLO	REF UFLO
PHN	217578±17568 *0.01.110e+006* (143)	21013±2292 *0.0-449647* (310)	12336±4545 *0.0-26708* (5)	27649±3863 *0.0-298820* (108)	37466±13793 *3175-279709* (21)
CHR	209828±13495 *0.0-563769* (130)	33323±2886 *0.0-527037* (302)	22114±4367 *8233-34366* (5)	45642±5829 *2088-470546* (108)	60557±21370 *11233-445400* (21)
NPH	129007±8599 *0.0-327733* (119)	14624±1327 *0.0-232576* (300)	9406±2899 *0.0-17512* (5)	28106±4181 *0.0-280206* (108)	34125±13682 *0.0-271692* (21)
PYR	763.1±89.40 *0.0-5567* (150)	272.3±36.75 *0.0-3961* (304)	87.02±52.22 *0.0-225.3* (5)	200.3±40.89 *0.0-2428* (108)	180.1±71.20 *0.0-1157* (21)
BAP	643.5±53.02 *0.0-4097* (150)	1140±75.09 *0.0-9904* (303)	582.3±185.8 *120.6-962.3* (5)	680.2±61.12 *0.0-3331* (108)	799.8±115.0 *78.13-1874* (21)

Differences between species indicated that *L. chamaeleonticeps* had significantly higher metabolite levels for most PAHs (PHN: $p<0.0001$, CHR: $p<0.0001$, NPH: $p=0.0006$, PYR: $p<0.0001$, and BAP: $p<0.0001$). Both *Urophycis* spp. had comparable metabolite levels in oiled sites, yet *U. cirrata* in reference locations had the lowest values (Figure 3A-E).

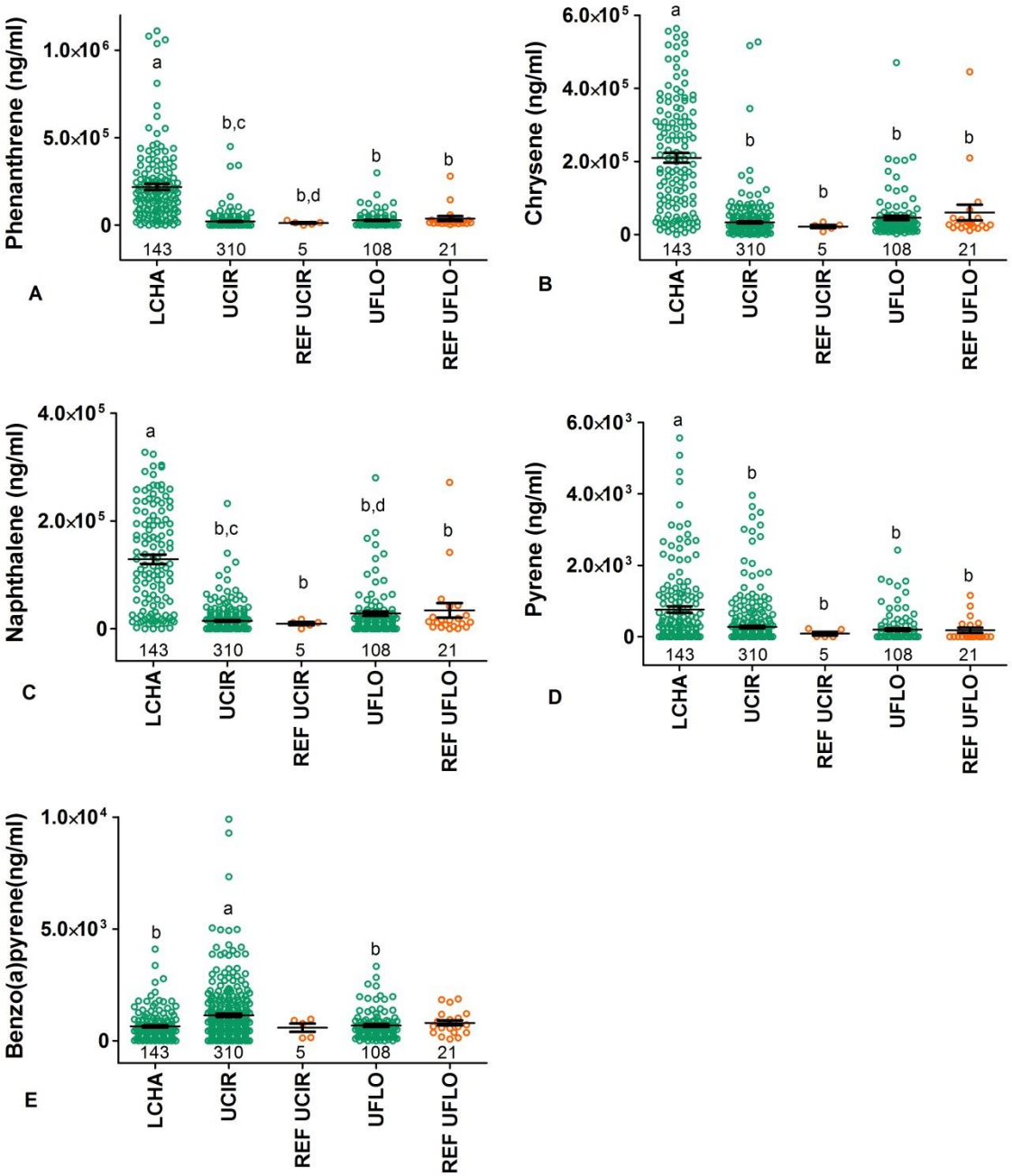

Figure 2.3 Bile Metabolites for Bony Fishes

PAH metabolite levels. A) Phenanthrene (ng/ml), B) Chrysene (ng/ml), C) Naphthalene (ng/ml), D) Pyrene (ng/ml), E) Benzo(a)pyrene (ng/ml) for individuals grouped by species (L. chamaeleonticeps- LCHA, U. cirrata- UCIR, U. floridana-UFLO). Means are displayed as central line with error bars representing SEM. Sample sizes are shown. Pairwise significant differences are denoted by letters

Temporal Differences

CYP1A activity in *L. chamaeleonticeps* from oiled sites (n=102) was significantly different by time of capture ($p<0.0001$). Levels of CYP1A activity in *L. chamaeleonticeps* were significantly higher in month 16 compared to later sampling periods, months 24, 39 and 42 (Figure 4A). Month 16 was also higher than month 12, yet sample size in month 12 is low. Significant differences (i.e., Month 27>16) with earlier sampling periods compared to later were also observed with GST activity, yet levels in subsequent periods increased (i.e., month 30) ($p<0.0001$, Figure 4B).

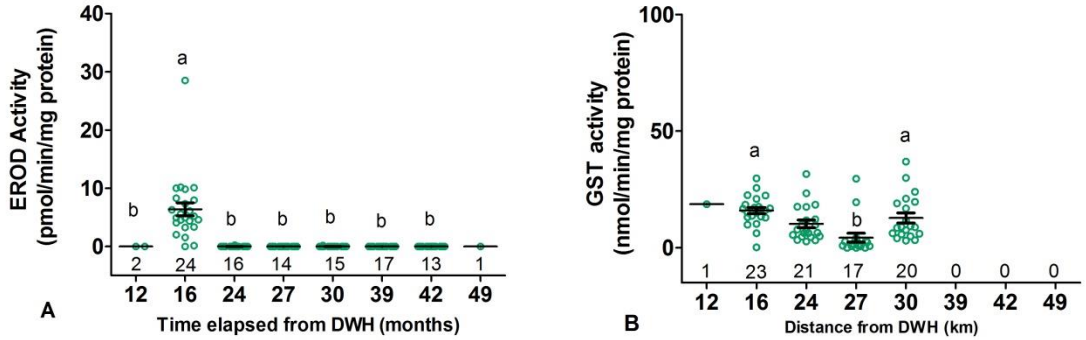

Figure2.4 Liver Enzymes for Lopholotilus chamaeleonticeps

A) CytochromeP4501a1 (EROD activity nmol*min^{-1}mg^{-1} protein) and B) Glutathione-S-Transferase (pmol*min^{-1}mg^{-1}) for individual *L. chamaeleonticeps* from northeast Gulf of Mexico sites captured 12-49 months after the Deepwater Horizon oil spill (DWH). Means are displayed as central line with error bars representing SEM. Sample sizes are shown. Pairwise significance is denoted by letters.

The concentrations of all PAH metabolites differed significantly by time of collection, with values observed in tilefish collected during Month 30 higher than those observed in fish from most other months ($p<0.0001$ for PHN, CHR, PYR, and BAP, $p=0.0006$ for NPH, Figure 5A-E).

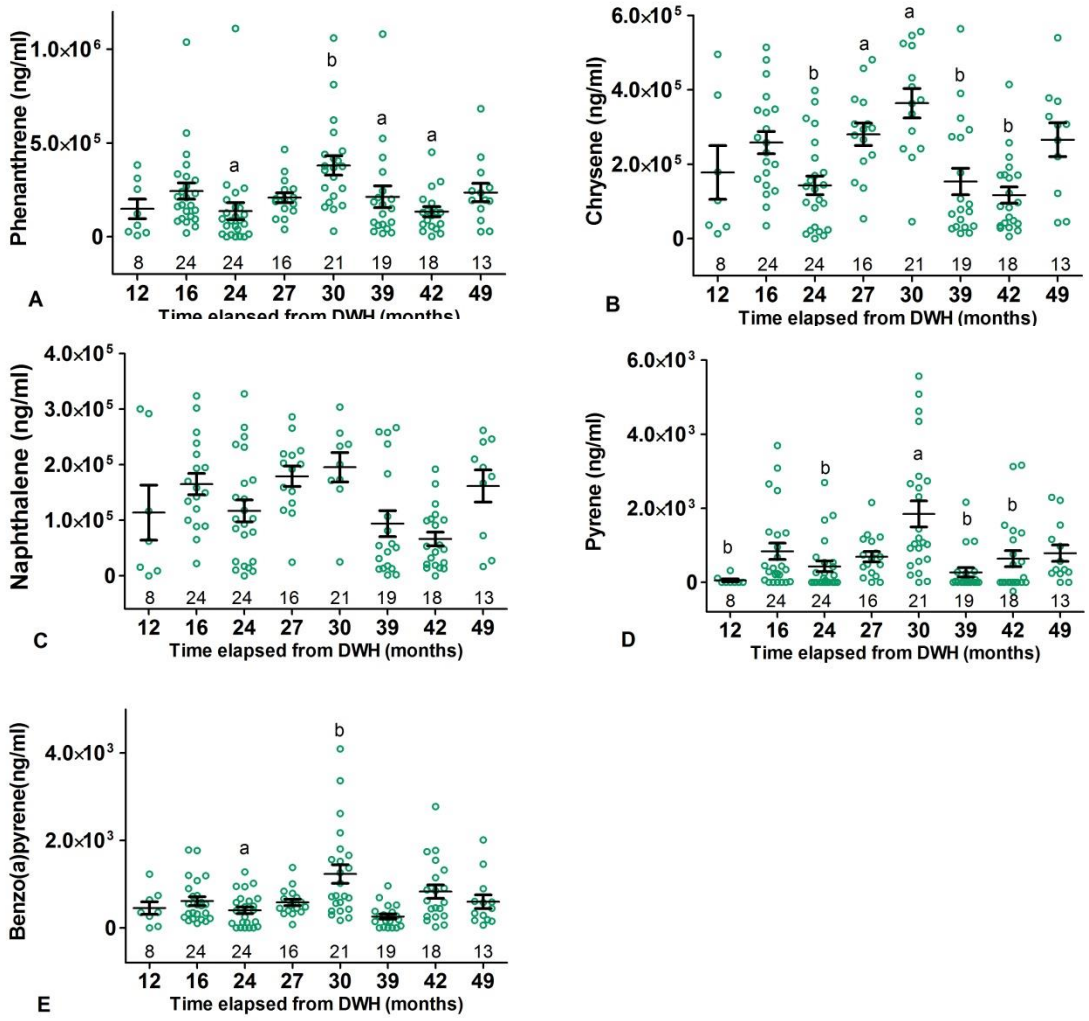

Figure 2.5 Bile Metabolites for Lopholotilus chamaeleonticeps

PAH metabolite levels. A) Phenanthrene (ng/ml), B) Chrysene (ng/ml), C) Naphthalene (ng/ml), D) Pyrene (ng/ml), E) Benzo(a)pyrene (ng/ml) for individual *L. chamaeleonticeps* from northeast Gulf of Mexico sites captured 12-49 months after the Deepwater Horizon oil spill (DWH).Means are displayed as central line with error bars representing SEM. Sample sizes are shown. Pairwise significance is denoted by letters.

CYP1A activity for *U. cirrata* (n=86) in oiled sites had more positive detections in earlier periods (i.e., month 16) compared to later as well as reference sites ($p<0.0001$, Figure 6A). No significance was observed for GST activity ($p=0.4556$), but this could be due to low sample size (N=20, Figure 6B).

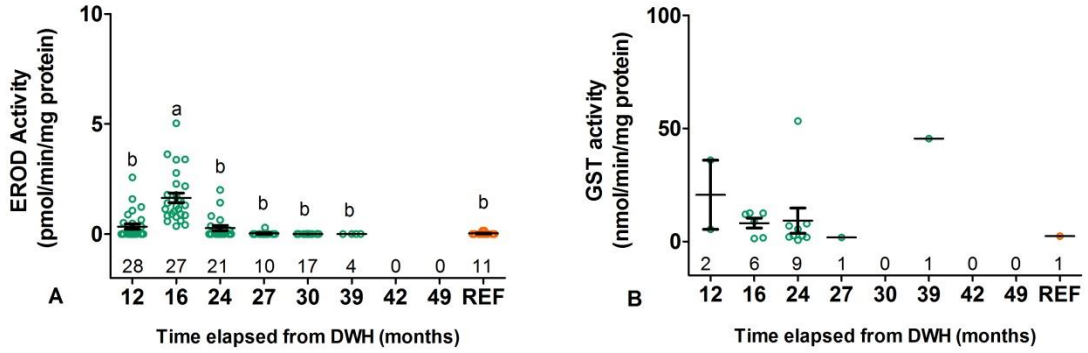

Figure 2.6 Liver Enzymes for Urophycis cirrata

A) Cytochrome P4501a1 (EROD activity nmol*min^{-1}mg^{-1} protein) and B) Glutathione-S-Transferase (pmol*min^{-1}mg^{-1} protein) for individual *U. cirrata* from northeast Gulf of Mexico sites captured 12-49 months after the Deepwater Horizon oil spill (DWH). Means are displayed a central line with error bars representing SEM. Sample sizes are shown. Pairwise significance is denoted by letters.

Levels of some PAH metabolites measured in *U. cirrata* collected from oiled sites differed significantly with time (PHN: p= 0.0162, CHR: p=0.0.674, NPH: p=0.1330, PYR: p=0.0032, and BAP: p= 0.0106) (Figure 7A-E). However, pairwise comparisons were not able to isolate individual groups that differed significantly.

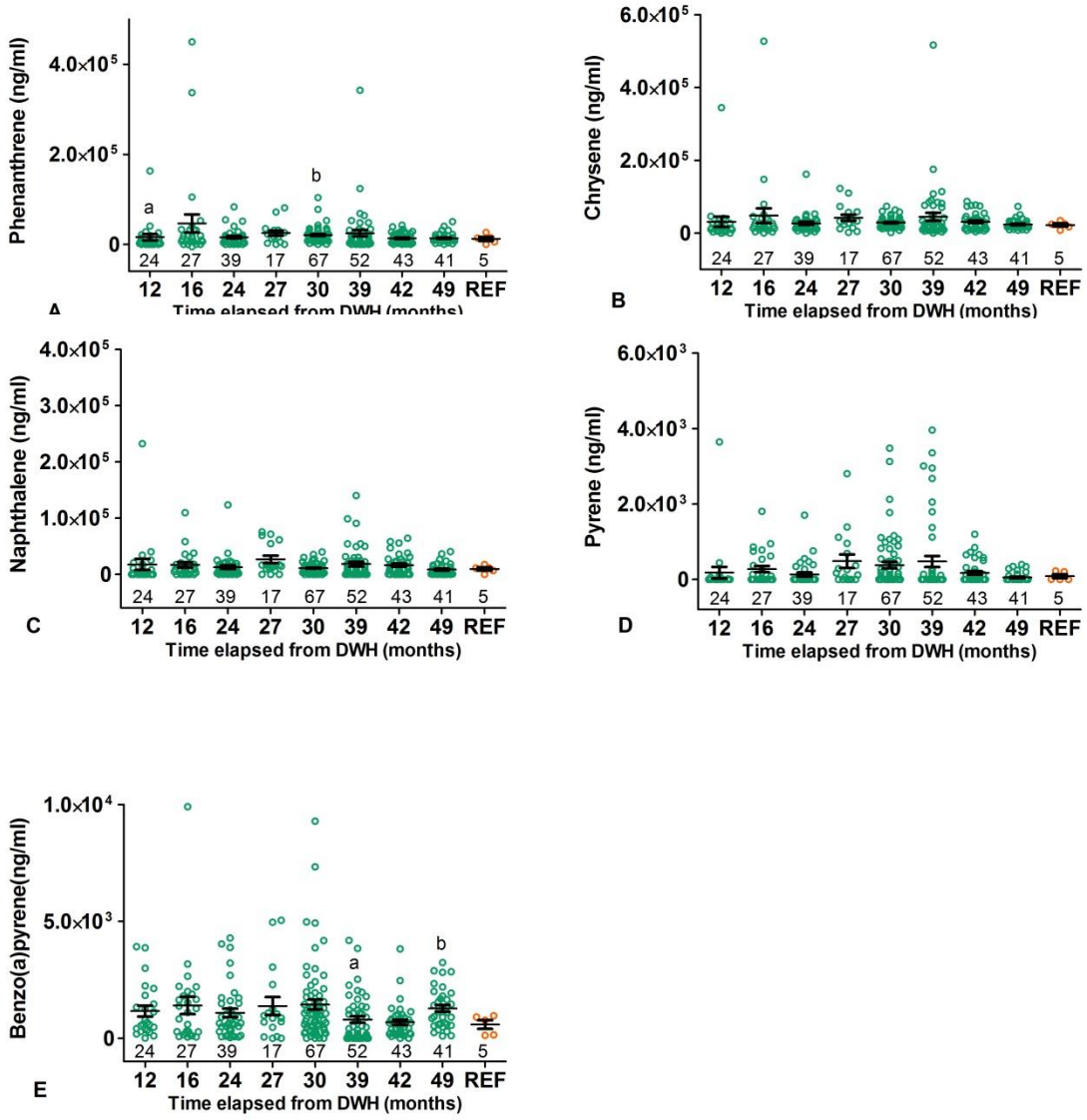

Figure 2.7 Bile Metabolites in Urophycis cirrata

PAH metabolite levels. A) Phenanthrene (ng/ml), B) Chrysene (ng/ml), C) Naphthalene (ng/ml), D) Pyrene (ng/ml), E) Benzo(a)pyrene (ng/ml) for individual *U. cirrata* from northeast Gulf of Mexico oiled sites captured 12-49 months after the Deepwater Horizon oil spill (DWH). Individuals collected from reference sites (REF) were captured 12-24 months after DWH. Means are displayed as central line and error bars represent SEM. Sample sizes are shown. Pairwise significance is denoted by letters.

CYP1A activity for *U. floridana* (n=77) had significant differences for sampling periods and site. Month 16 was statistically higher ($p<0.0001$) than other sampling periods in oiled sites and reference site (Figure 8). Further analysis of GST activity was not plausible due to low sample size.

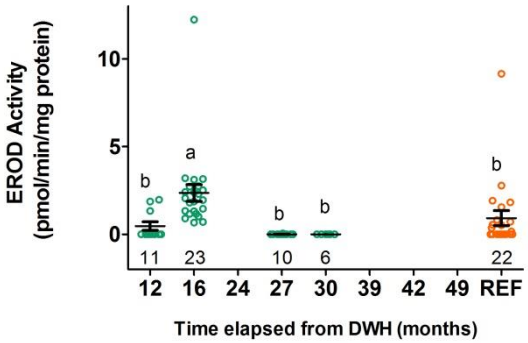

Figure 2.8 Liver Enzyme for Urophycis floridana

A) Cytochrome P4501a1 (EROD activity nmol/min/mg protein) and B) Glutathione-S-Transferase (pmol/min/mg protein) for individual *U. floridana* from northeast Gulf of Mexico sites captured 12-49 months after the Deepwater Horizon oil spill (DWH). Individuals collected from reference sites (REF) were captured 12-24 months after DWH. Means are displayed as central lines with error bars representing SEM. Sample sizes are shown. Pairwise significance is denoted by letters.

PAH metabolites did not differ by time of capture in *U. floridana*, except for PHN, (PHN: p= 0.0188, CHR: p=0.0806, NPH: p=0.1575, PYR: p=0.5332, and BAP: p= 0.3676) yet pairwise comparisons did not show differences for PHN (Figure 9A-E).

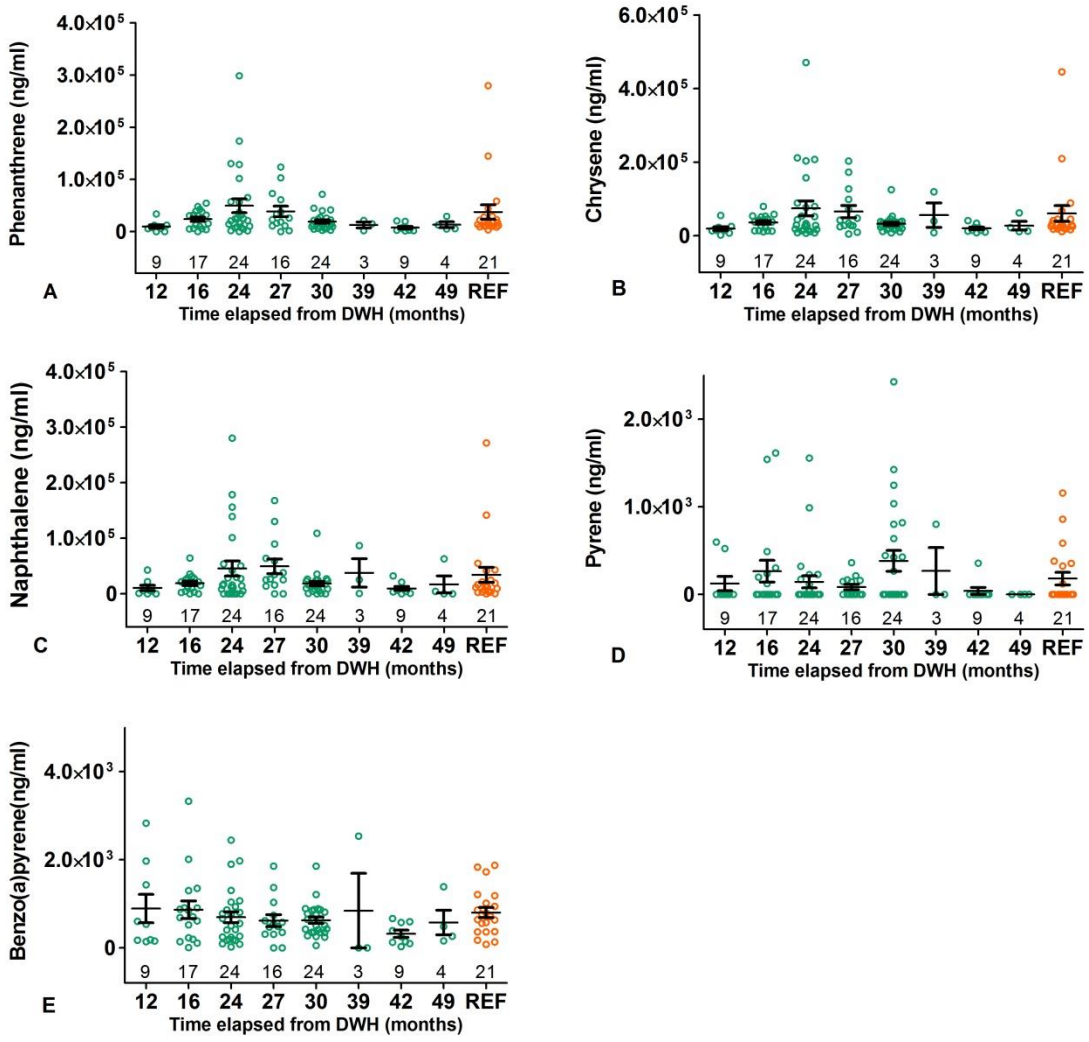

Figure 2.9 Bile Metabolites for Urophycis cirrata

PAH metabolite levels. A) Phenanthrene (ng/ml), B) Chrysene (ng/ml), C) Naphthalene (ng/ml), D) Pyrene (ng/ml), E) Benzo(a)pyrene (ng/ml) for individual *U. floridana* from northeast Gulf of Mexico sites captured 12-49 months after the Deepwater Horizon oil spill (DWH). Individuals collected from reference sites (REF) were captured 12-24 months after DWH. Means are displayed as central lines with error bars representing SEM. Sample sizes are shown. Pairwise significance is denoted by letters.

Spatial Differences

Levels of petrogenic biliary PAH metabolites in *U. cirrata* (n = 315) were also found to be significantly negatively correlated with distance from the location of the DWH oil spill. This was true for PHN ($r = -0.2768$, $p < 0.0001$), CHR ($r = -0.1517$, $p = 0.0079$) and NPH ($r = -0.1168$, $p = 0.0419$) (Figure 10A, B and C). In contrast, both PYR and BAP levels were not significantly correlated with distance from DWH ($r = 0.017$, $p=0.7954$ and $r = 0.0958$, 0.0932, respectively) (Figure 10D and E).

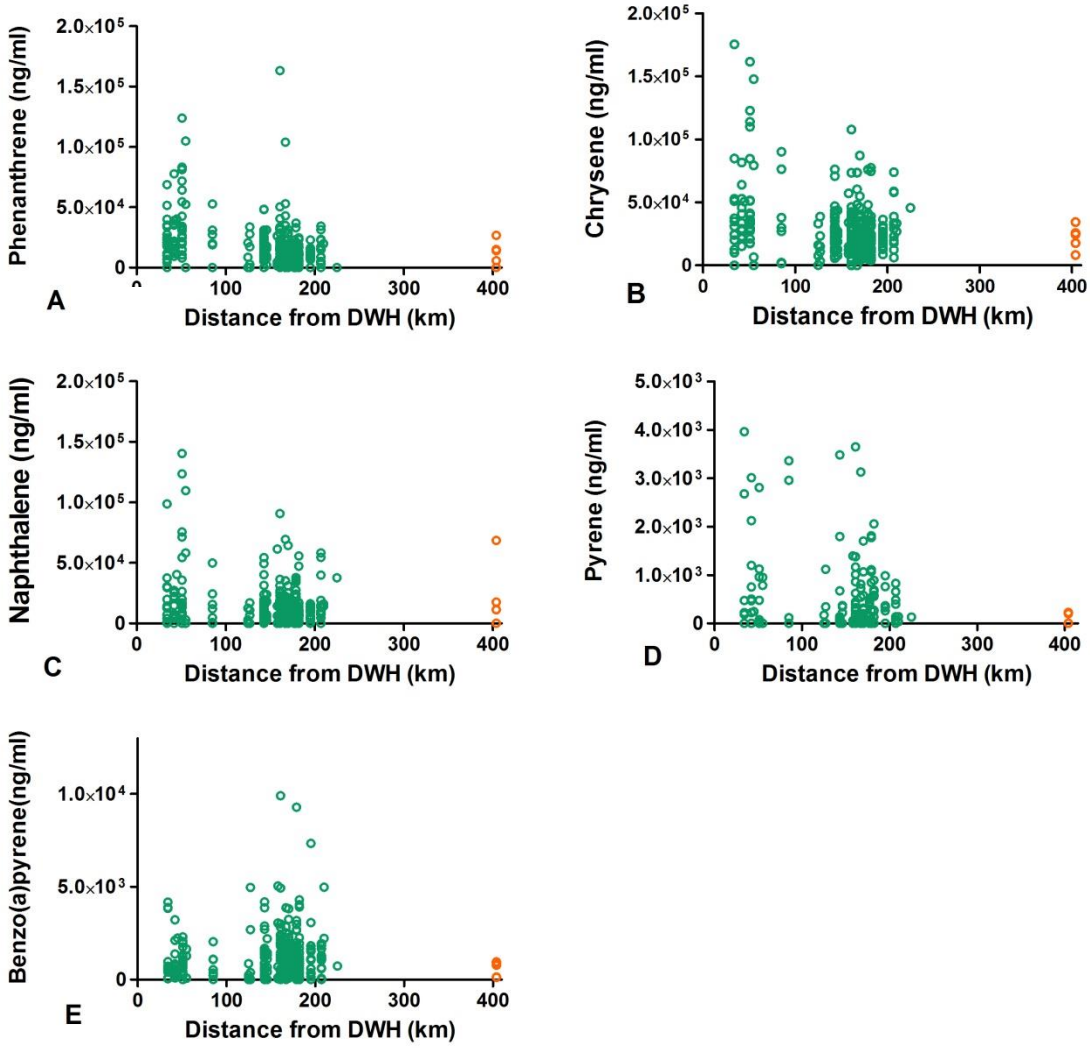

Figure 2.10 Bile Metabolites for Urophycis cirrata by Distance

A) Phenanthrene (ng/ml), B) Chrysene (ng/ml), C) Naphthalene (ng/ml), D) Pyrene (ng/ml), E) Benzo(a)pyrene (ng/ml) for individual *U. cirrata* (N=315) by distance from DWH (km). Individuals collected from reference sites located approximately 400km from the oil spill were captured 12-24 months after DWH, oiled sites are shown in blue and were captured throughout study (12-49 months post oil spill).

Discussion

The overall results of this study indicate species differences and some similarities with previous studies. CYP1A activity suggested temporal trends with relationship to the DWH, while GST appeared to be somewhat inconclusive. PAH metabolites not only provided some support for temporal trends but also spatial trends with proximity to the DWH.

CYP1A is considered to be one of the most robust PAH biomarkers, and one that the majority of oil spill studies have evaluated (Jung et al., 2011, Velando et al., 2010, Martinez-Gomez at al., 2009, Springerman et al., 2008, Jewett et al., 2002, Peters et al., 1999). The overall CYP1A activity in *L. chamaeleonticeps* is higher than other species in this study, perhaps suggesting higher exposure to PAHs in this species. Broad-spectrum differences among PAH metabolites were detected between the teleost species. Some PAH metabolites namely, phenanthrene, chrysene, naphthalene and pyrene were significantly higher in *L. chamaeleonticeps* compared to both *Urophycis* spp., suggesting higher metabolism of petrogenic PAHs. The burrowing habits of *L. chamaeleonticeps* situate it for increased exposure to lipophilic pollutants that will associate with the sediment, such as PAHs. Although metabolite concentrations were comparable between hake species, *U. floridana* had higher FAC levels than *U. cirrata* for PHN, CHR and NPH, yet metabolite levels for PYR and BAP were higher in *U. cirrata* than *U. floridana*, although neither of these differences were statistically significant.

Spatial trends indicate increased exposure in earlier sampling periods. Months 12 and 16 had CYP1A activity higher than later sampling periods and reference sites for the *L. chamaeleonticeps*. This suggests higher exposure to CYP-inducing contaminants, such as PAHs from oil in the Northeast Gulf or oiled sites compared to the west Florida Shelf or reference sites. This same trend was observed in *U. cirrata* and levels were also higher than reference sites. Overall CYP1A activity appears to be variable for different species from previous studies, ranging from 2-200pmol/min/mg protein (Raingeard et al., 2009, Sole et al., 2009, Aas et al. 2000). This may be an indicator of different metabolic pathways of PAHs in deep sea teleosts, a lower sensitivity to the induction of this enzyme by these compounds, or simply overall lower levels of enzyme activity due to minimal levels of typical exposure to CYP1A inducing compounds. The latter is considered because shallower-dwelling coastal species generally experience higher levels of pollutant exposure from anthropogenic activities. The two hake species had CYP1A activity levels that were slightly lower than previously reported for another hake species, *Merluccius merluccius*, after the Prestige Oil Spill (Raingeard at al., 2009). CYP levels in *M. merluccius* examined in Raingeard et al. (2009) ranged from about 0-25 pmol/min/mg protein. Depth ranges for animals in the Raingeard at el. (2009) study were 70-120m, which are much shallower than hakes in the current study, i.e., 191-760m. Depth has been paralleled with other enzyme activities in deep-sea fish, the deeper dwelling species had the lowest enzyme activity levels (Janssens et al., 2000). This depth difference may account for lower enzyme activity levels observed in the current study. Pollutant studies on other enzymes have indicated lower enzyme levels in deep sea fishes compared to shallow water counterparts

(Treberg et al., 2003; Janssens et al., 2000). One study indicated that *M. poutassou*, a benthic gladiform in the same order as *Urophycis* spp., collected at a depth of 660m in a highly anthropocentrically impacted site, had lower enzymes levels compared to other fish species in that study, i.e.,1-5 pmol/min/mg protein (Sole et al., 2009). CYP1A activity ranges in *U. cirrata* and *U. floridana* are similar for other deep dwelling, benthic gadiform fishes, such as the *M. poutassou* in Sole et al. (2009).

GST activity for this study was variable and did not exhibit significant spatial or temporal variations for any species. However, while it has often been used as a biomarker of oil exposure, GST levels have been shown to be an inconsistent reflection of pollutant exposure in past studies. For example, Gul et al. (2004) indicated lower GST activity in an area of higher pollution than in reference sites. In contrast, Napierka et al. (2006) observed significantly higher GST activity in the flounder in polluted sites compared to reference sites. GST plays a role in detoxification as well as other processes such as homeostasis and other physiological processes (Glisic et al. 2015) as well as involvement with immune protection (Arockiarajaet al., 2014), which will alter its abundance within an organism. GST is not as well studied with its regards to pollutants as CYP1A in fishes, yet its activity appears to be less sensitive to induction by pollutants (Andersson et al., 1985) and more variable due to other biological factors (Glisic et al. 2015, Arockiarajaet al., 2014). Additionally, GST activity levels in an organism will also be dependent of the availability of its cofactor, glutathione (Younes, Schlchting and Siegers, 1980).

Last, GST activity appears to be more sensitive to diet state of organism, e.g., Younes et al. (1980) found that GST activity was higher in fasting mice.

Biliary concentrations of petrogenic PAH metabolites in *U. cirrata* were higher in NE Gulf animals compared to reference animals collected from the west Florida Shelf. This suggests higher exposure and metabolism of PAHs from an oil-related source in this area, perhaps reflecting PAHs from the DWH. Both species of *Urophycis* had slightly lower metabolite levels compared to *M. merluccius* (the European hake) (Insuasti et al., 2009). However, Insausti et al. (2009) also observed lower metabolite levels in the deeper dwelling gadiforms, such as the *Phycis blennoides* and *Trachyrhynchus scabrous,* which are more similar to the ranges observed in the current study. The lack of increase in benzo(a)pyrene indicates metabolism of oil-related pollutants, potentially from the DWH. Additionally, concentrations of the petrogenic PAH, PHN, in *L. chamaeleonticeps* were found to be higher than previously reported levels in polluted sites for closely related species, *Pagellus bogavareo* a benthic fish with similar depth ranges as *L. chamaeleonticeps* and in the same order (Perciformes) (Insuasti et al., 2009). Other PAHs, namely NAPH, PYR and BAP, had slightly lower concentrations; CHR was not evaluated in this study (Insausti et al., 2009). Lastly, *U. cirrata* collected from sampling sites with closer proximity to the origin of the DWH had higher PAH metabolite concentrations, specifically petrogenic, than further sampling sites and reference sites. This suggests that *U. cirrata* experienced exposure and metabolism of oil related pollutants in the regions closest to the DWH.

Both temporal and spatial trends in relation to proximity to the DWH were observed. These results suggest exposure and metabolism of PAHs from a source, such as the DWH. Other significant differences observed may be attributed to changes of bioavailability of PAHs in environment. For example, there was an unexplained increase in biomarkers in month 30. Interestingly, from August 27-30, 2012 Hurricane Issac moved through the Gulf of Mexico, a period of time shortly prior to sampling month 30 (Berg, 2013). It is possible that this event could have redistributed PAHs settle within sediment. Additionally these biomarkers may fluctuate due to a variety of sources and have a fairly quick turn over time (Ferreira et al. 2006). This increase could also represent PAHs shifting into these fishes trophic level after being incorporated at much lower levels. As the sampling of this study started a year the DWH, the likely exposure route is diet. These compounds would have to be incorporated into the food web. The sampling periods initially after the DWH is absent in this study, which would likely indicate higher values than those reported in this study. The perceived delay may indicate that PAHs have been introduced into the food web and over time moved into higher trophic levels, where these teleost reside.

Laboratory studies support that these biomarkers provide a snapshot of recent exposure in an organism to PAHs. Ferreira et al. (2006) observed increased CYP1A levels up to 1 month after a single exposure to PAHs. Gorbi and Regoli (2004) found accumulation and excretion of bile metabolites after exposure to occur after about a week, and Ferreira et al. (2006) observed a significant decrease in metabolite levels 4 months after a single dose

exposure to PAHs. This would suggest that fishes in current study have been recently exposed to PAHs.

In conclusion, species within this study exhibited levels of PAH biomarkers that indicate exposure and metabolism of petrogenic PAHs likely from the DWH. Further studies are needed to determine cellular and organismal level effects that could lead to population alterations. *L. chamaeleonticeps* also require further attention, due to higher biomarker levels compared to other species collected in similar regions. The burrowing habits may be the cause for this species to experience increased exposure through the gills from the water column resulting in metabolism of PAHs. The route of exposure should be examined in future studies, possibly evaluating enzyme activity in the gills. Additionally, muscle should be evaluated or contamination, due the long line fishery and human consumption of this fish. A fishery management plan should also be consider for *L. chamaeleonticeps*, due to its longevity and uncertainty for chronic and/or population level effects.

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Vita

Education

Associates in Arts: Biology, Florida State Community College 2009
Bachelors' of Science: Coastal Biology, University of North Florida, 2012

Research Experience

2012-2014 MS Thesis: Multiple Polycyclic Aromatic Hydrocarbon (PAH) biomarker approach to determining oil exposure from Deepwater Horizon Oil Spill in deep sea fishes, processed over 1000 samples for several biomarkers.

2011-2012 Undergraduate: Evaluating effects of the Deepwater Horizon Oil Spill on coastal and epipelagic species in the Gulf of Mexico using fixed wavelength fluorescence (FF) of polycyclic aromatic hydrocarbon (PAH) metabolites in bile, over 400 samples analyzed.

Recent Research Presentations

2014 Society of Environmental Toxicology and Chemistry (Interactive)
American Elasmobranch Society (Platform)
Gulf of Mexico Oil Spill and Ecosystems (Poster)
Deep C Consortium Meeting (Platform)
UNF Biology, Chemistry and Physic Student Research Session (Poster)

Relevant Work Experience

2012-2014 Graduate Research Assistant
University of North Florida

2013 Graduate Teaching Assistant for General Biology 1 Lab
University of North Florida

Scholarships and Awards

2014 American Elasmobranch Society Travel Grant $600; Gulf of Mexico Research Initiative Scholar Award
2013 Guy Harvey FL Sea Grant: $5,000 ; UNF Coastal Biology Travel Grant $1,000; SETAC Travel Grant $500
2012 Smart Grant for Undergraduate Research: $1,500; Latin honors (B.S.)
2010 Dean's List and President's List (A.A.)

Outreach

Science Crew for OCEARCH Expedition Jacksonville 2013, and Fishing and Filming with National Geographic Monster Fish for an episode on
Bull Sharks