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Assessing the Effects of Pollutant Exposure on Sharks: A Biomarker Approach

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ASSESSING THE EFFECTS OF POLLUTANT EXPOSURE ON SHARKS: A BIOMARKER APPROACH

by

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CERTIFICATE OF APPROVAL

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Abstract

Many hydrophobic environmental pollutants have been shown to bioaccumulate and biomagnify at high levels in sharks due to their high liver lipid content, high trophic level, and life history characteristics. Studies have demonstrated that the levels of pollutants present in shark tissues can not only exceed the recommended levels for human consumption, but that, in some cases, they are also greater than the threshold for physiological effect in other aquatic species. However, few studies to date have investigated the biological effect of environmental exposure to contaminants in sharks. Therefore, the goal of this study was to investigate, through the use of biomarkers, if sharks are experiencing physiological effects due to exposure to 1) methylmercury (MeHg) and 2) polycyclic aromatic hydrocarbons (PAHs). The results of the first part of the study indicated that total mercury (THg) concentrations (μg/g w.w.) in *Sphyrna tiburo* muscle tissue were positively correlated with size of the animals, but that metallothionein (MT), a commonly used biomarker for toxic metal exposure, was not a valid biomarker for Hg exposure in this species, as no correlation between MT in muscle or liver and THg was found. The later portion of the study demonstrated that sharks off the coast of Alabama that were exposed to oil from the *Deepwater Horizon* oil spill (DHOS) in 2010 are exhibiting biochemical effects in the form of induced activity of the Phase I biotransformation enzyme, cytochrome P4501A1 (CYP1A1). Further research on the effects of both MeHg and PAH exposure in sharks should focus on the effects experienced by larger species and those at higher trophic levels, which are known to harbor higher levels of contaminants, and therefore be affected to a greater extent, than the species analyzed in this study (i.e. small species occupying lower trophic levels).

Introduction

Environmental pollutants have long been regarded as potential threats to the health of aquatic species, which often harbor high levels of many contaminants (Poulin et al., 2008; Van der Oost et al., 2003). Persistent organic pollutants (POPs), such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins (PCDDs), and organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT), pose the greatest threat to aquatic organisms due to their high lipid-solubility, which enables the contaminants to associate with organic materials and aquatic sediments (Gelsleichter and Walker, 2010). The association between POPs and substrates promotes the uptake of these compounds into aquatic organisms through the diet, resulting in their bioaccumulation or increase in pollutant concentrations in organisms over time as well as their biomagnification or increase up the food chain resulting in higher concentrations of chemicals in higher trophic levels.

As top marine predators, sharks are particularly susceptible to bioaccumulation and biomagnification due to their high lipid content, long life span, high trophic level, slow growth and metabolism (Endo et al., 2008; Hueter et al., 1995; Van der Oost et al., 2003). Additional factors such as the large-scale migrations of pelagic species and the utilization of near-shore areas for breeding and/or nursery grounds may allow for increased exposure to contaminants, including exposure to chemicals that have been previously banned in urbanized countries, but are still widely used in developing ones (Gelsleichter and Walker., 2010; Torres et al., 2008). For example, organochlorine contaminants and/or brominated flame retardants have been measured in sharks (e.g. bull sharks *Carcharhinus leucas*, Atlantic sharpnose sharks *Rhizoprionodon terraenovae*, bonnetheads *Sphyrna tiburo*, sandbar sharks *C. plumbeus*, and blacktip sharks *C. limbatus*) in

Florida and were found accumulate at levels that may elicit adverse biochemical responses in these species (Gelsleichter et al., 2005, 2007, 2008; Johnson-Restrepo et al., 2005, 2008) Additionally, in a review of pollutant exposure and effects in elasmobranchs by Gelsleichter & Walker (2010), it was found that several of the species, such as Greenland sharks *Somniosus microcephalus,* bull sharks *C. leucas,* and tiger sharks *Galeocerdo cuvier,* examined to date displayed levels of certain environmental pollutants that were close to or exceeded the amounts recommended for human consumption in some countries. For example, in the case of methylmercury (MeHg) exposure, approximately 70% of the 75 species that had been analyzed for MeHg showed mercury levels at or above the recommended limit for monthly consumption (i.e., 0.49-0.94 mg Hg/month) for a 70 kg person (i.e., the average weight of both male and female Americans; USEPA, 2004). Stride t al. (2007) also reported that while concentrations of PCDDs and polychlorinated dibenzofurans (PCDFs) on their own did not exceed limits for human consumption, the combined levels of the two compounds did. Although the remaining contaminant levels reported in the Gelsleichter & Walker (2010) review were not found to be above limits for human consumption, concentrations of most compounds were found to be highest in the larger species that occupy higher trophic levels, thereby confirming the bioaccumulation and biomagnification of some contaminants in sharks.

Due, in large part, to the growing concern for human exposure to environmental contaminants through fish consumption, the vast majority of studies investigating pollutant exposure in sharks and their relatives have focused primarily on the level of a contaminant present in muscle tissue of these animals rather than the effect of the contaminant on the health of the organism. However, some studies have demonstrated that the levels of pollutants present in shark tissues not only exceed the recommended levels for human consumption in some cases, but that they are also greater than the threshold for physiological effect in some teleosts species (Adams et al., 2010). Some of these effects include liver necrosis in spotted seatrout *Cynosciun nebulosus* (Adams et al., 2010) and altered behavior in golden shiners *Notemigonus crysoleucas*

(Webber & Haines, 2003) following exposure to mercury, as well as changes in the hypothalamic-pituitary-thyroid axis in adult fathead minnows *Pimephales promelas* exposed to polybrominated diphenyl ether-47 (PBDE-47), a widely used flame retardant (Lema et al., 2008). What is more, a study by Johnson-Restrepo et al. (2005) examined PBDE-47 levels in bull sharks *C. leucas* and found that PBDE-47 levels in bull shark muscle were approximately four times higher than the total body burden of the high-dose treatment of fathead minnows in the Lema et al. (2008) study. Exposure to addtional POPs, such as PAHs, has also been associated with the formation of DNA adducts, hepatic lesions and neoplasms, and cell death (Myers et al., 2008; Varanasi et al., 1987; Baumann et al., 1998). Still, with the exception of some laboratory studies on *in vitro* exposure to toxic metals (Redding, 1992; Kinne-Saffran, 2001), very few studies to date has investigated the biological effect of exposure to environmental contaminants in sharks (Fuentes-Rios et al., 2005).

Therefore, considering the evidence for bioaccumulation and biomagnification of environmental contaminants in sharks and the resulting adverse effects observed in various organisms, the goal of this study was to investigate if elasmobranchs are experiencing physiological effects due to exposure to select environmental contaminants. To accomplish this, I examined the use of biochemical markers, or biomarkers, as a method to detect the occurrence of adverse biological effects in elasmobranchs caused by exposure to two specific environmental toxicants, MeHg and PAHs. Biomarkers are specific biological features that may be used to characterize the biological effects that contaminants or diseases have on an organism. The use of cellular or molecular biomarkers has been suggested as an "early warning" tool for assessing pollutant availability and exposure effects in a variety of organisms (Van der Oost et al., 2003). It was hypothesized that, in the case of both MeHg and PAHs, biomarker responses would be higher in animals with increased levels of exposure to contaminants compared to animals that have had little to no exposure to these compounds.

Chapter One:

The effect of mercury exposure on bonnethead sharks

Introduction

Mercury (Hg) is a highly toxic, prevalent non-essential metal in aquatic environments (Poulin et al., 2008). It is deposited into aquatic environments primarily by anthropogenic activities (e.g., mining, waste incineration, combustion of fossil fuels) in its inorganic elemental form. Once Hg is deposited into an aquatic system, it undergoes bacterial methylation to produce the organic compound, monomethylmercury (MeHg; Storelli et al., 2002). Monomethylmercury is considered to be the most toxic form of mercury in the environment and is readily absorbed by aquatic organisms via the gills and digestive system (Huang et al., 2007). It is known to bioaccumulate and biomagnify in aquatic species and, in general, more than 90% of the total mercury content in fish muscle is in the form of MeHg (Adams & McMichael, 1999; Bebianno et al., 2007). Due to bioaccumulation and biomagnification, MeHg levels in fish muscle can range from one to ten million times greater than MeHg levels present in the corresponding aquatic environment, possibly resulting in adverse physiological effects such as behavioral changes, cerebral lesions, and impaired reproductive development (Wiener et al., 2003).

In a review by Gelsleichter & Walker (2010), it was found that mercury is the most widely studied toxicant in cartilaginous fish, with levels having been examined in a minimum of 75 species. Roughly 70% of these studies found muscle Hg concentrations that were considered to be within or exceed the maximum recommended limit for human consumption in most countries (i.e., 0.49-0.94 mg Hg/month for a 70 kg person in the United States; USEPA, 2004). Such high levels are primarily due to bioaccumulation and biomagnification of Hg in sharks, which is supported by the strong positive correlation between length and muscle Hg level in a number of elasmobranch species such as bonnetheads *S. tiburo* (Adams &

McMichaels, 1999; Evers et al, 2008). In addition, Jeffree et al. (2006) reported elevated levels of Hg and other toxic metals in the egg cases of the oviparous lesser-spotted dogfish *Scyliorhinus canicula*, indicating that embryos can begin to bioaccumulate these metals even before birth.

Despite the multitude of studies that have shown the presence of elevated Hg in sharks (Branco et al., 2004, 2007; Endo et al., 2008; McMeans et al., 2007; Storelli et al., 2002), few studies to date have attempted to explore the possible biological effects of exposure to Hg in these animals. Of the few that have, Redding (1992) and Kinne-Saffran and Kinne (2001) demonstrated that exposure to mercuric chloride resulted in decreased spermatogonial proliferation in the testis and inhibited chloride secretion in the rectal gland, respectively, in the spiny dogfish *Squalus acanthias*. The effects of MeHg exposure on teleosts, however, have been studied much more extensively, showing impaired gonadal development, emaciation, behavioral alterations, decreased sperm motility, and cerebral lesions following exposure (Hoffman et al., 2003; Khan & Weis, 1987; Jezierska et al., 1995). More recent studies have observed additional effects in teleosts due to mercury exposure, including renal and hepatic lesions, neurochemical alterations, and changes in blood-plasma chemistry (Adams et al., 2010).

Metallothioneins (MTs) are reliable molecular biomarkers for metal exposure in several species of fish and mammals (Atli & Canli, 2008; Cho et al., 2005; Ying et al., 2000), making them a possible biomarker for metal exposure in sharks as well. MTs are low molecular weight (6-7 kDa), cysteine-rich, intracellular proteins present in most vertebrates (Hylland et al., 1995; Cho et al., 2005). MTs display a high affinity for lipids resulting in high concentrations being found in the liver, however MTs are most frequently studied in muscle tissue due to concerns regarding human consumption of toxic metals (Bebianno et al., 2007). The functions of MTs include the detoxification and homeostasis of metal ions, roles in cellular growth and repair, and free hydroxyl radical scavenging (Thirumoorthy et al., 2007; Ying et al., 2000). Mercury increases MT gene transcription and synthesis (Alvarado et al., 2006, 2007; Cho et al., 2005; Ma et al., 2008). The inducible nature of MT makes it a potentially useful biomarker for heavy metal pollution in sharks and their relatives. A few laboratory-based experiments have confirmed the use of MT as a

biomarker for toxic metal exposure in some shark species (Betka & Callard, 1999; Cho et al., 2005), however it has not been extensively put to use in field studies on elasmobranchs.

The goal of this study is to evaluate the use of MT as a biomarker for detecting toxic metal exposure in field studies on sharks. In particular, this study will examine the reliability of using metallothionein as a biomarker for metal exposure in the bonnethead shark *Sphyrna tiburo*. *S. tiburo* was chosen as the target species for this study based on the available evidence for high mercury uptake in this species (Adams & McMichaels, 1999; Evers et al., 2008), the large database of accessible samples, as well as the extensive body of knowledge on the biology and movement of this species from the areas sampled (Carlson & Parsons, 1997; Cortes et al., 1996; Lombardi-Carlson et al. 2003). This will be accomplished by determining if muscle MT levels are correlated with *S. tiburo* muscle Hg concentrations and, if so, by comparing MT levels in *S. tiburo* populations from three estuaries on Florida's Gulf coast, chosen based on the availability of samples from these areas as well as evidence for differences in pollutant exposure in S. tiburo at these sites (Manire, 2002). It is hypothesized that based on the evidence for MT induction in response to heavy metal exposure, MT levels will increase proportionally along with increased Hg uptake and *S. tiburo* populations will reflect a difference in MT levels based on the catch location with populations from more Hg-contaminated sites having higher MT levels than populations from less Hg-contaminated sites.

Methods

-Animal Collection-

Bonnethead sharks *Sphyrna tiburo* (n = 50) were collected using gill nets between 1998 and 2001 from three Florida Gulf coast locations (Figure 1.1): Tampa Bay/Anclote Key (AK; n = 15), Florida Bay (FB; n = 20), and Apalachicola Bay (PC; n = 15). Sharks were weighed to the nearest 0.1 kg, measured to the nearest 1.0 cm, and further examined in order to determine sex. Specimens ranged in total length (TL) from 56 cm to 103 cm.

-Biological Sample Collection-

Following capture, sharks were rinsed with ambient seawater and packed in ice until arrival at the laboratory. Once at the lab, each shark was rinsed with running local tap water and 1 g of tissue was removed from the lateral muscle just below the dorsal fin and placed in a cryovial, which was subsequently placed in liquid nitrogen in order to prevent moisture loss during the freezing process. Samples were stored at -80ºC until time of analysis in 2009. Duplicate muscle samples were collected from every fifth animal and stored in the same manner. Liver samples were also collected (~1 g) from the ventral edge of the right lobe of the liver, placed in cryovials, frozen with liquid nitrogen, and then stored at -80ºC.

Samples were collected from the specimens at different laboratories and were rinsed using water from different municipal water supplies. Therefore there was some chance of sample exposure to trace Hg through rinsing, but this was not controlled for.

Figure 1.1. Map of Florida showing the location of the three study sites used for the collection of samples in this study (Apalachicola Bay = PC, Tampa Bay = AK, Florida Bay = FB).

-Mercury Analysis-

Muscle samples were dried for 48 hr at 60°C in an oven, using aluminum weight boats to minimize cross-contamination, homogenized using a mortar and pestle, and stored at 4 °C until analysis. Samples were weighed before and after drying in order to monitor water content and reduction throughout the process. Percent moisture was determined using the formula,

$$\%moisture = 100 - \left(\frac{W_d}{W_w}\right)x100 \quad ,$$

where W_d = weight of dry sample (g) and W_w = weight of weight sample (g).

Total mercury (THg; μg/g dry weight) was measured in *S. tiburo* muscle samples by the Florida Fish and Wildlife Commission - Fish and Wildlife Research Institute Indian River Field Lab (Melbourne, FL, USA) by EPA Method 7473 using a direct mercury analyzer (DMA-80) (Nam et al., 2011).

THg was presented in μg/g d.w. in all figures. However, because the majority of previous studies on THg in elasmobranchs have reported concentrations in μg/g wet weight (w.w.) (Gelsleichter and Walker, 2010), dry weight measurements have been converted to their wet weight equivalents, and are reported for comparative purposes.

-Western Blotting-

Western blot of MT was performed using a commercially available polyclonal rabbit anti-cod MT antibody (1:500; Biosense Laboratory, Norway), previously shown to cross-react with *S. tiburo* MT (Gelsleichter, unpublished data).

Muscle samples were homogenized in 3 volumes of buffer (100 mM Tris-HCl with 35 μl β-mercapethanol, pH 8.1) (Hylland et al., 1995) using the FastPrep-24 bead homogenizer (MP Biomedicals, Inc., US) and homogenates were centrifuged at 18,000 x g at 4 ºC for 1 hr. The resulting supernatant was stored at -80ºC in three 200-μl aliquots until the time of analysis. A standard Bradford protein assay, using 1/50 sample

dilutions, was used to determine the protein concentration (mg/ml) of each sample (Bio-Rad Laboratories, Hercules, CA, USA).

Proteins were separated via SDS-PAGE gel electrophoresis under denaturing and reducing conditions using 15% polyacrylamide gels and a Laemmli buffer system. Gels were fixed in a standard fixation solution (40% methanol, 10% glacial acetic acid) and stained with a fixation solution with 0.3% Coomassie blue in fixation solution.

For immunoblotting, proteins were electrotransferred to a PVDF membrane (Bio-Rad Laboratories**,** USA) and blocked in 10% nonfat dried milk in Tris-buffered saline (NFDM-TBS) overnight at 4ºC to prevent non-specific binding. Immunoreactive MT was detected using a polyclonal rabbit anti-cod MT primary antibody (Biosense Laboratory, Norway; diluted 1: 500 in 1% NFDM-TBS with 0. 05% Tween 20; incubated overnight at 4 °C), goat anti-rabbit IgG (whole molecule)-alkaline phosphatase secondary antibody (Sigma, USA; incubated 1 hour at room temperature), and the alkaline phosphatase chromogen 5-bromo-4-chloro-3'-indolyphosphate *p*-toluidine salt/nitro-blue tetrazolium chloride (BCIP/NBT) (Vector Laboratories**,** USA). Membranes were washed thoroughly in TBS with 0. 05% Tween 20 between each incubation. Following color reaction, membranes were rinsed in deionized water and air-dried. Signal intensity for each sample was acquired using the Gel Logic Imaging System (Carestream Heath, Inc., Rochester, NY, USA) and calculated using Kodak Molecular Imaging Software.

-Statistical Analyses-

Total Hg (THg; µg/g w.w.) was compared to the total length (cm) and to MT_{liver} and MT_{muscle} signal intensity for each animal using Spearman's Rank Order Correlation. Differences in mean THg and mean signal intensity between sites were analyzed using one-way ANOVA. Pearson's product-moment correlation was used to examine if MT_{liver} signal intensity and MT_{muscle} signal intensity were significantly correlated.

Results

-Total Hg (THg)-

Total mercury levels ranged from 0.86 to 6.58 μg/g dry weight (d.w.) (mean±sd = 3.00±1.82 μg/g d.w.), which correspond to wet weight (w.w.) values of 0.22-1.78 μg/g w.w. (mean±sd = 0.79±0.49 μg/g w.w.) in *S. tiburo* muscle tissue (Table 1.1). Of all the samples analyzed, 36% were found to have THg concentrations above the maximum recommended limit for monthly consumption in the USA (i.e., 0.94 mg Hg/month for a 70 kg person; USEPA, 2004; Gelsleichter and Walker, 2010).

A strong positive correlation between THg and TL of *S. tiburo* specimens was observed (Figure 1.2; Spearman's Rank Order Correlation, $\rho = 0.628$, $p < 0.001$). No significant differences between mean THg and site were observed (Figure 1.3; One-way ANOVA, $p = 0.603$).

-Metallothionein (MT)-

Western blot analysis of *S. tiburo* muscle and liver resulted in 2 signals corresponding to molecular masses of ~37kDa and ~12kDa, the later being equivalent to the expected molecular weight of MT, in all samples. Despite multiple attempts at separating putative MT (i.e., the 12 kDa band) from the 37 kDa band using ultracentrifugation, the high molecular weight band remained present (Figure 1.4). Consequently, density of only the low molecular weight species was analyzed.

Mean signal intensity of MT in *S. tiburo* muscle was significantly different between only Tampa Bay (AK) and Apalachicola Bay (PC) (Figure 1.5; One-way ANOVA, $p < 0.05$). No statistically significant differences between mean signal intensity of MT in *S. tiburo* liver were found between sites (Figure 1.5; One-way ANOVA, $p = 0.256$).

No correlation was observed between MT_{muscle} and MT_{liver} (Figure 1.6; Pearson's product-moment correlation, $r = -0.119$, $p = 0.438$). Additionally, no significant differences were found between mean signal

intensities of MT_{muscle} and MT_{liver} (116.84±51.92 and 128.67± 37.51, respectively) (Figure 1.7; t-Test, p = 0.210).

-THg vs. MT-

No correlation between the MT concentration in *S. tiburo* muscle and THg was observed (Figure 1.8A; Spearman's Rank Order Correlation, ρ = -0.0.18, p = 0.899). Additionally, there was no correlation between the MT concentration in *S. tiburo* liver and THg (Figure 1.8B; Spearman's Rank Order Correlation, ρ = 0.055, p = 0.721).

Table 1.1 Range and mean±SE of total mercury (THg) in *Sphyrna tiburo* muscle reported in µg/g dry weight (d.w.) and µg/g wet weight (w.w.) from three Florida Gulf coast locations (AK = Tampa Bay, FB = Florida Bay, PC = Apalachicola Bay), including the percentage of samples that contained THg levels that met or exceeded the maximum recommended limit for consumption in the United States (1.0 µg/g w.w.).

Location	Sample Number	THg (µg/g d.w.)		THg (µg/g w.w.)		
		Range	Mean±SE	Range	Mean±SE	% ≥1 µg/g
AK	15	1.17-6.14	2.94±1.68	0.31-1.53	0.77±0.43	40.0
FB	20	0.95-6.58	3.29±1.86	0.25-1.78	0.88±0.51	46.7
PC	15	0.86-6.36	2.67±1.94	0.22-1.64	0.71±0.51	33.3
Totals	50	0.86-6.58	3.00±1.82	0.22-1.78	0.79±0.49	36.0

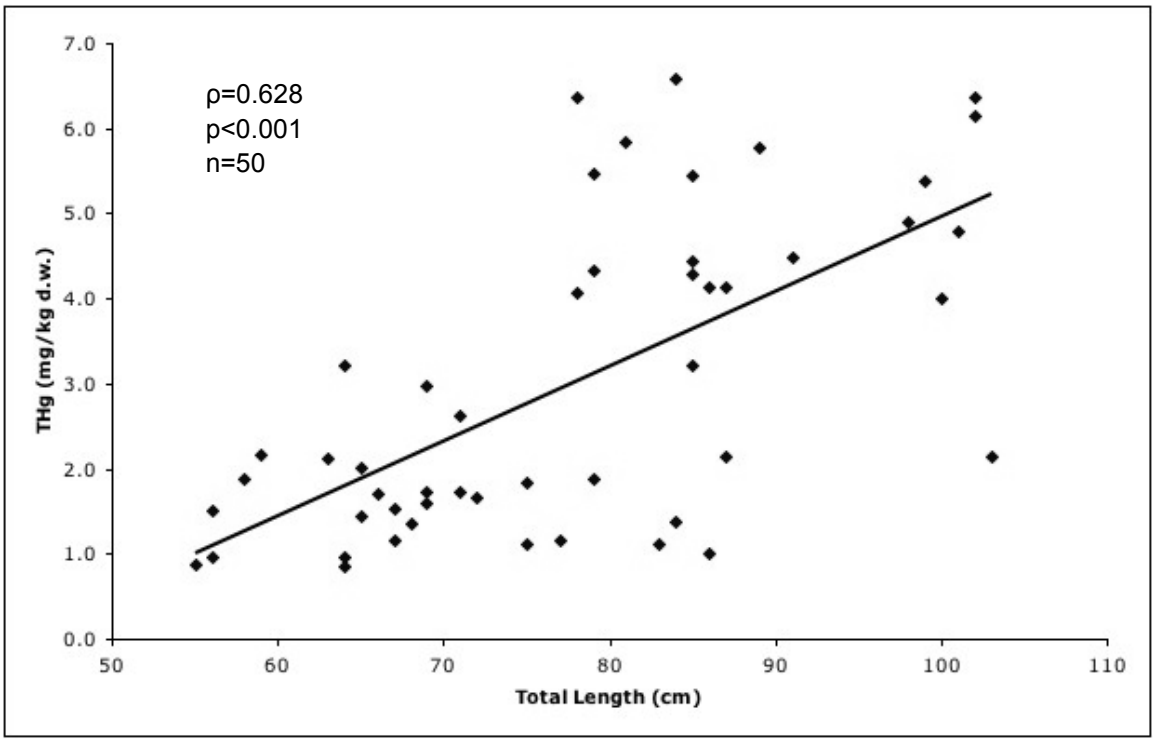

Figure 1.2 Total mercury (THg; dry weight mg/kg) measured in *Sphyrna tiburo* muscle compared to the total length (TL) for each specimen (n = 50). A significant positive correlation between length and THg was observed (Spearman's Rank Order Correlation; $\rho = 0.628$, $p < 0.001$).

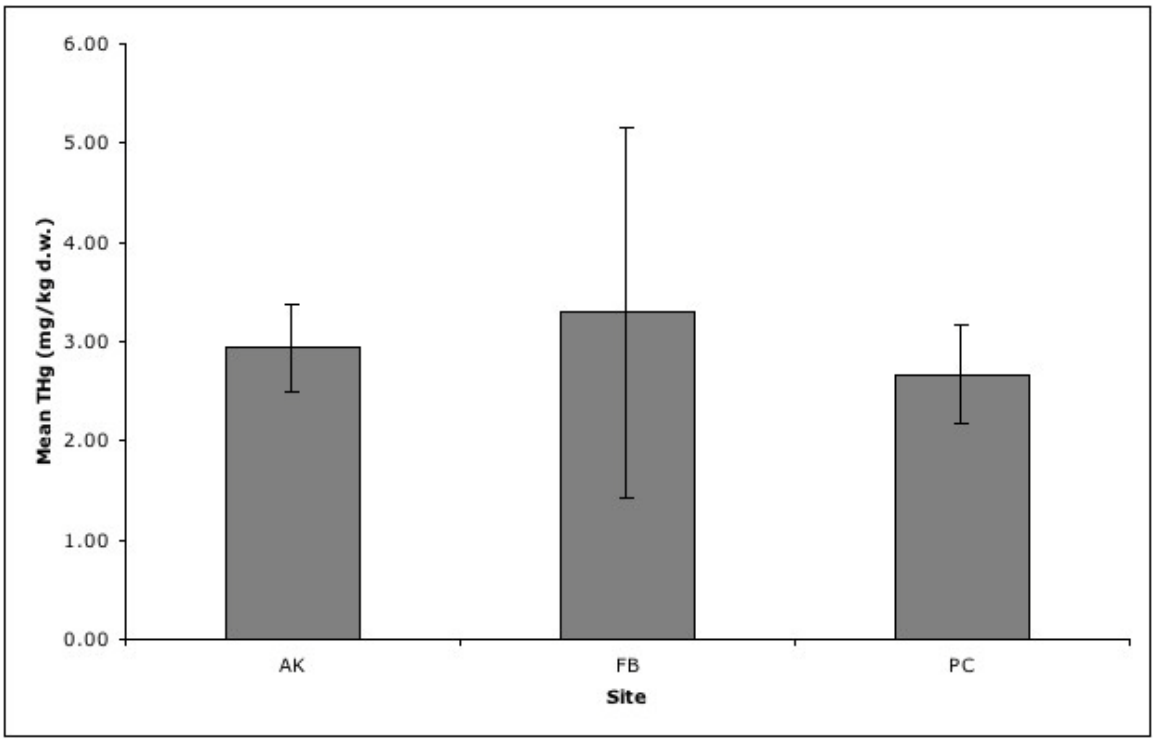

Figure 1.3 Mean THg in muscle (n = 50) from *Sphyrna tiburo* caught in three locations on the Florida Gulf coast (AK = Tampa Bay, FB = Florida Bay, PC = Apalachicola Bay). No significant differences between mean THg and site were found (One-way ANOVA, p = 0.603). Error bars indicate standard error.

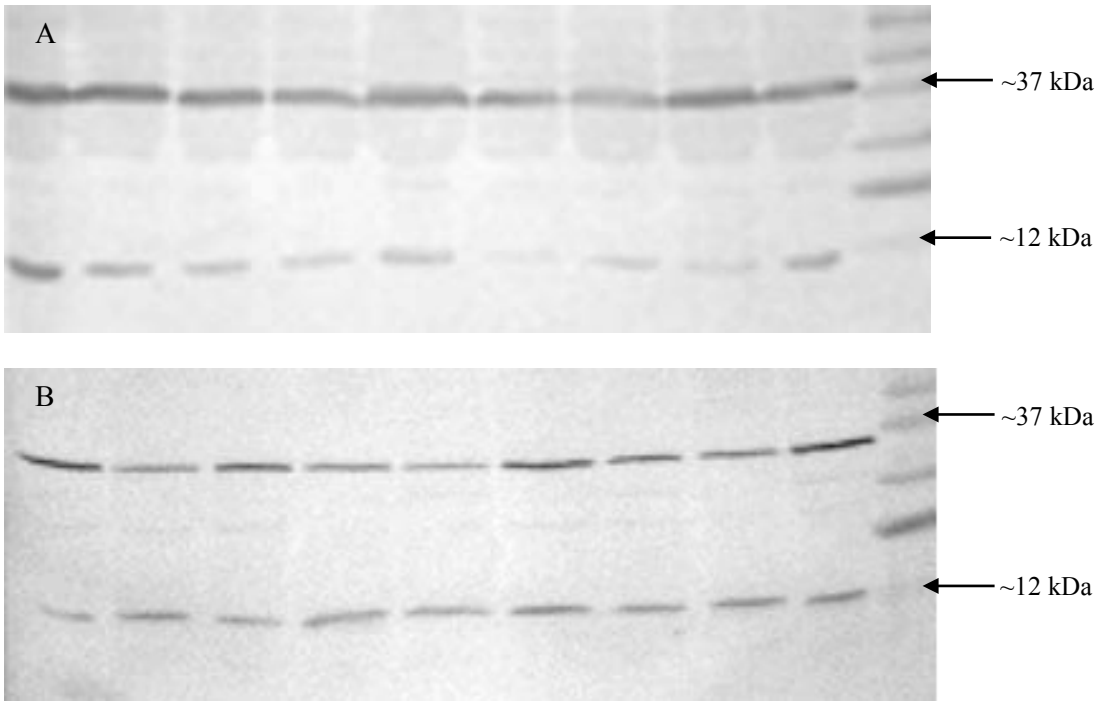

Figure 1.4 Western blot results of *Sphyrna tiburo* MT in muscle (A) and liver (B) tissue. Arrows indicate approximate size of MT protein signals. The low molecular weight signals represent a protein of the expected size for MT (~12 kDa). Due to the inability to reduce the upper bands by ultracentrifugation, only the lower weight signals were used during analysis. Signals were identified in 90 μg protein/sample using a polyclonal anti-cod MT antibody and visualized with a BCIP/NBT substrate kit. Kodak Molecular Imaging Software was used to acquire the images (0.05 s exposure, 5.6 F-stop) and to calculate the intensity of signals.

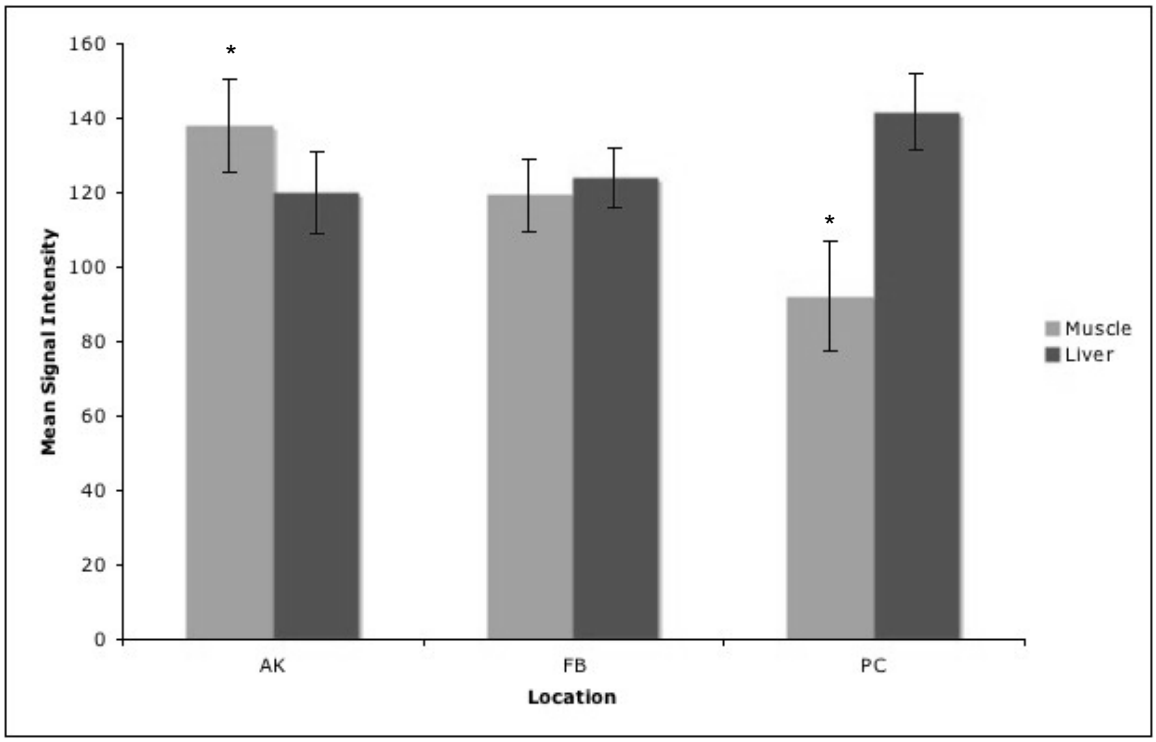

Figure 1.5 Mean band intensity of muscle and liver MT of *S. tiburo* at each catch location (AK = Tampa Bay, FB = Florida Bay, PC = Apalachicola Bay) as obtained through western blotting. Significance was determined using one-way ANOVA with Tukey's post-test. Asterisks represent significant differences in mean band intensity (AK_{muscle} vs. PC_{muscle}, $p < 0.05$). No other significant differenced were found. Error bars represent standard error.

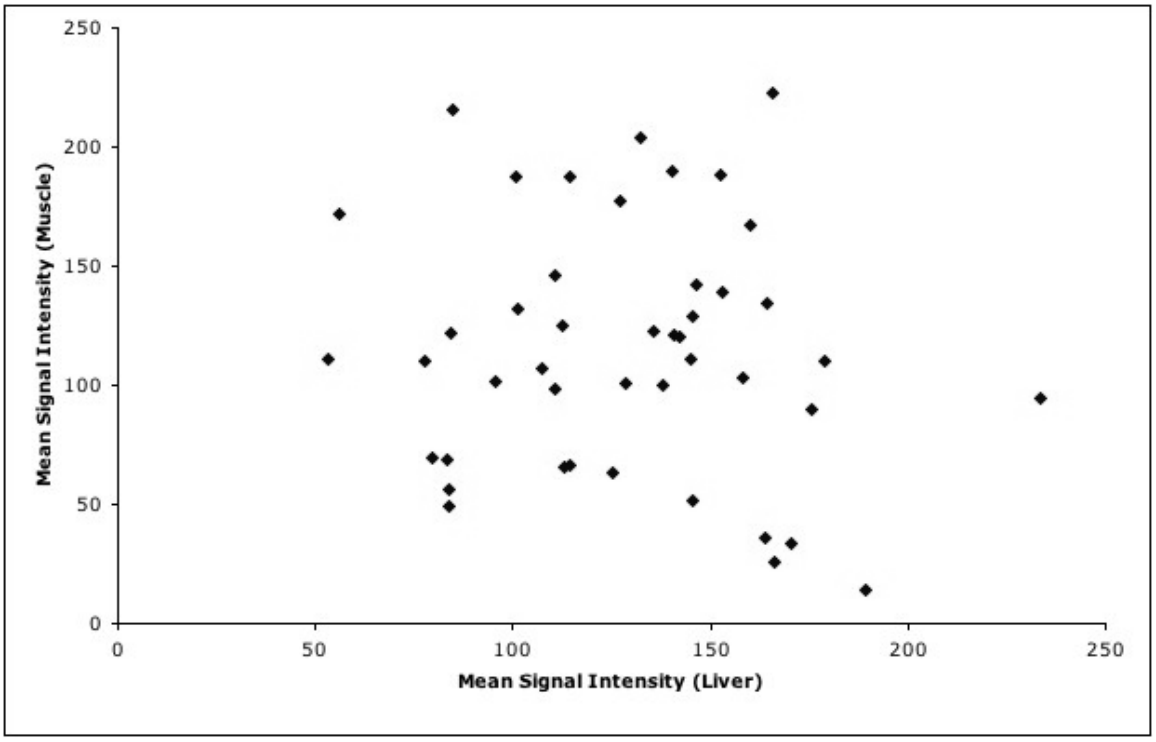

Figure 1.6 Western blot analysis of *Sphyrna tiburo* metallothionein (MT) in muscle compared to *S. tiburo* MT in liver (n = 45). Relative signal intensity was calculated by subtracting the mean background intensity from the mean signal intensity. No significant relationship was found between relative signal intensity of muscle and relative signal intensity of liver (Pearson's product-moment correlation; r = -0.119, p = 0.438).

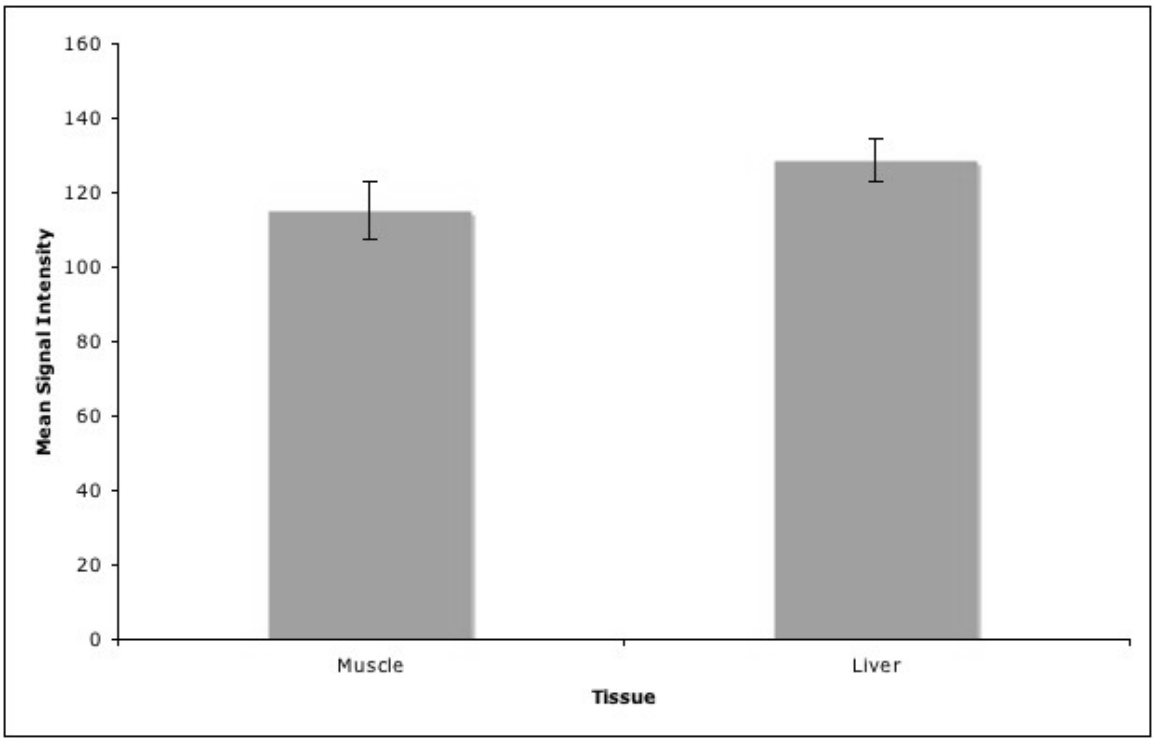

Figure 1.7 Mean signal intensity of muscle and liver MT of *Sphyrna tiburo* obtained via western blot. No significant differences in MT between muscle and liver were found ($p = 0.165$; t-Test). Error bars represent standard error.

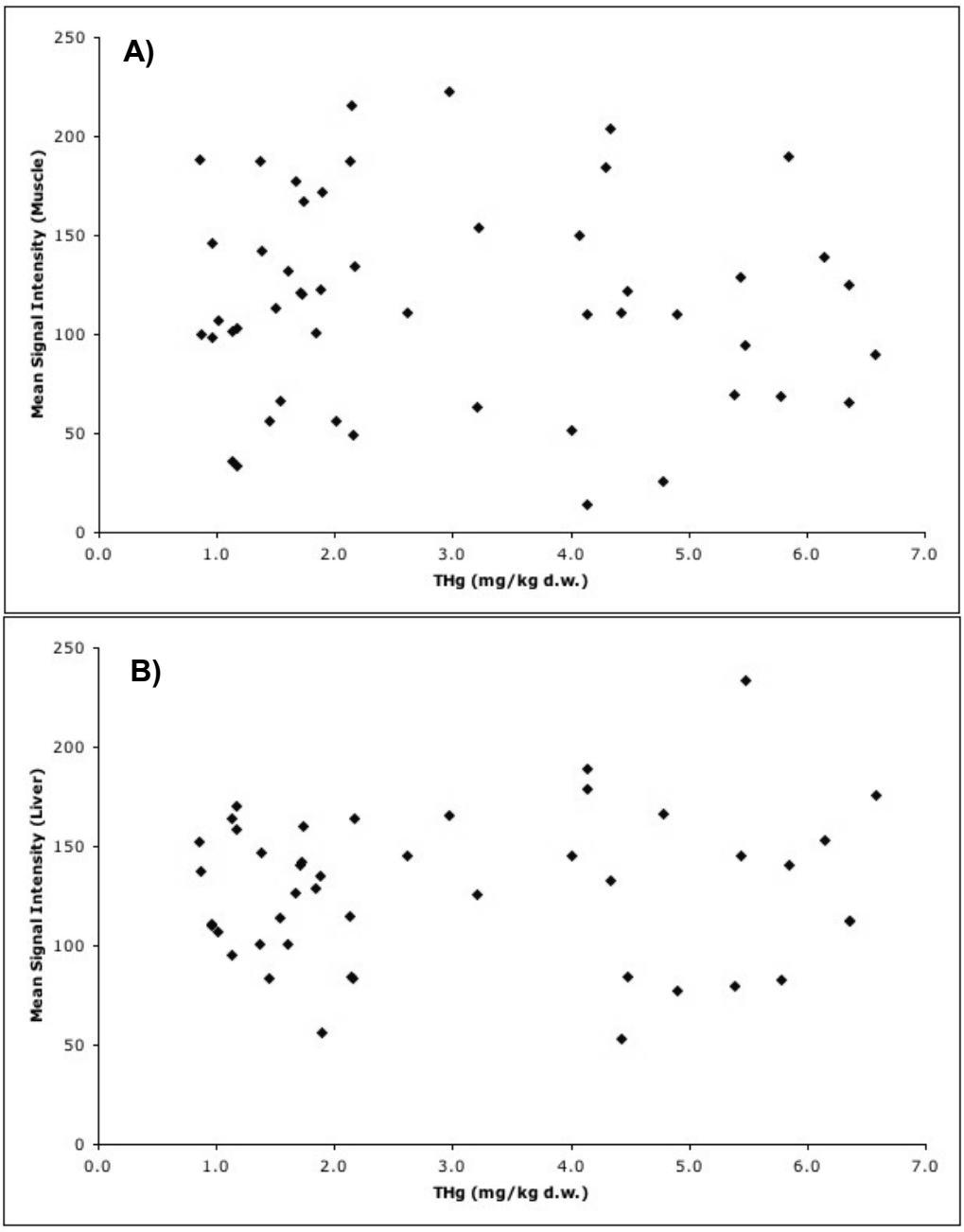

Figure 1.8 Western blot analysis of *Sphyrna tiburo* metallothionein in muscle (A; n=50) and in liver (B; n = 45) compared to total mercury (THg; µg/g dry weight) measured in muscle. Relative signal intensity was calculated by subtracting the mean background intensity from the mean signal intensity. No significant relationship was found between A) THg and relative signal intensity of muscle (Spearman's Rank Order Correlation; $\rho = -0.018$, $p = 0.899$), and B) THg and relative signal intensity of liver (Spearman's Rank Order Correlation; $\rho = 0.055$, $p = 0.721$).

Discussion

The results of the current study demonstrate that bonnethead sharks along the Florida Gulf coast accumulate Hg similar to those in other portions of their range (Adams & McMichaels, 1999; Evers et al., 2008). Animals in this study had muscle THg levels of 0.22-1.78 µg/g w.w. It was also found that 36% of the samples analyzed for THg in this study had THg muscle levels that exceeded the maximum recommended limit for human consumption in the United States (i.e., 0.94 mg Hg/month for a 70 kg person; USEPA, 2004)

The THg levels observed in this study are comparable to the concentrations reported by previous studies on THg in *S. tiburo* in Florida. In Evers et al. (2008), the mean THg in *S. tiburo* muscle from southeast Florida0 was 0.89±0.62 µg/g w.w, which is almost identical to the mean THg in *S. tiburo* muscle found in this study (0.79±0.49 µg/g w.w.). Adams & McMichael (1999) found THg levels to be 0.13-1.5 µg/g w.w. with a mean of 0.50±0.36 µg/g w.w. in *S. tiburo* from east-central Florida.

The total mercury (THg) concentrations (µg/g w.w.) in *S. tiburo* muscle tissue observed in this study were positively correlated with size of the animals, a result that has been confirmed by numerous other studies on THg concentrations in sharks (Branco et al., 2004, 2007; Marisco et al., 2007; Lacerda et al., 2000; Pinho et al., 2002; Adams & McMichael, 1999; Hueter et al., 1995; Evers et al., 2008), including Florida *S. tiburo* (Adams & McMichael, 1999; Evers et al., 2008). For example, Adams & McMichael (1999) reported a significant positive correlation between precaudal length (mm) and THg (ppm w.w.) for *S. tiburo* in east-central Florida ($p < 0.0001$; Pearson's product-moment correlation).

Although the THg reported in this study are comparable to previously published levels in *S. tiburo* from Florida, MT was not found to be a valid biomarker for Hg exposure in this species, as no correlation between MT in muscle or liver and THg was found. Additionally, while MT_{muscle} was found to differ between Tampa Bay specimens and Apalachicola Bay specimens, no differences between sites were observed for MT_{liver} or for THg.

Based on the conclusions of previous studies on the use of MT as a biomarker for toxic metal exposure in aquatic organisms, the results of the current study were unexpected. For example, following exposure to Hg, cadmium (Cd), copper (Cu), and zinc (Zn), MT levels have been shown to increase in both a dose- (Hogstrand and Haux, 1991) and/or a time-dependent manner (Beyer et al., 1997) in fish. This induction pattern has also been observed in field studies of bony fishes. For example, Hylland et al. (1992) reported that hepatic MT concentrations accurately reflected the levels of heavy metals measured in the liver of dab, *Limanda limanda,* from the German Bight. Similarly, MT levels in carp *Cyprinus carpio* were best induced by Hg in the gill as compared to MT induction by Cd, Cu, Zn, and silver (Ag).

One possible explanation for the conflicting results of this study is that MT induction may be more associated with exposure to other toxic metals or to the combined effect of exposure to several metals at once. For example, Atli & Canli (2008) found that MT levels in Nile tilapia *Oreochromis niloticus* significantly increased following exposure to any level of cadmium, and to high levels of copper and zinc while Dragun et al. (2009) found no correlation between Hg and MT in European chub *Squalus cephalus*. In accordance with Atli & Canli (2008), Alvarado et al. (2006) found that Cd, Cu, and Zn induced MT production in turbot *Scophthalmus maximus* as well.

Additionally, the mode of Hg exposure may partially explain the lack of a correlation in this study. Oliveira et al. (2010) found a positive correlation between hepatic MT in golden grey mullet *Liza aurata* and Hg and Cu in the water, suggesting that hepatic MT may be a better indicator of inorganic mercury in the water column rather than organic methylmercury in sediments. As reported in a number of studies, MT levels may also be tissue-specific, contributing to the variation in MT observed in the current study. For example, Creti et al. (2010) found that MT levels were highest in muscle compared to gut, liver, kidney, and gill in sea bream, *Sparus aurata*. Moreover, tissue specificity may also be related to age, as was reported in Bebianno et al. (2007), where young black scabbardfish *Aphanopus carbo* had the highest MT levels in gills, juveniles had highest MT in muscle, and adult fish has highest MT levels in liver.

MT levels may also be influenced by a number of conditions other than toxic metal exposure (Stegeman et al., 1992). For example, MT can be induced by glucocorticoid and peptide hormones (Muto et al., 1999) while estradiol and estrogenic polychlorinated biphenyls seem to inhibit MT induction (Gerpe et al., 2000). As well, animals that have been exposed to increased levels of organic pollutants may exhibit lower-than-expected MT levels due to a higher demand for cysteine residues, a major component of MT, for the synthesis of Phase II biotransformation enzymes, specifically glutathione (Van der Oost et al., 2003). Constitutive MT, which is always present due to its homeostatic function in organisms, may also vary with season, sex, reproductive status, and age of the animals (Amiard et al., 2006; Hamza-Chafai et al., 1995; Oliveira et al., 2010).

Furthermore, the role MT plays in Hg detoxification has been questioned in some species. For example, Decataldo et al. (2004) investigated the association between Hg and MT-like proteins in two species of stranded dolphins (*Stenella coeruleoalba* and *Tursiops truncates*) and identified Hg in the insoluble fraction of the liver and kidneys. Therefore, Hg could not interact with or induce MT. These findings confirmed that, in dolphins, MT may not be as involved in Hg detoxification as previously thought.

Finally, the threshold of physiological effect for Hg exposure is unknown for elasmobranchs, but has been determined to be between 0.5-1.2 μg/g w.w. for sublethal, physiological effects and between 6-20 μg/g w.w. for lethal effects in adult bony fishes (Wiener et al., 2003). Therefore, it may be likely that the THg concentrations observed in this study (0.22-1.78 μg/g w.w.) may be below the threshold of effect for bonnethead sharks, possibly explaining the absence of a correlation between THg and MT levels in these animals. This explanation is supported by Dragun et al. (2009) in which European chub (*Squalis cephalus*) were found to exhibit high variability of constitutive MT under conditions of low dissolved metals concentrations. Therefore, examination of sharks with higher Hg levels is needed in order to determine at what level MT is induced, if at all.

In conclusion, while Hg does accumulate in *S. tiburo* muscle to levels that exceed the maximum recommended limits for monthly human consumption, the levels do not seem to correlate with MT levels in

either liver or muscle. Therefore, this study indicates that there is limited use for MT as a biomarker for the moderate exposure to toxic metals in bonnethead sharks. Future studies should aim to evaluate the relationship between MT levels and exposure to a broader panel of heavy metals and should examine the use of additional biomarkers (e.g., oxidative stress biomarkers) in determining the effect of exposure to toxic metals. Additionally, future studies should attempt to investigate the relationships between MT and metal exposure in species of sharks occupying higher trophic levels than *S. tiburo* (e.g., blue sharks, tiger sharks), thereby accumulating higher levels of metals than observed in this study.

Chapter Two:

The effect of the *Deepwater Horizon* oil spill on sharks in the Gulf of Mexico

Introduction

On 20 April 2010, the *Deepwater Horizon* oil-drilling rig, located in the Gulf of Mexico (GOM), exploded. For 87 days, until the well was successfully sealed on 15 July 2010, crude oil leaked from the well at an estimated rate of 5.6×10^4 barrels/day, resulting in a total release of over 4 million barrels of oil into the GOM (Crone & Tolstoy, 2010; Camilli et al., 2010). The amount of oil released during the *Deepwater Horizon* oil spill (DHOS) was one order of magnitude higher than the amount of oil released during the *Exxon Valdez* spill in 1989 (Crone & Tolstoy, 2010), making it the largest offshore oil spill in the history of the United States. As a result, the environmental quality of the GOM has been significantly impacted and the oil contamination now threatens the health and survival of many species, especially those that inhabit the areas within and/or close to the principal DHOS zone. It is also critical to monitor the impacts of oil exposure on near-shore species because of the movement of the oil from the primary DHOS zone to >600 miles of inshore habitats in Louisiana, Texas, Alabama, and Florida.

There is substantial evidence of organism- and population-level effects of exposure to polycyclic aromatic hydrocarbons (PAHs), one of the most toxic constituents of oil, that has been presented by studies performed after past oil spills (i.e., *Exxon Valdez* oil spill, *Prestige* oil tanker accident) (Boehm et al., 2007; Martinez-Gomez et al., 2006). For example, following the *Exxon Valdez* oil spill, Peterson et al. (2003) described increased mortality of fish, sea otters *Enhydra lutris*, and harlequin ducks *Histrionicus histrionicus* immediately following the spill and continuing for up to 4 years after. In fact, as of 1997 the harlequin duck population was still experiencing a ~ 5% annual population decline. Sublethal effects including compromised growth and reproduction were also observed in a variety of other species (i.e.,

salmon *Oncorhynchus gorbuscha*, black oystercatchers *Haematopus bachmani*), resulting in decreased growth rates, decreased breeding and egg size, as well as decreased survivorship of juveniles, for up to 3 years after the spill (Peterson et al., 2003). Lethal and sublethal biological effects have also been described for mangrove communities and coral reefs near the Panama Canal up to 2 years after the release of approximately 50,000 barrels of oil from a storage tank in 1986 (Jackson et al., 1989).

The PAHs present in oil tend to associate with organic material in aquatic environments and therefore persist in sediments and may become bioavailable to aquatic organisms via diet (Al-Hassan et al., 2000). Although these compounds tend to only bioaccumulate in species without the ability to metabolize them (e.g., mollusks), PAHs have been examined in marine fish populations inhabiting contaminated areas and levels ranging from 1 to 4 μg/g wet weight (w.w.) were reported in lipid-rich tissues, such as liver (Kannan and Perrotta, 2008). Top marine predators (e.g., dolphins, sharks) tend to have higher levels of PAHs present in their tissues due to their high trophic level, large size, slow metabolism, and a tendency to bioconcentrate these chemicals (Marsili et al., 2001; Al-Hassan et al., 2000). For example, following the Kuwait oil fires in 1991, when 6 to 8 million barrels of crude oil were purposefully released into the Arabian Gulf, a study by Al-Hassan et al. (2000) reported that sharks from this area had total liver PAH concentrations ranging from 0.13 to 33 μg/g w.w. Teleosts from this same area had comparable, but lower, total liver PAH concentrations ranging from 1.69 to 10.79 μg/g w.w. Due to their tendency to accumulate high levels of these toxins, large predators such as sharks may face special health risks from these compounds.

The use of biomarkers is an effective technique for detecting physiological responses in an organism exposed to pollutants and can be used as an "early warning tool" in determining the risk of higher-level effects in animals exposed to PAHs (Schreiber et al., 2006; Van der Oost et al., 2003). The activity of the Phase I biotransformation enzyme cytochrome P4501A1 (CYP1A1) is a reliable biomarker for exposure to PAHs, polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and dioxin-like polychlorinated biphenyls (DL-PCBs) in bony fishes (Van der Oost et al., 2003). Additional biomarkers may be further utilized to identify the specific contaminants an organism was exposed to. For example,

assays designed to detect PAH-DNA adducts and bile fluorescent aromatic compounds may be used to confirm exposure to PAHs (Aas et al., 2000). A laboratory study by Hahn et al., (1998) confirmed both the presence and inducible nature of CYP1A1 in sharks while Fuentes-Rios et al. (2005), in the only field study to date to examine CYP1A1 in sharks, confirmed that CYP1A1 activity was greater in cat-sharks (*Schroederichthys chilensis*) from areas of high PAH contamination in comparison to reference site animals. Due to the dependability of CYP1A1 as a biomarker for pollutant exposure, it has been widely used as a tool for monitoring the effects of oil spills on organisms (Lee & Anderson, 2005). For instance, elevated CYP1A1 levels were reported in teleost fish following the *Exxon Valdez* oil spill (Carls et al., 1996) as well as after the *Braer* oil spill (Ritchie & O'Sullivan, 1994) and the *Prestige* spill (Martinez-Gomez et al., 2006).

In addition to elevated levels of CYP1A1, exposure to PAHs may also lead to several higher-level effects in organisms. The increase of this biotransformation enzyme can cause the production of more highly toxic PAH metabolites, which tend to form DNA adducts and/or reactive oxygen species (ROS) (Gelsleichter and Walker, 2010). The formation of DNA adducts can result in hepatic lesions and neoplasms, whereas the formation of ROS may lead to DNA damage and cell death (Myers et al., 2008; Varanasi et al., 1987; Baumann et al., 1998).

Therefore, utilizing liver samples from a variety of species obtained between 2006 and 2010, the goal of this study was to assess the impacts of the *Deepwater Horizon* oil spill on several species of elasmobranchs inhabiting GOM waters. Specifically, this study set out to (1) determine baseline measurements of CYP1A1 activity in fifteen species of elasmobranch species collected off the coast of Alabama, sampled up to 4 years prior to the DHOS, and (2) determine the impacts of the DHOS on the same species sampled from the same locations following the arrival of oil from the DHOS in Alabama waters (June 2010; NEDIS Anomaly Analysis 01-June-2010 Composite, http://www.GeoPlatform.gov/gulfresponse). The availability of samples for up to 4 years before the occurrence of the DHOS allowed for the comparison of "pre-oil" and "post-oil" samples, a comparison that is often not possible to determine following environmental disasters due to the lack of baseline data available (Kirby & Law, 2010).

Methods

-Sample Collection-

Fourteen species of sharks (blacktip shark *Carcharhinus limbatus*, blacknose shark *C. acronotus*, finetooth shark *C. isodon*, sharpnose shark *Rhizoprionodon terraenovae*, angelshark *Squatina dumeril*, bonnethead *Sphyrna tibruo*, bull shark *C. leucas*, great hammerhead *S. mokarran*, narrowfin smooth-hound *Mustelus norrisi*, scalloped hammerhead *S. lewini*, smooth dogfish *M. canis*, spinner shark *C. brevipinna*, tiger shark *Galeocerdo cuvier*, sandbar shark *C. plumbeus)* and 1 species of stingray (southern stingray *Dasyatis americana*) were collected using gill nets and long-lines from the Gulf of Mexico between the years 2006-2010, off the coast of Alabama (Figure 2.1), by the Dauphin Island Sea Lab Center for Ecosystem Based Fishery Management on Dauphin Island, AL using gillnets, and bottom and pelagic long-lines. Once caught, the sharks were weighed to the nearest 0.1 kg, measured to the nearest 1.0 cm, and further examined in order to determine sex. Livers (~1-2 g) were excised and frozen with liquid nitrogen in cryovials and stored at -80°C until analysis in 2010 and 2011.

The total number of samples analyzed for this study was 686; of which 394 were considered to be "pre-oil" (i.e., caught prior to June 2010, when oil from the DHOS reached Alabama waters) and 292 were considered "post-oil" (i.e., caught during or after June 2010, following the arrival of DHOS oil in Alabama waters). The number of samples per species is as follows: *C. limbatus*: $n_{pre\text{-}oil} = 36$, $n_{post\text{-}oil} = 24$; *C. acronotus*: $n_{pre\text{-}oil} = 43$, $n_{post\text{-}oil} = 92$; *C. isodon*: $n_{pre\text{-}oil} = 43$, $n_{post\text{-}oil} = 67$; *R. terraenovae*: $n_{pre\text{-}oil} = 183$, $n_{post\text{-}oil} = 65$; *S. dumeril*: $n_{pre\text{-}oil} = 2$, $n_{post\text{-}oil} = 0$; *S. tiburo*: $n_{pre\text{-}oil} = 14$, $n_{post\text{-}oil} = 7$; *C. leucas*: $n_{pre\text{-}oil} = 10$, $n_{post\text{-}oil} = 1$; *S. mokarran*: $n_{pre\text{-}oil} = 1$, $n_{post\text{-}oil} = 2$; *M. norrisi*: $n_{pre\text{-}oil} = 2$, $n_{post\text{-}oil} = 0$; *S. lewini*: $n_{pre\text{-}oil} = 9$, $n_{post\text{-}oil} = 1$; *M. canis*: $n_{pre\text{-}oil} = 14$, $n_{post\text{-}oil} = 0$; *C. brevipinna* : $n_{pre\text{-}oil} = 24$, $n_{post\text{-}oil} = 10$; *G. cuvier*: $n_{pre\text{-}oil} = 1$, $n_{post\text{-}oil} = 21$; *C. plumbeus*: $n_{pre\text{-}oil} = 2$, $n_{post\text{-}oil} = 0$; *D. americana*: $n_{pre\text{-}oil} = 1$, $n_{post\text{-}oil} = 0$.

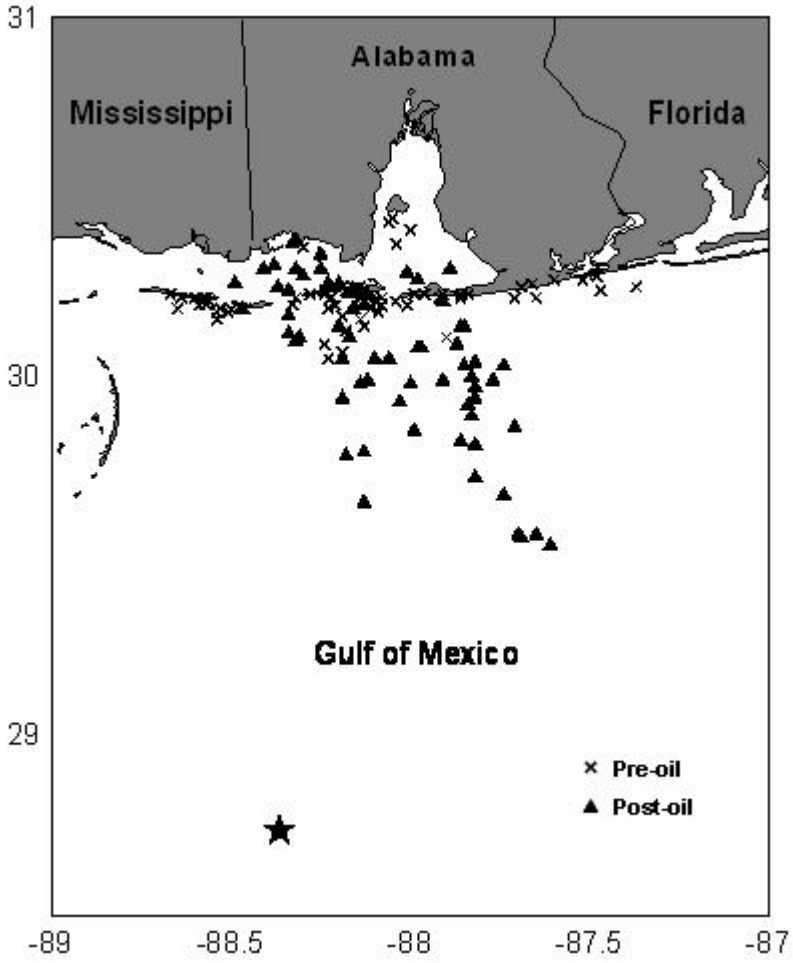

Figure 2.1 Map of locations of samples analyzed in this study caught before (Pre-oil) and after (Post-oil) oil reached Alabama waters analyzed in this study. The star indicates the location of the *Deepwater Horizon* oil rig.

-Sample Preparation-

Liver samples (~0.1 g) were homogenized in a 1:5 w:v tissue:buffer (10 mM Tris-base, 250 mM sucrose, 1 mM EDTA, 0.2 mM dithiotreitol, 0.1 mM phenylmethylsulfonyl fluoride) ratio using a FastPrep©-24 bead homogenizer (MP Biomedicals, Inc., Solon, OH, USA). The homogenate was centrifuged for 10 minutes at 8,000g and then the resulting supernatant was centrifuged again for 20 minutes at 12,000g. The supernatant (S9 fraction) was stored at 4 °C until used for further analysis. Samples were run within 4 days of homogenization in order to avoid enzyme and protein degradation.

-EROD Assay-

Hepatic CYP1A1 activity was measured in S9 fractions using the ethoxyresorufin O-deethylase (EROD) method described by Sepulveda et al. (2004) with appropriate modifications. The assay was run in triplicate in 96-well fluorescence microtiter plates. Each well contained 5 µL sample homogenate with 2 µl 10 mM ethoxyresorufin, 193 µl 0.1 M phosphate buffer, and 10 ul 5 mM β-NADPH. The kinetic reaction was read on a fluorescent plate reader at 37°C (excitation: 530/25 nm, emission: 590/35 nm) once a minute for 10 minutes including an initial reading, resulting in 11 measurements. A standard curve was created using serial dilutions of resorufin (20, 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 pmol) and was measured using the same parameters as the EROD assay described above. The standard curve was used to convert change in fluorescence per minute of each sample to the rate of resorufin produced during the assay (pmol/min). Protein concentration (mg/mL) was measured using a standard Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) using homogenates, diluted as necessary. The rate of resorufin produced was adjusted for the amount of protein in each sample to present EROD activity in pmol /min/mg protein.

-Statistics-

Data was grouped by sampling period (Pre-Oil, Post-Oil), by month in 2010, and by species. Each grouping was analyzed using either a Fisher Exact Test, a one-way Analysis of Variance, or Student's t-Test. When fitting, the equivalent non-parametric tests will be applied.

Results

The effect of PAH exposure, caused by the *Deepwater Horizon* oil spill of 2010, was assessed in various species of elasmobranchs by measuring the activity of a Phase I biotransformation enzyme, cytochrome P4501A1 (CYP1A1), as detectable EROD activity in both "pre-oil" and "post-oil" animals. No animals caught in the years 2006-2009, prior to the occurrence of the DHOS, displayed detectable EROD activity (Table 2.1). Of the animals caught following the spill, 3.4% exhibited detectable levels of EROD activity (n = 23) with 21 of those animals having been caught after oil had contaminated AL waters (*Rhizoprionodon terraenovae*: n = 1, *Carcharhinus acronotus*: n = 10, *C. isodon*: n = 2; *C. brevipinna*: n = 1; *Galeocerdo cuvier*: n = 7) (Figure 2.2). The 2 animals caught after the spill occurred, but prior to the arrival of oil offshore of AL were *Dasyatis americana* and *R. terraenovae*. EROD activity for all samples ranged from 0 to 3.17 pmol/min/mg except for one "post-oil" *G. cuvier* specimen with EROD activity of 16.75 pmol /min/mg protein.

Table 2.1 The number of specimens analyzed, EROD activity per year of sampling, and the number of samples with positive EROD activity for elasmobranchs species caught 6-10 miles offshore of Alabama. Size ranges provided are precaudal length (cm)*

Species	Year	n	Mean±SE	Range	# of Positives
Squatina dumeril	2006	2	0	-	0
(39-69 cm)	2007	0	-	-	-
	2008	0	-	-	-
	2009	0	-	-	-
	2010	0	-	-	-
Rhizoprionodon terraenovae	2006	59	0	-	0
(24-47 cm)	2007	7	0	-	0
	2008	25	0	-	0
	2009	1	0	-	0
	2010	159	0.03±0.021	0-3.17	2
Carcharhinus acronotus	2006	4	0	-	0
(41-71 cm)	2007	22	0	-	0
	2008	8	0	-	0
	2009	0	-	-	0
	2010	101	0.01±0.005	0-0.31	10
C. limbatus	2006	4	0	-	0
(43-112 cm)	2007	28	0	-	0
	2008	1	0	-	0
	2009	0	-	-	-
	2010	27	0	-	0
Sphyrna tiburo	2006	9	0	-	0
(27-79 cm)	2007	5	0	-	0
	2008	0	-	-	-
	2009	0	-	-	-
	2010	7	0	-	0
C. leucas	2006	0	-	-	-
(63-180 cm)	2007	5	0	-	0
	2008	5	0	-	0
	2009	0	-	-	-
	2010	1	0	-	0
C. obscurus	2006	1	0	-	0
(149 cm)	2007	0	-	-	-
	2008	0	-	-	-
	2009	0	-	-	-
	2010	0	-	-	-

* EROD activity is presented in pmol/min/mg protein.

Table 2.1 (Continued) The number of specimens analyzed, EROD activity per year of sampling, and the number of samples with positive EROD activity for elasmobranchs species caught 6-10 miles offshore of Alabama. Size ranges provided are precaudal length (cm)*

Species	Year	n	Mean±SE	Range	# of Positives
C. isodon	2006	3	0	-	0
(33-82 cm)	2007	30	0	-	0
	2008	10	0	-	0
	2009	0	-	-	-
	2010	67	0.04±0.028	0-1.58	2
S. mokarran	2006	1	0	-	0
(118-155 cm)	2007	0	-	-	-
	2008	0	-	-	-
	2009	0	-	-	-
	2010	2	0	-	0
Mustelus norrisi	2006	2	0	-	0
(NA)	2007	0	-	-	-
	2008	0	-	-	-
	2009	0	-	-	-
	2010	0	-	-	-
C. plumbeus	2006	0	-	-	-
(145 cm)	2007	1	0	-	0
	2008	1	0	-	0
	2009	0	-	-	-
	2010	0	-	-	-
S. lewini	2006	0	-	-	-
(60-175 cm)	2007	7	0	-	0
	2008	1	0	-	0
	2009	0	-	-	-
	2010	2	0	-	0
M. canis	2006	14	0	-	0
(NA)	2007	0	-	-	-
	2008	0	-	-	-
	2009	0	-	-	-
	2010	0	-	-	-
Dasyatis americana	2006	0	-	-	-
(NA)	2007	0	-	-	-
	2008	0	-	-	-
	2009	0	-	-	-
	2010	1	0.27	-	1

* EROD activity is presented in pmol/min/mg protein.

Table 2.1 (Continued) The number of specimens analyzed, EROD activity per year of sampling, and the number of samples with positive EROD activity for elasmobranchs species caught 6-10 miles offshore of Alabama. Size ranges provided are precaudal length (cm)*

Species	Year	n	Mean±SE	Range	# of Positives
C. brevipinna	2006	7	0	-	0
(19-137 cm)	2007	9	0	-	0
	2008	7	0	-	0
	2009	0	-	-	-
	2010	11	0.23±0.227	0-2.49	1
Galeocerdo cuvier	2006	0	-	-	-
(60-270 cm)	2007	0	-	-	-
	2008	1	0	-	0
	2009	0	-	-	-
	2010	21	1.03±0.796	0-16.75	8

* EROD activity is presented in pmol/min/mg protein.

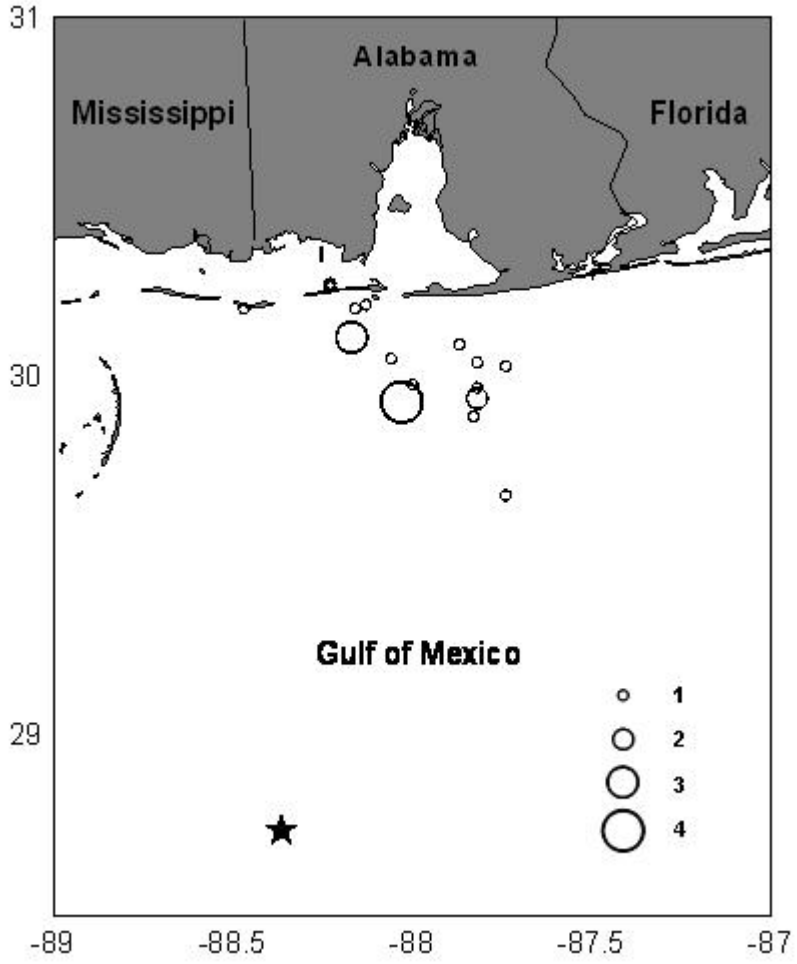

Figure 2.2 Catch locations of the samples that had detectable levels of EROD activity. The sizes of the circles correspond to the number of animals with detectable EROD activity from that location. The star indicates the location of the *Deepwater Horizon* oil rig.

-Pre-Oil vs. Post-Oil-

Overall, the numbers of samples with detectable levels of EROD activity were significantly higher for "post-oil" samples (n = 21) than for "pre-oil" samples (n = 2; $p < 0.0001$; Fisher Exact Test; Figure 2.3). Likewise, mean EROD activity was significantly higher in "post-oil" samples (0.107±0.059) than in "pre-oil" samples (0.003±0.002; $p < 0.05$; Welsh's t-test; Figure 2.4).

-By Month in 2010-

The EROD activity measurements for every species sampled during each month in 2010 are summarized in Table 2.2. The number of positive EROD activity detections measured in October (n = 11) is significantly higher than the number of detections measured in June (n = 1; $p < 0.0001$), July (n = 2; $p < 0.0001$), August (n = 1; $p < 0.0001$), September (n = 1; $p < 0.001$), and November (n = 5; $p < 0.05$; Fisher's Exact Test; Figure 2.5). The number of detections in November was significantly higher than the number in May ($p < 0.05$) and August ($p < 0.05$) as well (Fisher's Exact test).

Similar results were found when comparing mean EROD activity between the months sampled during 2010 (Figure 2.6). The mean EROD activity in sharks collected during October was significantly higher than that of all other months sampled in 2010 except for December (May: $p < 0.001$, June: $p < 0.001$; July: $p < 0.001$; August: $p < 0.001$; September: $p < 0.001$; November: $p < 0.05$; December: $p > 0.05$; Kruskal-Wallis and Dunn's Multiple Comparisons Test). No significant differences in mean EROD activity between any other months were observed ($p > 0.05$ for all comparisons; Kruskal-Wallis and Dunn's Multiple Comparisons Test).

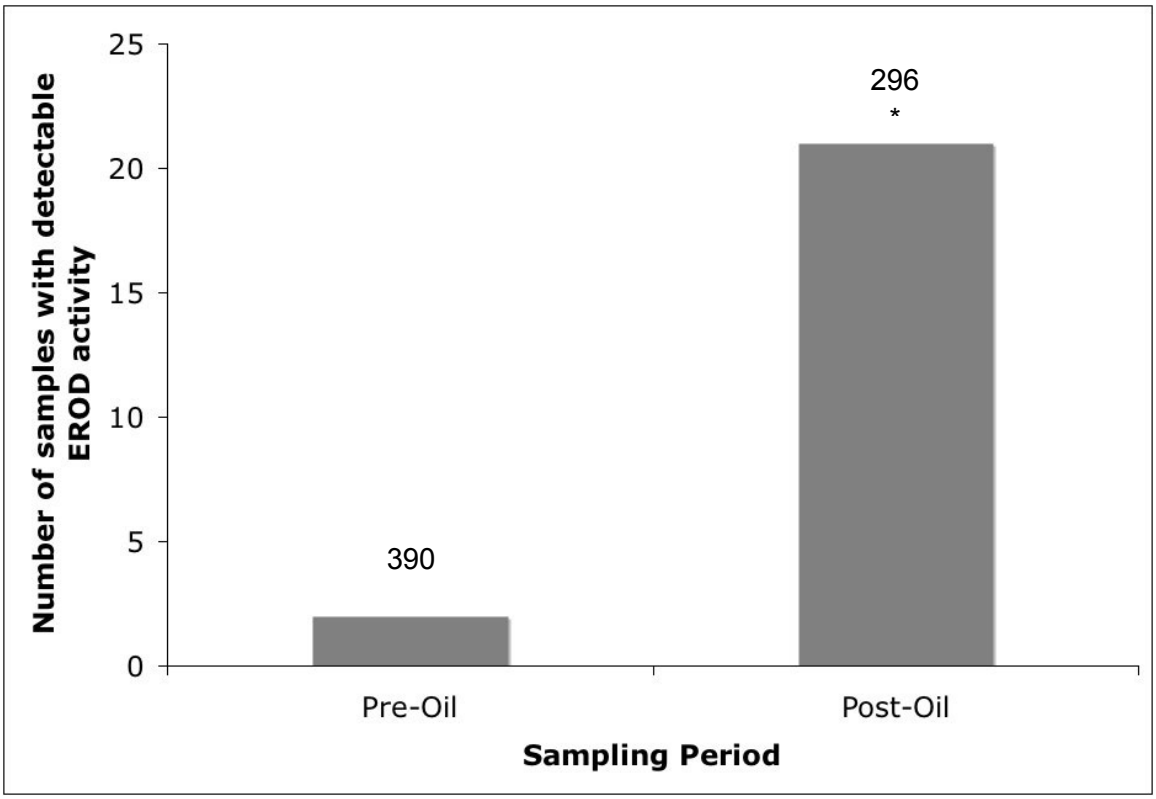

Figure 2. 3 Frequency o f s amples t hat d isplayed p ositive E ROD act ivity (pmol/min/mg p rotein) for a ll species s ampled b efore (Pre-Oil) a nd a fter (Post-Oil) o il f rom t he D eepwater H orizon o il s pill r eached Alabama waters. Sample size is indicated above each bar. The asterisk indicates significance ($p < 0.0001$; Fisher Exact Test).

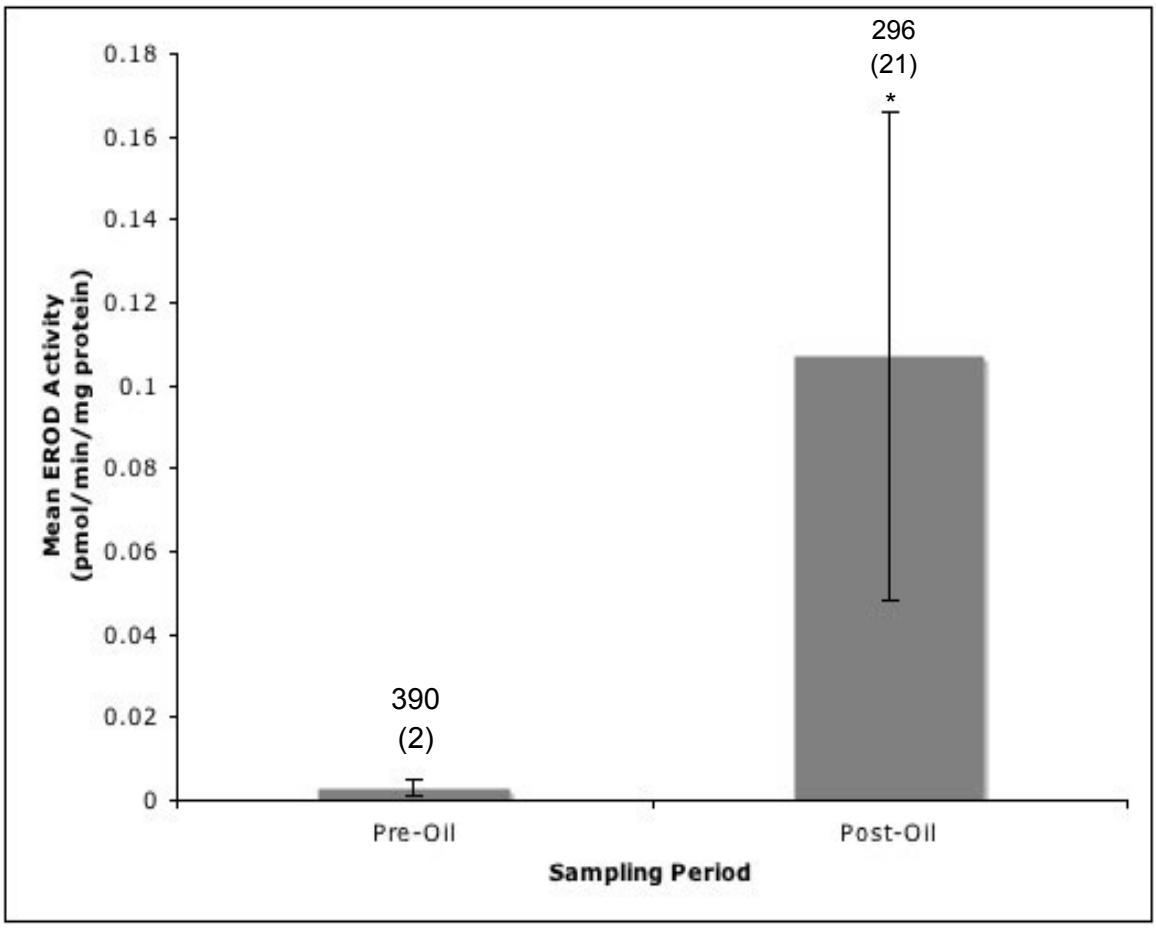

Figure 2.4 Mean EROD activities of all species sampled before (Pre-Oil) and after (Post-Oil) oil from the Deepwater Horizon oil spill reached Alabama waters. Sample sizes are indicated above each bar, with the number of samples with positive EROD activity in parentheses. Significance is indicated by the asterisk ($p < 0.05$; Welsh's t-Test). Error bars indicate standard error.

Table 2 .2 The n umber of samples a nalyzed, E ROD a ctivity, a nd t he num ber o f s amples with pos itive EROD activity per month in 2010*

Species	Month	n	Mean±SE	Range	# of positives
Rhizoprionodon terraenovae	May	94	0.01	0-0.92	1
	June	12	0	-	0
	July	33	0.096	0-3.17	1
	August	13	0	-	0
	September	4	0	-	0
	October	1	0	-	0
	November	1	0	-	0
	December	0	-	-	-
Carcharhinus acronotus	May	9	0	-	0
	June	14	0	-	0
	July	16	0	-	0
	August	11	0	-	0
	September	11	0	-	0
	October	17	0.075±0.026	0-0.32	9
	November	20	0.006	0-0.11	1
	December	4	0	-	0
C. limbatus	May	3	0	-	0
	June	1	0	-	0
	July	6	0	-	0
	August	10	0	-	0
	September	6	0	-	0
	October	0	-	-	-
	November	0	-	-	-
	December	0	-	-	-
Sphyrna tiburo	May	0	-	-	-
	June	0	-	-	-
	July	0	-	-	-
	August	5	0	-	0
	September	0	-	-	-
	October	1	0	-	0
	November	0	-	-	-
	December	0	-	-	-
C. leucas	May	0	-	-	-
	June	0	-	-	-
	July	0	-	-	-
	August	0	-	-	-
	September	0	-	-	-
	October	1	0	-	0
	November	0	-	-	-
	December	0	-	-	-

* EROD activity is presented in pmol/min/mg protein.

Table 2.2 (Continued) The number of samples analyzed, EROD activity, and the number of samples with positive EROD activity per month in 2010*

Species	Month	n	Mean±SE	Range	# of positives
C. isodon	May	0	-	-	-
	June	3	0.528	0-1.58	1
	July	2	0	-	0
	August	39	0.025	0-0.97	1
	September	10	0	-	0
	October	4	0	-	0
	November	9	0	-	0
	December	0	-	-	-
S. mokarran	May	0	-	-	-
	June	0	-	-	-
	July	0	-	-	-
	August	1	0	-	0
	September	1	0	-	0
	October	0	-	-	-
	November	0	-	-	-
	December	0	-	-	-
S. lewini	May	1	0	-	0
	June	0	-	-	-
	July	1	0	-	0
	August	0	-	-	-
	September	0	-	-	-
	October	0	-	-	-
	November	0	-	-	-
	December	0	-	-	-
Dasyatis americana	May	1	0.274	-	1
	June	0	-	-	-
	July	0	-	-	-
	August	0	-	-	-
	September	0	-	-	-
	October	0	-	-	-
	November	0	-	-	-
	December	0	-	-	-

* EROD activity is presented in pmol/min/mg protein.

Table 2.2 (Continued) The number of samples analyzed, EROD activity, and the number of samples with positive EROD activity per month in 2010*

Species	Month	n	Mean±SE	Range	# of positives
C. brevipinna	May	1	0	-	0
	June	0	-	-	-
	July	9	0.277	0-2.49	1
	August	0	-	-	-
	September	1	0	-	0
	October	0	-	-	-
	November	1	0	-	0
	December	0	-	-	-
Galeocerdo cuvier	May	0	-	-	-
	June	0	-	-	-
	July	0	-	-	-
	August	0	-	-	-
	September	6	0.034	0-0.20	1
	October	6	0.077±0.074	0-0.45	2
	November	9	2.331±1.822	0-16.75	4
	December	0	-	-	-

* EROD activity is presented in pmol/min/mg protein.

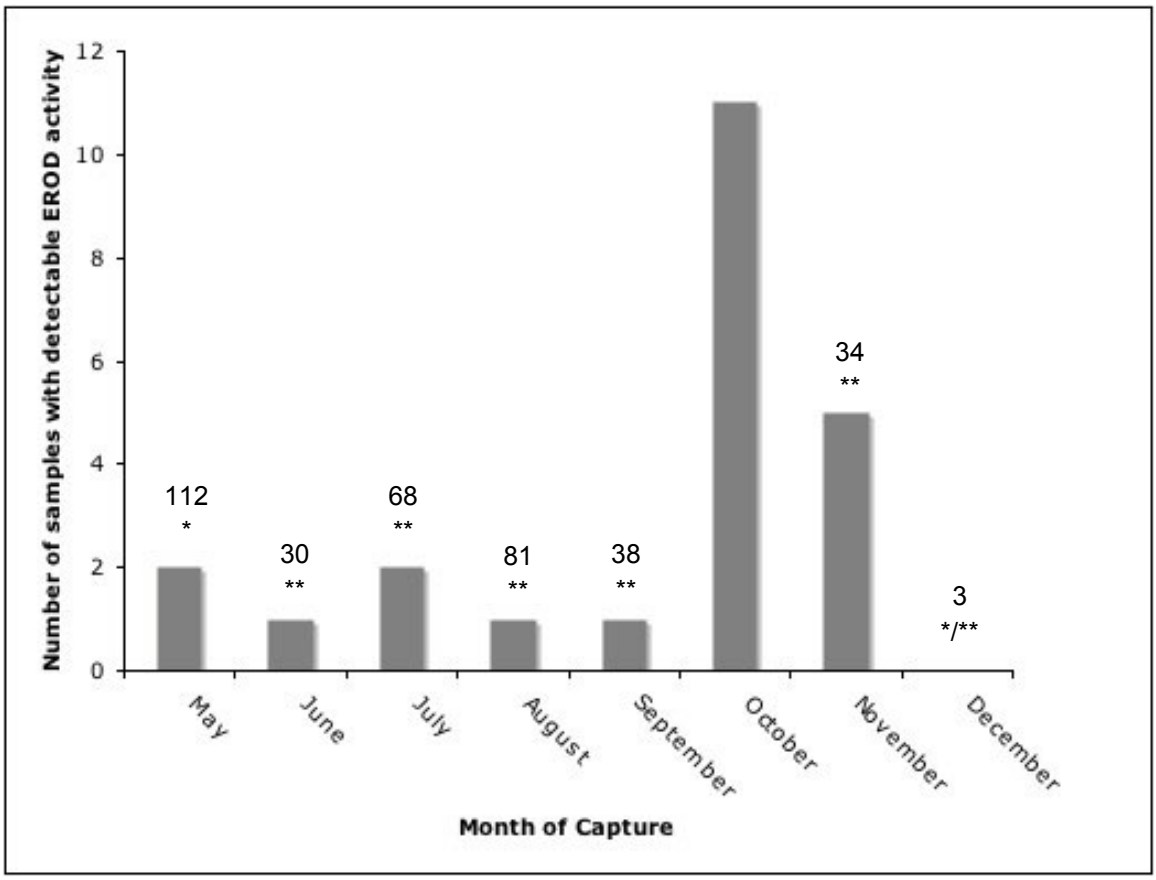

Figure 2. 5 Number o f a nimals, al l s pecies included, pe r month i n 2010 with pos itive E ROD a ctivity (pmol/min/mg p rotein). S ample s ize i s i ndicated ab ove each b ar. The as terisks i ndicate s ignificant differences be tween months (October v s. J une: p < 0. 0001, J uly: p < 0. 0001, A ugust: p < 0. 0001, September: p < 0.001, and November: p < 0.05; November vs. May: p < 0.05 and August: p < 0.05; Fisher Exact Test).

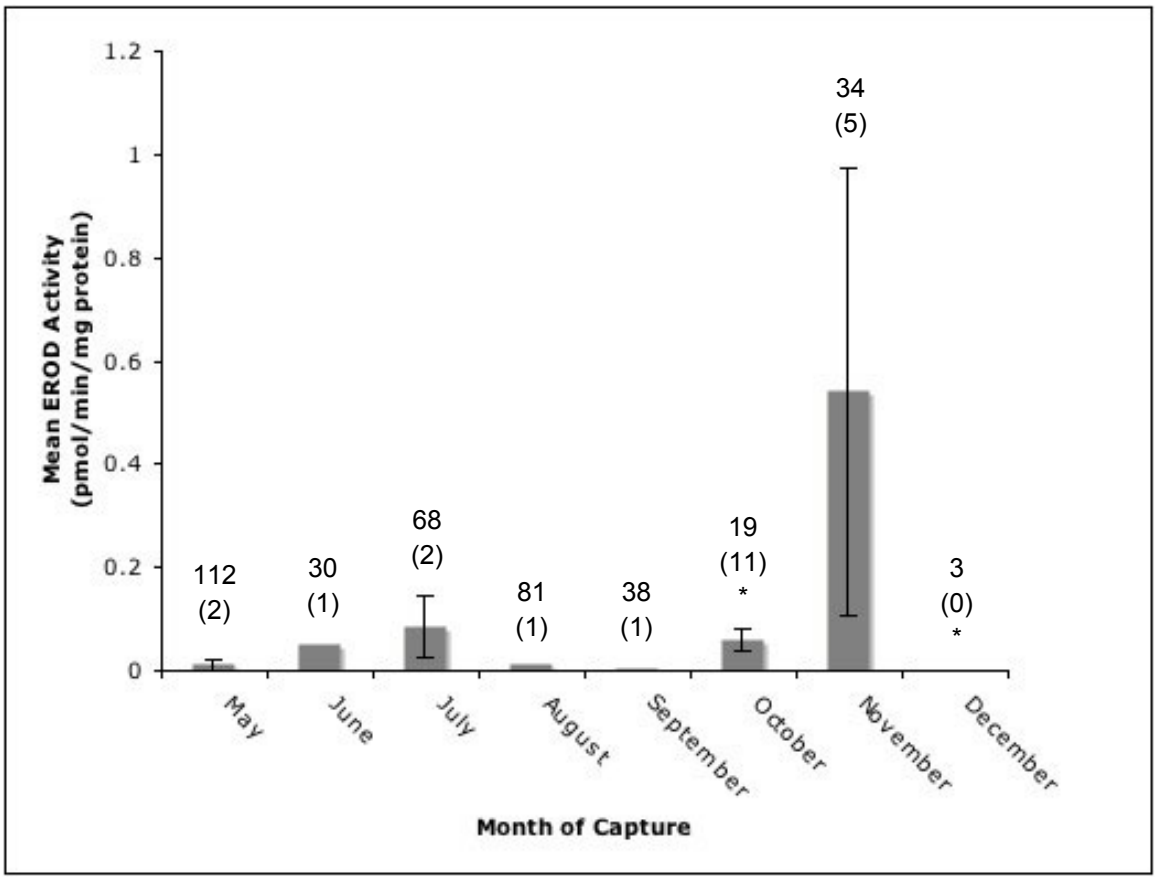

Figure 2.6 Mean EROD activity (pmol resorufin/min/mg protein) by month for all species. Sample sizes are indicated above each bar, with the number of samples with positive EROD activity in parentheses. Significance is shown by the asterisk (October vs. May: $p < 0.001$, June: $p < 0.001$; July: $p < 0.001$; August: $p < 0.001$; September: $p < 0.001$; November: $p < 0.05$; Kruskal-Wallis/Dunn's Multiple Comparisons Test). Error bars indicate standard error.

-By Species-

Due to the low number of samples with detectable EROD activity, comparisons could not be performed for each species analyzed (Figure 2.7). Only those species having more than one positive EROD detection were used for comparisons (i.e., *Rhizoprionodon terraenovae*, *Carcharhinus acronotus, C. isodon*, *C. brevipinna*, and *Galeocerdo cuvier*). Of the five species having two or more samples with detectable EROD activity, *C. acronotus* proved to be the best indicator species for EROD induction following oil exposure. "Post-oil" *C. acronotus* samples had both a significantly higher number of EROD detections ($p < 0.01$; Fisher's Exact Test; Figure 2.8) and a significantly higher mean EROD activity ($p < 0.01$; Welsh's t-test; Figure 2.9) than "pre-oil" *C. acronotus* samples. Significant differences in mean EROD activity for *C. acronotus* were also found between October 2010 and every month sampled during 2010 except for December (May: $p < 0.001$, June: $p < 0.001$; July: $p < 0.001$; August: $p < 0.001$; September: $p < 0.001$; November: $p < 0.001$; December: $p > 0.05$; Kruskal-Wallis/Dunn's Multiple Comparisons Test; Figure 2.10).

A single high EROD activity value (16.75 pmol resorufin/min/mg protein) was repeatedly measured for a *G. cuvier* sample, however the other EROD activity levels for this species fall within the range of EROD activities measured for the other species in this study (0.02-3.17 pmol/min/mg protein).

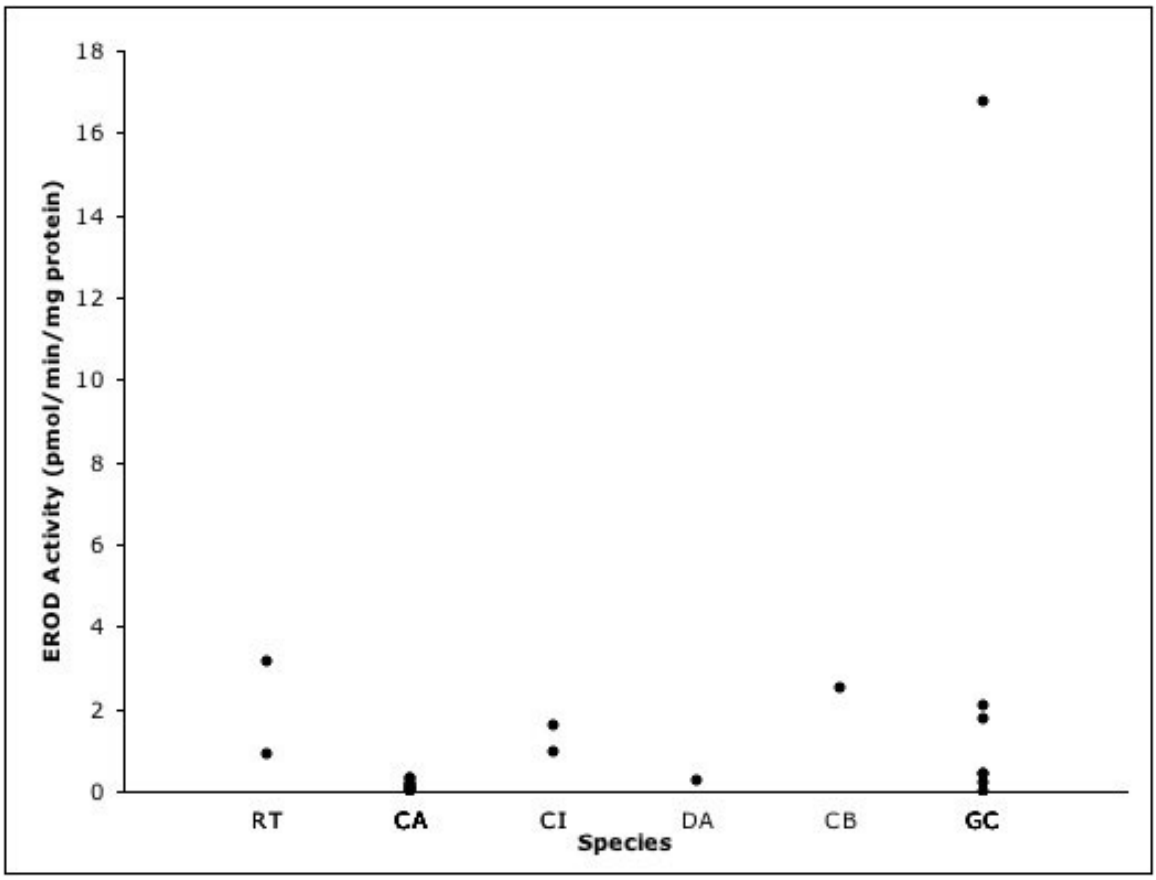

Figure 2.7 EROD activity measurements (pmol/min/mg protein) for all species that displayed detectable levels (RT = *Rhizoprionodon terraenovae*, CA = *Carcharhinus acronotus*, CI = *C. isodon*, DA = *Dasyatis americana*, CB = *C. brevipinna*, GC = *Galeocerdo cuvier*).

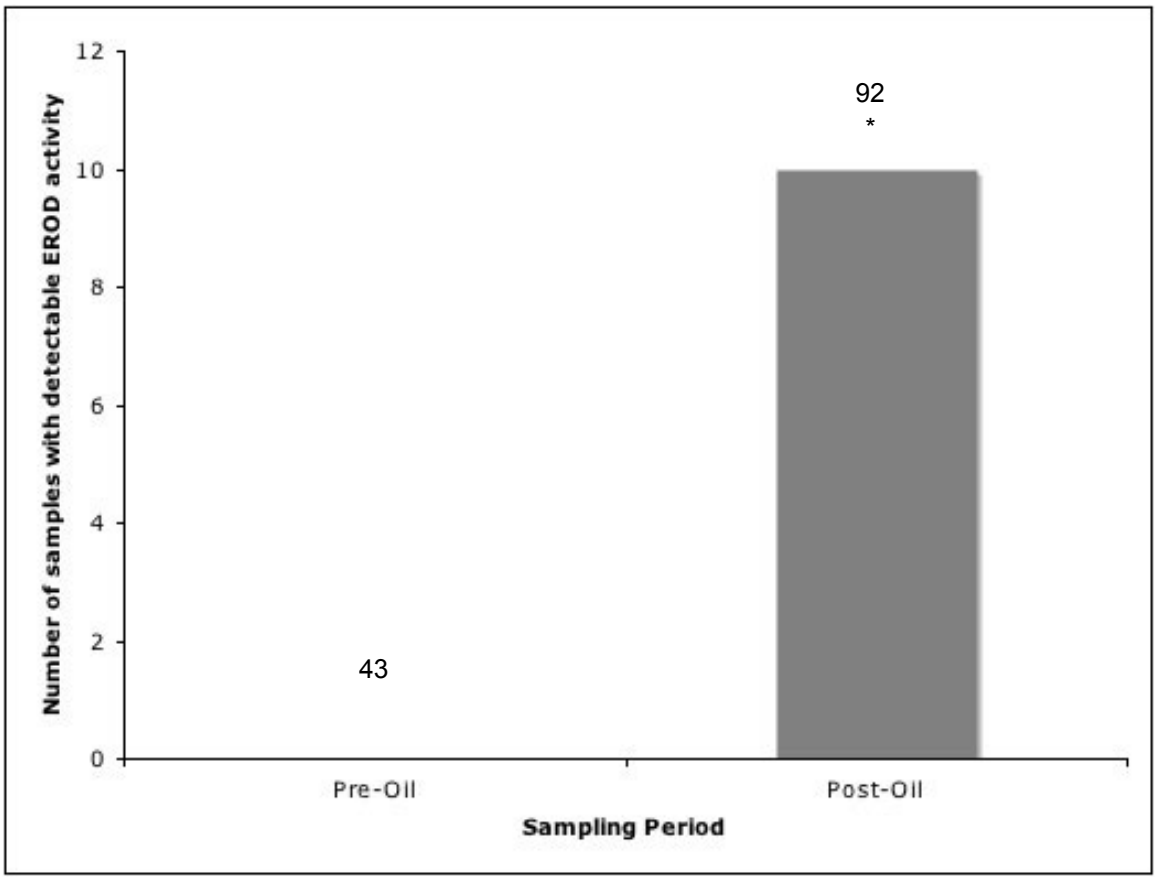

Figure 2.8 Number of *Carcharhinus acronotus* samples with detectable EROD activity before and after oil from the Deepwater Horizon oil spill reached Alabama waters. Sample sizes are indicated above each bar. Significance is shown by the asterisk ($p < 0.01$; Fisher Exact Test).

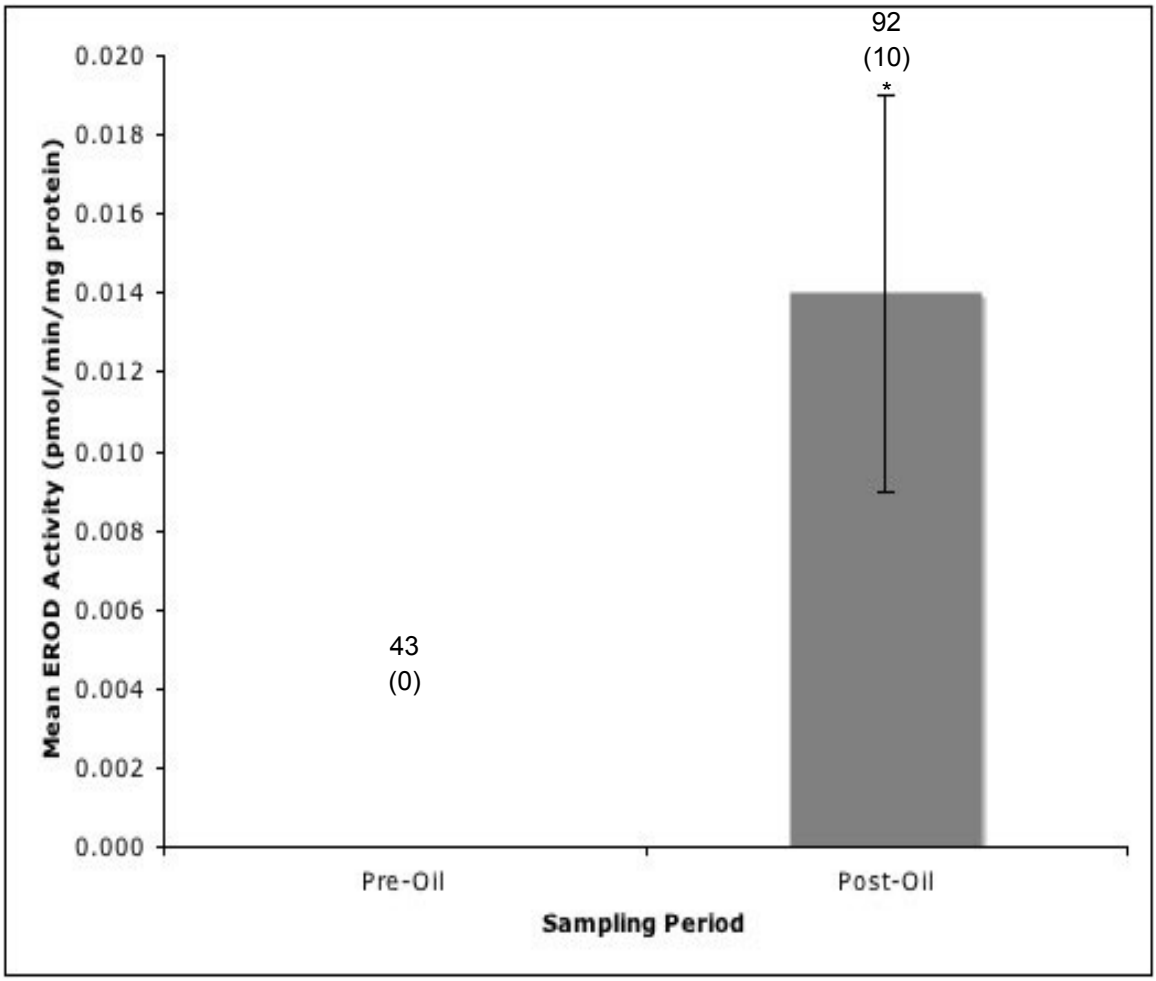

Figure 2.9 Mean EROD activity for *Carcharhinus acronotus* samples obtained before and after oil from the Deepwater Horizon oil spill reached Alabama waters. Sample sizes are indicated above each bar, with the number of samples with positive EROD activity in parentheses. Significance is shown by the asterisk ($p < 0.01$; Welsh's t-Test). Error bars indicate standard error.

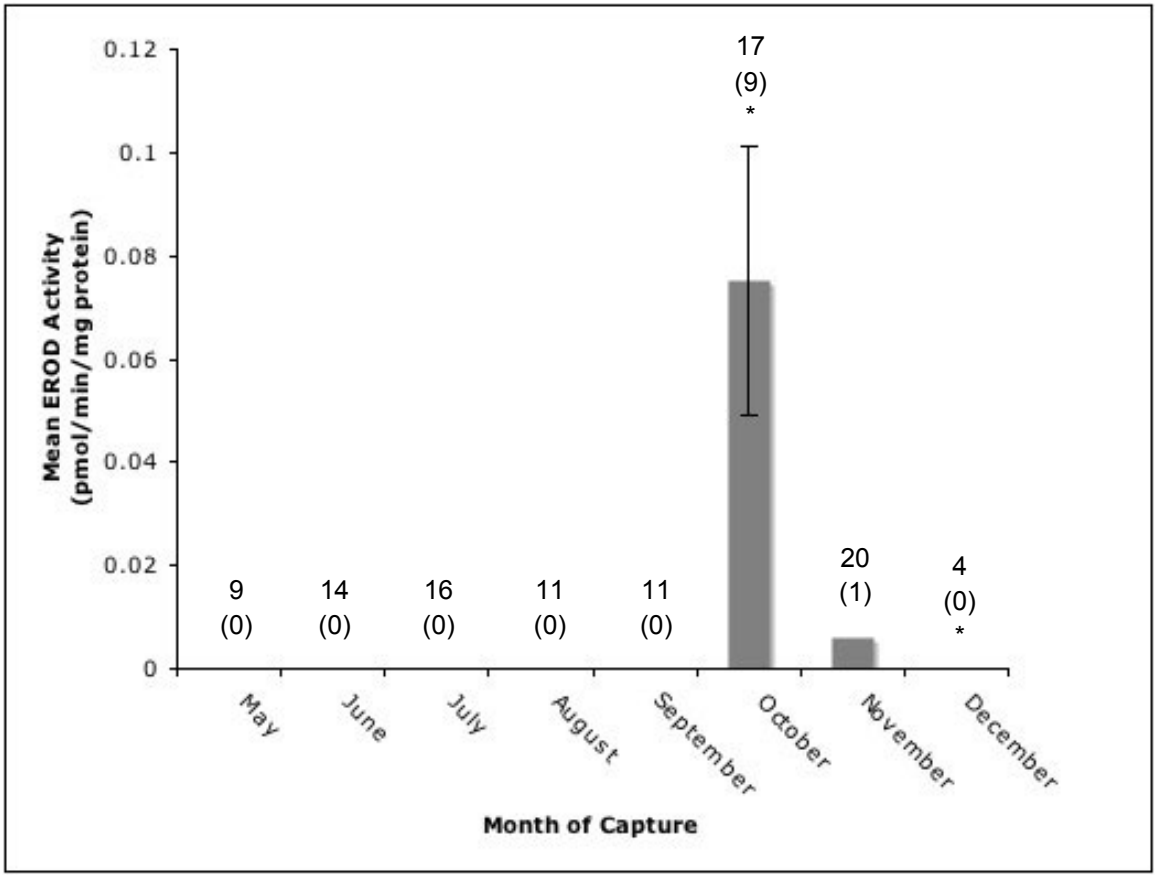

Figure 2.10 Mean EROD activity per month in 2010 for *Carcharhinus acronotus* samples. Sample sizes are indicated above each bar, with the number of samples with positive EROD activity in parentheses. Significance is shown by the asterisk (October 2010 vs. May: $p < 0.001$, June: $p < 0.001$, July: $p < 0.001$, August: $p < 0.001$, September: $p < 0.001$, November: $p < 0.001$; Kruskal-Wallis/Dunn's Multiple Comparisons Test). Error bars indicate standard error.

Discussion

Overall, the results of this study suggest that sharks residing off the coast of Alabama, in a region that was eventually oiled, are exhibiting biochemical effects in the form of induced activity of the Phase I biotransformation enzyme, cytochrome P 4501A1 (CYP1A1) likely due to exposure to oil from the *Deepwater Horizon* oil spill (DHOS) in 2010. CYP1A1 activity has previously been shown to be induced in a variety of organisms (Van der Oost et al., 2003; Newman, 2010), including sharks (Fuentes-Rios et al., 2005), following exposure to the toxic component of oil, polycyclic aromatic hydrocarbons (PAHs), and the results of this study agree with these findings. Sharks obtained after oil reached Alabama waters in June 2010 had significantly higher EROD activity, and therefore higher CYP1A1 activity, than sharks sampled in the years prior to the occurrence of the DHOS as well as the months before oil reached Alabama waters. Also, the EROD activity levels observed in this study, with the exception of one extremely high value from a tiger shark, are within the range of EROD activity levels reported by Fuentes-Rios et al. (2005) for cat-sharks exposed to PAHs. Therefore, based on the elevated EROD activity levels observed in "post-oil" samples, it is likely that these sharks were exposed to higher concentrations of PAHs than the "pre-oil" samples were. Although exposure to other chemicals present in the GOM may also induce CYP1A1 activity, the effects due to exposure to these additional pollutants would have been detectable before 2010, which was not observed in this study.

The DHOS occurred on 20 April 2010 and before it was sealed 87 day later, over 4 million barrels of oil had been released into the GOM (Crone & Tolstoy, 2010). Beginning 31 May 2010, oil began encroaching into the areas where samples for this study were collected (NEDIS Anomaly Analysis 31-May-2010 Composite, http://www.GeoPlatform.gov/gulfresponse). By June 2010, this oil had reached the shores of Alabama (Webb et al., 2011), putting all of the marine organisms in these areas at an increased risk of adverse health effects due to exposure to oil and PAHs. Previous studies on the effects of PAH exposure on marine organisms following oil spills have demonstrated that exposure to these contaminants can result not only in biochemical and physiological changes (Collier et al., 1996; Kirby et al., 1999; Bodkin et al., 2002), but organism- and population-level effects as well (Peterson et al., 2003; Jackson et al., 1989). The

increased level of CYP1A1 activity observed in this study following the oiling of Alabama waters represents a biochemical change in those sharks, which could lead to the production of more highly toxic PAH metabolites, DNA adducts and/or reactive oxygen species (ROS) (Gelsleichter and Walker, 2010), and possibly cell- or organ-level damage (Myers et al., 2008; Varanasi et al., 1987; Baumann et al., 1998). Ultimately, these effects may manifest themselves in ways similar to the reproductive changes, slowed growth, and population declines observed by Peterson et al. (2003) and Jewett et al. (2002) in pink salmon *Oncorhynchus gorbuscha,* masked greenling *Hexagrammos octogrammus*, and crescent gunnel *Pholis laeta* following the *Exxon Valdez* oil spill in Alaska.

Of all the species examined individually in this study, blacknose sharks *Carcharhinus acronotus* appeared to be the best indicator for effects due to PAH exposure, as *C. acronotus* was the only species to show a significant increase in EROD activity in "post-oil" samples when compared to "pre-oil" samples. Blacknose shark populations also undergo limited seasonal migrations in the GOM, which allowed for samples to be obtained year-round (Sulikowski et al., 2007), a necessity for accurate monitoring of effects in fishes due to pollutant exposure (Van der Oost et al., 2003). It was also observed that the highest frequency of EROD detections occurred during October 2010, four months after oil reach Alabama waters. This timing in peak EROD activity is similar to the timing of CYP1A1 induction observed in fish from oil-spill sites, in which peak activities are reached within 2-3 months following oil exposure (Ritchie & O'Sullivan, 1994; Lindstrom-Seppa, 1988). These studies also found that within one year of the exposure, CYP1A1 activities had returned to background levels. In order to determine the length of effect in sharks, it is suggested that sampling and testing of *C. acronotus* off the coast of Alabama should continue until CYP1A1 is no longer detected in these animals.

Tiger sharks *Galeocerdo cuvier* may also prove be a useful indicator species for exposure due to the high frequency of EROD activity detections observed in "post-oil" samples, however the lack of "pre-oil" samples prevented an accurate comparison from being made. The EROD activity, after repeated measurements, in one *G. cuvier* sample was extremely high (16.75 pmol/min/mg protein) in comparison to other EROD values in this study as well as in the Fuentes-Rios et al., (2005) study. This may be a result of

the diet of *G. cuvier*, which typically consists of much larger prey than do the diets of the other species in this study (Wetherbee & Cortes, 2004). This high level of EROD activity may also indicate the presence of higher-level physiological effects in this animal, including the formation of DNA adducts. Future work should attempt to measure EROD activity in additional tiger sharks as well as to investigate the possibility of higher-level effects in these animals.

Of the 23 samples with detectable EROD activity levels, two, a southern stingray *Dasyatis americana* and a sharpnose shark *Rhizoprionodon terraenovae,* were obtained prior to oil reaching Alabama waters. These results may be a function of the movement of these animals to deeper, offshore water during the DHOS event. *R. terraenovae* have been observed offshore at depths greater than 30 m, possibly utilizing these deeper waters for parturition (Parsons, 1983; Drymon et al., 2011). Similarly, although *D. americana* typically prefer shallow waters, they have been observed at depths of 53 m of water (Grubbs et al., 2006). Any migration of these animals to deep, offshore waters may have brought them into contact with oil from the DHOS sooner than those animals that remained nearshore, explaining the induction of EROD activity before Alabama waters were contaminated with oil.

In conclusion, it appears that sharks were potentially exposed to PAHs from the DHOS, are experiencing significant biological effects. Sharks obtained after the arrival of oil from the DHOS in Alabama displayed an obvious induction of EROD activity as compared to animals caught prior to the arrival of the oil indicating that exposure to the PAHs present in the oil resulted in the observed increases in EROD activity in exposed animals. Future work should continue to investigate the degree of effects of exposure to oil on sharks while focusing on effects due to exposure on larger sharks (i.e., *G. cuvier*) as well as to determine the duration of effects experienced by sharks following exposure to oil.

Conclusions

The results of this study demonstrate that sharks have the ability to accumulate environmental contaminants at levels that may be detrimental to their health. Although numerous studies have been done on pollutant levels in the tissues of sharks, this is one of the first studies to investigate the effects of exposure to these pollutants in these animals. Using biomarkers as an indicator of physiological effect in elasmobranchs, this study attempted to determine the extent at which sharks are adversely affected by exposure to mercury and polycyclic aromatic hydrocarbons (PAHs).

This study reported that, while bonnethead sharks *Sphyrna tiburo* along the west coast of Florida can accumulate levels of mercury that exceed the recommended limit for monthly consumption for the average person in the United States (i.e., 0.49-0.94 mg Hg/month for a 70 kg person; USEPA, 2004), the levels of mercury do not seem to correlate with metallothionein (MT) levels in either liver or muscle, suggesting that the use for MT as a biomarker for mercury exposure in *S. tiburo* may be limited. The lack of correlation may be explained by the influence of other contaminants on MT induction, induction associated with exposure to other toxic metals or the combined effect of exposure to several metals at once, the mode of Hg exposure, or the tissues used for analysis as MT and Hg concentration can vary between tissues. Another possible explanation may be that the THg concentrations observed in this study may be below the threshold of effect for bonnethead sharks. It is suggested that further research be conducted in the following areas: 1) the relationship between MT levels and exposure to a broader panel of heavy metals, 2) the use of additional biomarkers (i.e., oxidative stress parameters) in determining the effect of exposure to toxic metals, and 3) the relationships between MT and metal exposure in species of sharks occupying higher trophic levels than *S. tiburo* (e.g., blue sharks, tiger sharks).

Additionally, this study found that exposure to the PAHs present in the oil released by the *Deepwater Horizon* oil spill (DHOS) in 2010 may have resulted in adverse biochemical effects in a variety of shark

species in the Gulf of Mexico. While blacknose sharks *Carcharhinus acronotus* seemed to be the best indicator of biological effect following PAH exposure, it is possible that, had an adequate number of samples from before the DHOS been available, tiger sharks *Galeocerdo cuvier* would have also shown to be a reliable indicator of effects related to oil exposure. I propose that monitoring of the effects of PAH exposure caused by the DHOS on sharks should continue. In particular, future studies should investigate the duration of these effects, the possibility of higher-level effects occurring in these animals, as well as the effects experienced by larger, longer-lived species that occupy higher trophic levels based on their ability to accumulate much higher levels of this contaminant.

It is possible that the migrations and movement patterns of some of the shark species in this study may have had an influence on the extent and timing of pollutant exposure, and therefore the effects, observed. For example, tiger sharks have been found to undergo long-range, offshore movements interrupted by time spent in near-shore areas (Heithaus et al., 2007; Holland et al., 1999). While the reasons for these movements are largely unknown (Heithaus et al., 2007), it is possible that, if the sharks in this study had moved into the deep water closer to the site of the *Deepwater Horizon* oil rig, they may have experienced larger bouts of exposure to the oil as compared to some of the other species in this study (i.e., blacktip sharks *C. limbatus*, bonnetheads *Sphyrna tiburo*, blacknose sharks *C. acronotus*). However, the limited movements of *C. acronotus* populations, both offshore and along the coast, in the GOM supports the species use as an indicator for effects due to exposure to environmental contaminants (Sulikowski et al., 2007).

Overall, the results of this study clearly show that sharks are at risk of accumulating environmental pollutants at levels that may result in adverse health effects. However, to date, very few studies have investigated the nature of these effects in sharks. Therefore, future work should further investigate the effects of exposure to not only the contaminants investigated in this project, but to the other persistent organic pollutants and heavy metals as well. These studies should make use of biomarkers as tools for determining the types of effects experienced by sharks after exposure as well as the potential for higher-level effects in these species.

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