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Mercury Contamination and Its Relation to Trophic Ecology and Anthropogenic Pollution in Coastal and Deep Sea Shark Communities

Johanna Leigh Imhoff

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MERCURY CONTAMINATION AND ITS RELATION TO TROPHIC ECOLOGY AND ANTHROPOGENIC POLLUTION IN COASTAL AND DEEP SEA SHARK COMMUNITIES

By

JOHANNA LEIGH IMHOFF

A Dissertation submitted to the Department of Biological Science in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

The effect of changing anthropogenic mercury emissions on marine wildlife is of broad interest. Methylmercury can cause reproductive and neurological damage and biomagnifies in food webs. Mercury availability in the Pacific Ocean has been increasing and therefore could be expected to increase in marine fishes. In Hawaii, tunas have shown increases proportional to increasing oceanic mercury over several decades. Historical data on shark muscle mercury is also available for Hawaii, presenting an opportunity for comparison in a long-lived upper trophic level cartilaginous fish. Muscle samples were opportunistically collected from sandbar sharks in 2003-2005 for mercury analysis and comparison with published data from 1971. Mercury contamination was similar in sandbar sharks collected in the two time periods, in contrast to tunas. Sandbar sharks collected in 2003-2005 had a higher observed rate of stillborn embryos than previously documented. Since mercury can cause reproductive toxicity, muscle mercury contamination was quantified in available sandbar shark embryos. Contamination was similar in stillborn and viable embryos, indicating that mercury toxicity was likely not the cause of embryo death. Mercury does not appear to have changed in sandbar sharks and the observed frequency of stillborn sharks does not appear to be due to increasing mercury emissions over three decades.

Deep sea chondrichthians comprise nearly half of global chondrichthyan fauna but have been researched relatively less than their coastal and pelagic counterparts. As long-lived mesopredators and apex predators in their food webs, deep sea shark can bioaccumulate high levels of mercury in their tissues. Mercury was measured in six species of relatively abundant deep sea sharks in the GOM that inhabit the continental shelf edge to the continental slope. To attempt to address whether the Deepwater Horizon (DwH) oil spill indirectly affected bioavailability of methylmercury in GOM, the same or closely related species were also analyzed from regions not affected by the oil spill. Overall, Mustelus sinusmexicanus had the lowest mercury among the GOM sharks analyzed, and Squalus clarkae had the highest. Mustelus canis and Centrophorus granulosus were similar to each other in their contamination. Males differed from females for both S. cubensis and S. clarkae, as well as C. uyato Eleuthera S. cubensis had significantly different mercury contamination to females of GOM S. clarkae, to which they were closer in size than GOM S. cubensis. GOM Centrophorus granulosus had slightly but not significantly higher mercury contamination than Virginia C. granulosus. Regional comparisons
in lieu of prespill samples did not suggest that there was an effect of the DwH oil spill on mercury in sharks, though these are necessarily challenging comparisons.

Since mercury bioaccumulation in organisms is tightly linked to biomagnification, mercury studies are often coupled with trophic ecology studies using light stable isotopes as tracers. Since animals vary in the quantity of lipids that they store in their tissues, and since the presence of lipids can affect $\delta^{13}C$ signatures, it is necessary to explore these effects to correctly interpret $\delta^{13}C$ results. A particular challenge for chondrichthyan fishes is that they store isotopically light nitrogenous wastes in their tissues for osmoregulation, and this can affect the interpretation of $\delta^{15}N$, sometimes leading to inaccurate interpretation of trophic level. The effects of lipids and nitrogenous wastes on stable isotope analysis of chondrichthyan fishes has been an important aspect of trophic ecology research on this group. The effects of lipid extraction, urea extraction, and combined lipid and urea extraction compared to bulk tissue were tested on a coastal and deep sea teleost and a coastal and deep sea elasmobranch as representatives that might be included in coast to deep sea food web studies. Since mercury analysis requires the same initial muscle tissue handling steps as stable isotope analysis (SIA), the effects of treatments on mercury quantification were also tested. The results suggest that chemical lipid extraction is necessary for accurate interpretation of SIA results in coastal and deep sea teleosts and elasmobranchs and that urea was extracted sufficiently during the lipid extraction, therefore additional urea extraction steps are not necessary. Additionally, it may be possible for researchers to use leftover treated muscle from SIA to obtain accurate mercury results. Future research on trophic ecology including SIA informed by this research may help to provide context for mercury findings in GOM deep sea sharks.
CHAPTER 1

INTRODUCTION

In aquatic and marine fishes, mercury has often been investigated from a food safety perspective (e.g., Bloom 1992). It is monitored in common food fishes so that regulatory bodies can make recommendations for safe consumption choices. Fishes like sharks are sometimes consumed by humans, but not all species are commercially harvested or monitored for mercury contamination. Mercury both bioaccumulates or increases in concentration as an animal grows larger or longer, and biomagnifies, or increases with trophic level in a food web. Since sharks are generally mesopredators and apex predators, they have high potential for biomagnifying mercury, and since they are long-lived, slow-growing, and some species attain large sizes, they have the potential bioaccumulate high levels of mercury. A consequence of this is that sharks can be swimming reservoirs of mercury for long periods of time, even decades, carrying methylmercury in their tissues until they are consumed by predators or scavengers. There are many angles from which to explore the dynamics of mercury in elasmobranch fishes, including speciation and bioaccumulation curves (e.g., Bloom 1992, Hueter et al. 1995, Gelsleichter and Walker 2010), link with trophic ecology (Domi et al. 2005, Newman et al. 2011, Pethybridge et al. 2012, Le Bourg et al. 2014, Endo et al. 2016), relation to habitat (e.g., Hornung et al. 1993, Pethybridge et al. 2010), and maternal offloading (e.g., Childs et al. 1973, Adams and McMichael 1999, Le Bourg et al. 2014, Frías-Espericueta et al. 2015, van Hees and Ebert 2017, de Souza-Araujo et al. 2020). This dissertation investigates some of the dynamics of mercury contamination in elasmobranch fishes.

Chapter 2 of this dissertation explores temporal dynamics of mercury in sharks as well as aspects of maternal offloading. Long time series of mercury data are relatively rare in elasmobranch fishes (Greig et al. 1977, Taylor et al. 2014), are often opportunistic, and are not set up as long-term studies, but rather comparisons are made to previously published research. For example, the investigation of mercury in sandbar sharks in Hawaii in Chapter 2 was an opportunistic study made possible by sample collections from other projects (Romine et al. 2006, Daly-Engel et al. 2007). The temporal comparison provided by Thieleke's (1973) thesis on mercury in sandbar and tiger sharks was also opportunistic, as sharks were taken in Hawaii’s
Shark Research and Control programs, which were intended to minimize and prevent shark-human interactions as well to learn about shark biology.

Maternal offloading of mercury has been investigated in terms of comparing embryo mercury to mother mercury (Adams and McMichael 1999, Le Bourg et al. 2014, van Hees and Ebert 2017, Dutton and Venuti 2019, de Souza-Araujo et al. 2020), mechanisms of maternal transfer (Lyons and Lowe 2013a, Frías-Espericueta et al. 2015, van Hees and Ebert 2017), but the effects of mercury on embryos is not well understood. Chapter 2 investigates whether it is likely that observed embryo death in sandbar shark litters was caused by maternal offloading of mercury, or whether increased mercury contamination could be the cause of an apparent increase in rate of litters containing stillborn embryos.

Chapter 3 investigates mercury contamination in deep sea sharks in the Gulf of Mexico. Mercury has not been previously reported for these species in this region, though their trophic ecology (Churchill et al. 2015b) and stress physiology (Prohaska et al. 2021) have been described, and recent advances have been made in understanding their taxonomy and population genetics (Veríssimo et al. 2014, Giresi et al. 2015, Pfleger et al. 2018). Investigation of mercury in deep sea sharks in the Gulf of Mexico was driven not by the need to determine if they can be consumed safely, as these species are not commercially harvested or commonly consumed by humans, but by the observation that the 2010 Deepwater Horizon oil spill had strong effects on microbial community dynamics and population sizes, and the hypothesis that this could have led to increased methylation of mercury in situ, and therefore higher bioavailability (Joye et al. 2014, Kleindienst et al. 2015). Testing this hypothesis is hindered by a lack of pre-spill data, so muscle mercury contamination in deep sea sharks in the Gulf of Mexico was compared to mercury from the same or closely-related species in disparate regions which were not affected by oil spills.

Effects of depth on mercury concentration were examined because some fishes have displayed patterns in mercury contamination with depth habitat or biome (Choy et al. 2009, Pethybridge et al. 2010, 2012), and sharks living and feeding in deeper waters may be accessing deeper benthic food webs as well as the mesopelagic food web. Effects of proximity to the site of the Deepwater Horizon oil spill were also examined by comparing mercury contamination between the east (closer to the oil spill) and west (farther from the oil spill) sides of De Soto Canyon.
Understanding of the patterns observed in mercury contamination in deep sea sharks of the Gulf of Mexico will be greatly improved with trophic ecology data in the form of stomach contents and stable isotopes analyses, which are ongoing but not included in this dissertation. Chapter 4 focuses on the effects of lipid and urea extraction on stable isotopes analysis of coastal and deep sea teleosts and elasmobranchs. This was important to investigate prior to conducting stable isotope analysis because of the confounding effects that stored lipids and nitrogenous wastes can have on the interpretation of δ¹³C and δ¹⁵N data (Post et al. 2007, Logan and Lutcavage 2010, Hussey et al. 2012, Kim et al. 2012, Li et al. 2015). In the years following the Deepwater Horizon oil spill, there was interest in large scale food web research from the coast to the deep sea (Coleman et al. 2014) and this drove the decision to analyze representative coastal and deep sea teleosts and elasmobranchs. Since mercury analysis and stable isotope analysis share the same initial tissue processing steps, the effect of lipid and urea extraction treatments on mercury analysis were also tested.
CHAPTER 2

AN EVALUATION OF MERCURY IN SANDBAR SHARKS
(CARCHARHINUS PLUMBEUS) IN HAWAII: STABILITY OVER A
THREE DECADE PERIOD

2.1 Introduction

Mercury, particularly in the form of methylmercury (MeHg), is an anthropogenic pollutant of global concern in aquatic systems (Mason et al. 1994) because it is toxic to humans and wildlife (Driscoll et al. 2013) and has the ability to biomagnify in food webs (Wang 2002, Chen et al. 2008). Mercury is known to cause neurological damage in humans, particularly in children whose mothers consumed mercury-contaminated fish or shellfish while pregnant (Driscoll et al. 2013). Mercury contamination has been extensively investigated in fishes because consumption of fish is the primary way that humans are exposed to dietary mercury (Driscoll et al. 2013). The harmful effects of mercury on these taxa have also been studied. Deleterious effects on bony fishes caused by mercury contamination can include decreased hatching success (Latif et al. 2001), decreased weight, length and gonad to body weight ratio (Friedmann et al. 1996), decreased appetite and death (Niimi and Kissoon 1994), behavioral changes that reduce survival (Matta et al. 2001), and impaired reproduction (Matta et al. 2001). Shark muscle mercury is often higher than levels recommended for human consumption and increases with increasing shark size (e.g. Boush and Thieleke 1983; Hornung et al. 1993; Hueter et al. 1995; Adams and McMichael 1999; de Pinho et al. 2002; Pethybridge et al. 2010; Taylor et al. 2014; Gilbert et al. 2015; Endo et al. 2016). While mercury contamination in elasmobranch fishes (sharks, skates, and rays) has been well-documented, the effects on the health of the animal are not well understood, particularly for free-ranging animals, and are probably mostly sublethal. Similarly, effects of mercury on birds are often sublethal, which can make it difficult to understand population-level consequences of mercury contamination (Whitney and Cristol 2017).

Aspects of mercury transfer from mothers to embryos have been described for some elasmobranch species. Embryos have lower mercury than their mothers (Adams and McMichael 1999, Le Bourg et al. 2014, van Hees and Ebert 2017), for example, Adams and McMichael (1999) found that embryos of bull sharks, Atlantic sharpnose, bonnethead, and blacktip sharks,
all yolk-sac placental live-bearing species, contained 8.3% to 60.4% of their mothers’ mercury loads. Mercury may transfer from mothers to embryos across the placenta and through the umbilical cord, as documented in the Pacific sharpnose shark (Frías-Esparicueta et al. 2015), or through embryo consumption of yolk in oophagous species (Lyons and Lowe 2013b). If mercury is offloaded from mothers to embryos, embryo mercury should increase with mother mercury, as observed in leopard sharks and thornback rays, and the amount of mercury contamination in the mother may be important in determining whether and how much mercury is offloaded to the embryos (van Hees and Ebert 2017). Resources stored in the mother’s liver are transferred to the embryos, so liver mercury levels can be more similar between mothers and embryos than muscle mercury levels in some cases (Lyons and Lowe 2013b), but maternal muscle mercury is sometimes a better predictor of embryo mercury in others (van Hees and Ebert 2017). The effects of mercury accumulated during development on elasmobranch embryos is not well understood, though organic contaminants have been shown to reduce embryo growth (Lyons and Wynne-Edwards 2018).

There has been some interest in understanding patterns in mercury contamination of fishes over decadal time scales because mercury contamination in the marine environment is hypothesized to increase with increasing atmospheric mercury (Kraepiel et al. 2003). Both increases and decreases in mercury contamination of fishes in the Savannah River, Georgia, were observed over a 30 year period and were associated with reductions in point source pollution inputs, changes in atmospheric deposition, and river flow (Paller and Littrell 2007). Museum specimens of glacier lanternfish (*Benthosema glaciale*) collected between 1936 and 1993 showed significant differences in mercury contamination between years, but overall similar values over the long time series (Martins et al. 2006). Tuna caught 1970-1971 and canned for human consumption did not have significantly different mercury levels from museum specimens of tuna and swordfish from 62 to 93 years earlier (Miller et al. 1972), but only eight specimens comprising three species of tuna and one individual swordfish were examined. Yellowfin tuna collected in the central Pacific in 1998 (Kraepiel et al. 2003) were found to have similar mercury levels to those collected in 1971 (Thieleke 1973), but the addition of a third dataset from 2008 (Choy et al. 2009) revealed an increase in mercury contamination (Drevnick et al. 2015). While most long-term studies of mercury accumulation in fishes have focused on freshwater fishes and some on marine teleost fishes that are of interest for human consumption (e.g., tunas), decadal
scale studies of mercury contamination in sharks are rare. Comparison of mercury in spiny dogfish (*Squalus acanthias*) caught off of Rhode Island, USA between 2009 and 2012 showed a decrease in mercury compared to spiny dogfish caught from a similar but broader area of the western Atlantic (Gulf of Maine to New York Bight) in 1972 (Greig et al. 1977, Taylor et al. 2014).

The availability of life history data and muscle tissues of sandbar sharks (*Carcharhinus plumbeus*) in nearshore Hawaiian waters made this species ideal for a decadal-scale investigation of mercury contamination as well as an investigation into the effects of mercury on shark embryo viability. Multiple shark control and research programs were carried out in the state of Hawaii between 1959 and 1976, with objectives of removing sharks from nearshore areas and preventing interactions with humans (Wetherbee et al. 1994). Thieleke (1973) analyzed mercury in sandbar shark and tiger shark (*Galeocerdo cuvier*) muscle samples from the 1971 Shark Control and Research Program (Fujimoto and Sakuda 1971), as well as commercially important fish species including yellowfin tuna (*Thunnus albacares*) collected from a commercial fish market. Life history research sampling in the early 2000s (e.g. Romine et al. 2006; Daly-Engel et al. 2007) presented an opportunity to collect tissues from sandbar sharks again, allowing for the comparison of mercury contamination in this species over three decades. Additionally, it was observed that while average litter sizes in 2002-2005 (5.51; Daly-Engel et al. 2007) were similar to average litter sizes in 1969-1979 (5.5; Wass 1973), rates of observed stillbirth apparently increased from 2.2% of litters and 0.04% of embryos in 1967-1969 (based on data from Tester 1969) to 26.1% of litters and 5.3% of embryos in 2003-2005 (Fig. 1). Since mercury can be transferred to embryos and cause damage to reproductive systems without killing the mother, and since sandbar sharks have been observed to carry high levels of mercury (Thieleke 1973, Hueter et al. 1995, Gilbert et al. 2015) it was hypothesized that mercury contamination of embryos in utero could be a cause of embryo death, and therefore could be the cause of the apparent increase in rate of stillbirth observed in 2003-2005. The objectives of this research were: 1) to determine if muscle total mercury (THg) in sandbar sharks was higher in specimens sampled in 2003-2005 than those sampled in 1971 and 2) to determine if muscle THg in sandbar sharks was higher in stillborn embryos than in viable embryos.
Figure 2.1. Examples of sandbar shark embryo mortality: A) an embryo deceased by umbilical strangulation, B) two deceased embryos within a litter of five other viable embryos. Photographs by R. Dean Grubbs.
2.2 Methods

Sandbar sharks were caught in a fishery-independent longline survey between 2002 and 2005 outside of Kaneohe Bay, Hawaii (Fig. 2). The details of this survey were described elsewhere (Romine et al. 2006). White muscle was removed anterior to the caudal peduncle and frozen for later analysis. The muscle tissue was freeze-dried and ground to a fine powder using a ball mill grinder. Mercury was extracted from dried, ground muscle by acid digestion and measured using cold vapor atomic absorption spectroscopy (EPA Method 1630) on a Tekran 2600 (FSU Department of Earth, Ocean, and Atmospheric Science) or a Tekran 2700 (National High Magnetic Field Laboratory) Mercury Analyzer. Sample analysis was shifted from the Tekran 2600 to the Tekran 2700 in the interest of analyzing MeHg in addition to total mercury. Muscle (0.047-0.320 g) was mixed with 5 mL of 6M HNO₃ in acid-cleaned glass vials and heated at 70 °C for 8 to 12 hours. The vials were allowed to cool and were then stored at 6-7 °C until derivatization. Samples analyzed on the Tekran 2700 were centrifuged and 1 mL of the supernatant was recovered and diluted with 10mL of ultrapure deionized water (or 5mL for embryos) to prepare for derivatization. Blanks were not diluted. Twenty to 40 µl of diluted sample (for juveniles or adults, and embryos, respectively) was derivatized with 40 µl of 1% sodium tetraethylborate in 30 ml of ultrapure deionized water. Solutions of known monomethyl mercury (MMHg) and inorganic mercury (IHg) concentrations made from standards were used to generate a calibration curve for each analysis. Blanks and certified reference materials (Dogfish Muscle NRCC DORM-3, Dogfish liver NRCC DOLT-4, Tuna muscle ERM-CE 464) were run every 10 samples. Replication of samples and standards was performed at the levels of extraction and volatilization. The precision of the method was typically within 5% relative standard deviation and the limit of quantification was less than 0.05 ng/L MeHg and 0.2 ng/L
IHg mercury. Samples were often run in tandem with other researchers using the same calibration curves and certified reference materials (Mickle 2016, Malinowski 2019). Additionally, a subset of goliath grouper samples were sent to another laboratory for comparison, which produced similar mercury quantification, confirming the accuracy of the method (Malinowski 2019). Thieleke’s samples (1973) were analyzed using cold vapor atomic absorption spectroscopy. However, he used different tissue homogenization, acid digestion and volatilization protocols as dictated by the standards of the time and available mercury analyzer (Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer).

Figure 2.2. Sampling area for sandbar sharks caught in 2002-2005. The inset shows Kaneohe Bay off Oahu. Sharks from 1971 Shark Control and Research Program were caught all around Hawaii, within 20 miles.
The relationship between shark length and total mercury contamination was examined for past (1971, n = 26; Thieleke 1973) and recent (2003-2005, n = 27) samples. Past sandbar shark total lengths were converted from inches to centimeters and regressed against total mercury to facilitate comparison with recent samples. Recent samples included sharks that were smaller than the range of shark total lengths analyzed in the past, so a separate regression was performed on recent samples excluding this smaller size range. Total mercury contamination in recent samples was compared to that of past samples using analysis of covariance (ANCOVA) with shark total length as a covariate. Mean total mercury contamination in stillborn embryos was compared to that of viable embryos using one-way analysis of variance (ANOVA). Where possible, embryo total mercury contamination was expressed as a percentage of maternal mercury. All total mercury concentrations are reported in ppm on a wet weight basis. Statistical analyses were performed in R version 3.5.3 using the car package (Fox and Weisberg 2019).

2.3 Results

Sandbar shark muscle samples collected between 2002 and 2005 (recent samples) were composed of a mean 80.1% water (± 6.2% SD). Methylmercury represented 77.7 to 88.0% of total muscle mercury in viable embryos that were analyzed for mercury speciation (n = 9), with a mean of 83.3% MeHg (Table 2.1). All available embryo samples were analyzed for mercury contamination, and only two females were available. Samples were haphazardly selected for mercury speciation analysis and that all were males was coincidental. Juveniles and adults that were analyzed for mercury speciation (n = 6) had MeHg that ranged from 74.9 to 92.7% of total mercury, with a mean of 87.5%, and concentrations that ranged from 0.02 to 3.15 ppm with a mean of 0.80 ppm (Table 2.1).

Total mercury in juvenile and adult sandbar shark muscle generally increased with shark total length for recent samples when sharks smaller than the size range of the past samples (Thieleke 1973) were included ((y = 0.01x – 0.85, Adj. R² = 0.41, p < 0.001; Fig. 2.3) and when they were excluded (y = 0.02x – 1.58, Adj. R² = 0.20, p = 0.04; Fig. 2.3). Mean mercury
contamination was only slightly higher in past samples (0.0051 ± 0.0019, n = 26) than in recent samples (0.0048 ± 0.0042, n = 23), and not significantly so (Fig. 2.3, Table 2.2, 2.3). Time period (past vs. present) did not have a significant effect on THg and there was no interaction between shark total length and time period, regardless of whether sharks smaller than the past size range were excluded (ANCOVA; Table 2.2, 2.3). The recent samples showed greater variability in mercury contamination for larger sharks, with a high concentration of 3.4 ppm THg in a 177 cm female, and low concentrations of 0.02 and 0.05 ppm THg in a 65 cm female and 48 cm male, respectively.

Table 2.1. Mercury speciation in sandbar sharks caught in the mid-2000s. These samples represent a subset of the total samples analyzed (n = 27), for which reliable speciation data were obtained. MeHg and THg are reported in ppm on a wet weight basis and shark total length (TL) in cm is provided.
Total mercury concentrations in embryos were low, ranging from 0.02 to 0.12 ppm, with a mean of 0.06 ppm. While stillborn embryos had slightly higher mean THg (0.0052 ± 0.0073, n = 4) than viable embryos (0.0045 ± 0.0062, n = 12; Fig. 2.4), the difference was not significant (ANOVA; p = 0.62; Table 2.4). Samples for both mothers and their viable embryos were available for two litters. In one litter, embryo THg ranged from 0.0353 to 0.054 ppm, and represented 3.38 to 6.16% of maternal THg (n = 6 embryos). For the other litter, embryo THg ranged from 0.054 to 0.107 ppm and represented 4.46 to 8.87% of maternal THg (n = 2 embryos).

Table 2.2. ANCOVA results for comparison of THg in ppm on a wet weight basis for past (1971) and recent (2003-2005) sandbar sharks off Hawaii. The covariate was shark total length (TL) in cm.

<table>
<thead>
<tr>
<th>ANOVA (Type II):</th>
<th>SS</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>7.78</td>
<td>1</td>
<td>38.31</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time Period (past or recent)</td>
<td>0.00</td>
<td>1</td>
<td>0.01</td>
<td>0.91</td>
</tr>
<tr>
<td>TL: Time Period</td>
<td>0.02</td>
<td>1</td>
<td>0.11</td>
<td>0.74</td>
</tr>
<tr>
<td>Residuals</td>
<td>9.14</td>
<td>45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3. ANCOVA results for comparison of THg in ppm on a wet weight basis for past (1971) and recent (2003-2005) sandbar sharks off Hawaii. Recent sharks smaller than the size range of past sharks were excluded. The covariate was shark total length (TL) in cm.

<table>
<thead>
<tr>
<th>ANOVA (Type II):</th>
<th>SS</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>4.13</td>
<td>1</td>
<td>18.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Time Period (past or recent)</td>
<td>0.03</td>
<td>1</td>
<td>0.14</td>
<td>0.71</td>
</tr>
<tr>
<td>TL: Time Period</td>
<td>0.20</td>
<td>1</td>
<td>0.88</td>
<td>0.35</td>
</tr>
<tr>
<td>Residuals</td>
<td>8.81</td>
<td>39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4. ANOVA results for comparison of THg in ppm on a wet weight basis for viable and stillborn sandbar shark embryos collected off Hawaii. Viability is viable or stillborn. TL is embryo total length in cm.

<table>
<thead>
<tr>
<th>ANOVA (Type II):</th>
<th>SS</th>
<th>df</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TL</td>
<td>0.000032</td>
<td>1</td>
<td>0.04</td>
<td>0.84</td>
</tr>
<tr>
<td>Viability</td>
<td>0.00021</td>
<td>1</td>
<td>0.26</td>
<td>0.62</td>
</tr>
<tr>
<td>Residuals</td>
<td>0.010</td>
<td>13</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 2.3. Total mercury (ppm, wet weight basis) in sandbar sharks from 1971 (past samples) and analyzed by Thieleke (1973) are depicted with open circles and a dashed line (y = 0.01x – 0.66, Adj. R² = 0.56, p < 0.001). Sandbar sharks collected in 2003-2005 (recent samples) are depicted with solid circles and solid lines, with sharks in the same size range as the 1971 samples in black and sharks smaller than that size range in grey. The black solid line shows the regression of all recent samples (y = 0.01x – 0.85, Adj. R² = 0.41, p < 0.001) and the grey line shows the regression with smaller sharks excluded (y = 0.02x – 1.58, Adj. R² = 0.20, p = 0.04).
Figure 2.4. Total mercury contamination (ppm, wet weight basis) in viable and stillborn embryos. Mean contamination was not significantly different between the two embryo conditions (ANOVA, p = 0.62).
2.4 Discussion

The MeHg portion of THg in sandbar sharks was slightly less than the expected 95% MeHg which is widely accepted for fishes (Bloom 1992) and the 91% reported for 9 demersal shark species off Australia (Pethybridge et al. 2010), but still conforms to the generalization that most of the total mercury in fish muscle tissue is represented by MeHg. Total mercury is often used as a proxy for MeHg in fishes, but the observation of lower portions of MeHg in this and other studies suggest that this should not be taken for granted and mercury speciation should be characterized in at least a subset of samples for species and locations that have not previously been assessed. Variable portions of MeHg and portions much lower than 90% of have been reported for gummy sharks (Mustelus antarcticus, Walker 1976) and dusky smoothhound (Mustelus canis, de Pinho et al. 2002) and this was attributed to feeding on invertebrates rather than primarily on fishes. Since MeHg contamination is closely linked to trophic ecology, it is possible that foraging in deeper environments on the continental slope may be responsible for portions of MeHg lower than 90%, and even the phenomenon of lower portions of MeHg than IHg. Sandbar shark diets include teleosts, crustaceans and cephalopods, and cephalopods and other elasmobranchs are more common in larger sandbar sharks (McElroy et al. 2006, Papastamatiou et al. 2006).

The relationship between sandbar shark length and mercury contamination is in agreement with the well-documented trend of increasing mercury contamination as animals increase in size (e.g. Hueter et al. 1995; Adams and McMichael 1999; Gelsleichter and Walker 2010; Taylor et al. 2014; Gilbert et al. 2015) and similar to the samples analyzed by Thieleke (1973). Variation in mercury contamination could indicate differences in feeding habits or differential ability of individual sharks to offload mercury. The steep continental slope off the Hawaiian Islands provides sandbar sharks with access to deeper foraging habitats, where prey may have different mercury contamination than in shallower coastal habitats. In Hawaii, deeper dwelling prey have been found to have higher mercury content than shallower dwelling prey (Choy et al. 2009).

Mercury contamination of other fishes caught in Hawaii has also been compared over decadal time scales. Kraepiel et al. (2003) did not find an increase in mercury from past (Thieleke 1973) to more recent (1998) yellowfin tuna samples, despite their expectation that oceanic MeHg should have increased in the region. However, when the datasets from Thieleke
(1973) and Kraepiel et al. (2003) were reanalyzed with a third, more recent, dataset from 2008, an increase in mercury was observed, and found to correlate to increases in anthropogenic mercury in the Pacific Ocean (Drevnick et al. 2015). All three years had similar slopes, or rates of bioaccumulation in tunas, but the intercept for the 2008 samples was significantly higher than the intercepts for 1971 and 1998. A similar increasing trend in mercury contamination was also found for bigeye tuna in Hawaiian waters (Drevnick and Brooks 2017). Contrastingly, Atlantic bluefin tuna (Thunnus obesus) were observed to decline in mercury contamination during a time period in which mercury emissions also declined (Lee et al. 2016). Given that studies have demonstrated that mercury in tunas can increase (Drevnick et al. 2015, Drevnick and Brooks 2017) or decrease (Lee et al. 2016), and these changes correlated with increases or decreases in anthropogenic mercury in the same geographic area, it might have been expected that mercury in Hawaii-caught sandbar sharks would have been higher in recent samples than in past samples. Given the variability of mercury contamination in recent samples, it is possible that a larger sample size would reveal higher mercury contamination than demonstrated in the present study. It should also be noted that the tunas analyzed in the Kraepiel et al. (2003) study were collected outside 50 miles around Hawaii, rather than in the same location as those analyzed by Thieleke (1973). In the present study, sandbar sharks were collected from nearshore areas around the same island (Oahu) as those collected by Thieleke (1973).

Mercury contamination in embryos was lower than that previously reported for sandbar, blacktip and Atlantic sharpnose shark embryos, and comparable to the lower end of the contamination range reported for bonnethead shark embryos (Hueter et al. 1995, Adams and McMichael 1999). No pattern in THg concentration with sandbar shark embryo total length was observed but a wide range of embryo sizes was not available for analysis and variation in mercury contamination among embryos within the same litter is not necessarily expected because mercury in embryos is maternally-derived (Lyons and Lowe 2013b) and embryos within the same litter are similar in size.

Embryos of species that exhibit placental viviparity, such as sandbar sharks, may continue to accumulate mercury throughout gestation while they are receiving maternal nourishment (Pethybridge et al. 2010). Early in gestation embryos are nourished by yolk, later by uterine secretions including histotroph and finally through a highly vascularized placental connection where the remains of the yolk sac attach to the uterine lining (Hamlett 1999).
Ingestion of yolk by embryonic oophagous common thresher sharks \( (Alopias vulpinus) \) has been demonstrated as a mechanism of maternal transfer of mercury (Lyons and Lowe 2013b). It is theoretically possible to see a shift in maternal offloading of mercury in placental viviparous sharks between stages when embryos are nourished with yolk and when they are nourished with histotroph, but that phenomenon was not observed here. Likewise, higher maternally-derived mercury was not observed in the yolk-sac viviparous shark species \( (Triakis semifasciata) \), which exhibits limited histotrophy, than in a yolk-sac viviparous batoid \( (Platyrhinoidis triseriata, \) van Hees and Ebert 2017), which exhibits no histotrophy.

The percent of maternally-derived mercury for embryos in this study was less than that documented from sandbar shark embryos off the coast of Florida (12.1-92% Hueter et al. 1995) and generally less than percentages reported for other placentally viviparous sharks (Adams and McMichael 1999, Endo et al. 2016), but is comparable to the low end of the contamination ranges reported for Atlantic sharpnose sharks and bonnethead sharks (Adams and McMichael 1999). Embryo mercury levels in this study were also comparable to (Childs et al. 1973, Greig et al. 1977, Paiva et al. 2012, Lyons and Lowe 2013b, Le Bourg et al. 2014, van Hees and Ebert 2017, Dutton and Venuti 2019), or lower than (Hueter et al. 1995, Pethybridge et al. 2010, Paiva et al. 2012, Le Bourg et al. 2014) embryos of yolk sac viviparous species.

If maternally derived mercury is a cause of embryo death, we would have expected to see higher contamination levels compared to other studies, but the level of mercury contamination that would cause stillbirth is unknown. The stillborn embryos analyzed in this study were from four separate litters for which maternal muscle samples were not collected. It would be useful to know how mercury contamination in stillborn embryos relates to that of their mother, however, since mercury in stillborn embryos in general was not higher than mercury in viable embryos, this study does not support mercury contamination as a cause of embryo death or of the apparent increase in stillborn rate observed in sandbar shark litters between 1967-1969 and 2003-2005.

Mercury may indeed be harmful to developing shark embryos, but its precise effect on embryos remains unknown (van Hees and Ebert 2017). Sublethal effects of mercury on reproductive success are widespread in terrestrial, aquatic and marine birds (Whitney and Cristol 2017). High contamination (3.86 ppm in black ducks, \( Anas rubripes \)) can cause egg death, but some birds also have the apparent ability to adapt to high contamination and have higher egg survival in subsequent years after initial contamination (Finley and Stendell 1978).
While this study did not provide evidence that mercury in sandbar shark tissues has increased between the 1970s and the mid-2000s, it is possible that a larger sample size and even more recent sampling could reveal a different pattern, similar to that observed in yellowfin tuna (Drevnick and Brooks 2017). Alternatively, mercury in sandbar sharks may be more variable than in yellowfin tuna due to feeding preferences, inclusion of deeper-living prey in the diet, movement patterns, or detoxification processes including offloading mercury to offspring. Understanding the causes of stillbirth in sharks is difficult because stillborn embryos are documented infrequently and could be more widespread than reported here. Neither the levels of embryo contamination nor the percentage of maternal mercury support the hypothesis that mercury played a role in the apparent increase in stillbirth rate over three decades.
CHAPTER 3

MERCURY CONTAMINATION IN DEEP SEA SHARKS IN THE GULF OF MEXICO

3.1 Introduction

Deep sea chondrichthians are relatively poorly known compared to their coastal and epipelagic counterparts, despite representing 48.4% of global chondrichthians (Musick and Cotton 2015). Deep sea sharks and other chondrichthians exhibit low productivity and have very low potential to recover from exploitation, and this pattern becomes stronger with increasing depth (Simpfendorfer and Kyne 2009). Among deep sea chondrichthians, the fauna of the Gulf of Mexico received relatively less attention than those in other regions until recent years (Benz et al. 2007, Churchill et al. 2015b, 2015a, Giresi et al. 2015, Pfleger et al. 2018, Daly-Engel et al. 2019, Larsen et al. 2020, Prohaska et al. 2021). These studies represent strides in species records, accurate taxonomic identification of those species, population genetics, physiology, and trophic ecology, but there is much yet to learn on all these topics as well as life history, movement ecology and toxicology.

As long-lived mid- to upper trophic level predators (Cortes 1999, Musick 1999), chondrichthians are at risk of bioaccumulating mercury to high levels of contamination and of serving as reservoirs of that mercury for long periods of time. Different tissue types can vary in their mercury accumulation (e.g., Lyons et al., 2017; Pethybridge et al., 2010; van Hees and Ebert, 2017), but muscle tissue is where the majority of the body burden of mercury accumulates in sharks (Pethybridge et al. 2010, Kazama et al. 2020).

Muscle mercury bioaccumulation in sharks can be influenced by trophic ecology, including dominant prey types, trophic level, and food web affinity. For example, mercury contamination in deep sea sharks was shown to be higher in species that consumed more benthic prey than species that consumed more pelagic prey (Newman et al. 2007). Differences in mercury concentrations among deep sea sharks have been observed based on depth habitat and geographic region. Higher mean mercury concentrations and higher portions of MeHg were observed in demersal sharks inhabiting shelf waters than deeper mid-slope waters off Australia (Pethybridge et al. 2010). Likewise, bathyal and epipelagic sharks had higher mercury contamination than coastal sharks from the southwest Indian Ocean even though bathyal sharks...
were more similar to their coastal counterparts in terms of both size and trophic level (Le Bourg et al. 2019). Deep sea shark muscle mercury was found to be higher in sharks from the eastern Mediterranean than in the same species caught off the west coast of Italy (Hornung et al. 1993).

Anthropogenic mercury enters the marine environment through a variety of pathways but does not become available for bioaccumulation in food webs until it is methylated by sulfate-reducing bacteria, often in coastal seagrass beds (Gilmour et al. 2003). Specifically, mercury enters the Gulf of Mexico primarily from the Atlantic Ocean via the Loop Current, deposition from the Mississippi and Atchafalaya Rivers, and from the atmosphere (Harris et al. 2012). Natural oil seeps or cold seeps could also increase the bioavailability of MeHg if they have increased biological activity, specifically where bacteria are converting inorganic mercury to methylmercury (Brown et al. 2013). Oil spills, such as the 2010 Deepwater Horizon Oil Spill, can indirectly affect methylmercury (MeHg) availability by providing a pulse of food for the bacteria that methylate mercury, leading to sudden changes in their abundance and community structure (Joye et al. 2014, Kleindienst et al. 2015). Gulf of Mexico waters are predicted to have high MeHg bioaccumulation factors because they are low in dissolved organic carbon and high in chloride compared to freshwater systems (Harris et al. 2012).

The objectives of this study were to examine mercury contamination in demersal deep sea sharks in the Gulf of Mexico within and between species, and to assess whether the 2010 Deepwater Horizon Oil Spill could have caused an increase in mercury contamination in demersal deep sea sharks. Since no pre-oil spill samples from the northern Gulf of Mexico were available, pre-spill reference samples from another region were used (Norfolk Canyon, Virginia, Atlantic Ocean) as a temporal comparison and post-spill samples from an area that has not been affected by an oil spill (Eleuthera, Bahamas) were used as a regional comparison (Fig. 3.1).

3.2 Methods

Six species of demersal sharks that inhabit a range of depths from the continental shelf edge to the continental slope were chosen for analysis. These species comprise three pairs of congeneres, occupy a gradient of depth habitats, and four of the six were relatively abundant in our research longline survey in the northern Gulf of Mexico. The dusky smoothhound (*Mustelus canis*) and Gulf smoothhound (*M. sinusmexicanus*) inhabit shallow coastal depths to the edge of the continental shelf down to about 300 meters. The Cuban dogfish (*Squalus cubensis*) and
Genie’s dogfish (*S. mitsukurii*) inhabit deeper water from about 250m to 450m. The little gulper (*Centrophorus uyato*) and gulper shark (*C. granulosus*) are among the deepest-living chondrichthyan species in the Gulf of Mexico, on average between 500 and 800m. Species differ significantly from one another in their mean capture depth, except for *M. canis* and *S. cubensis* (Prohaska et al. 2021).

![Map of sampling regions for deep sea sharks in the Gulf of Mexico, Norfolk Canyon and Eleuthera Sound.](image)

**Figure 3.1.** A) Map of sampling regions for deep sea sharks in the Gulf of Mexico, Norfolk Canyon and Eleuthera Sound. B) Map of sampling stations in the Gulf of Mexico only, with FSU stations indicated by circles, Dauphin Island Sea Lab stations indicated by squares, and NOAA stations indicated by triangles.
M. canis, or dusky smoothhound, is represented by two subspecies, M. canis canis and M. canis insularis, and the Gulf of Mexico sharks belong to the former subspecies (Heemstra 1997). M. canis canis inhabits both coastal and continental shelf edge habitats in various parts of its range. Studies of the biology of smoothhound sharks in the Gulf of Mexico have been hindered by the difficulty of differentiating between the three sympatric species: M. norrisi, M. sinusmexicanus and M. canis (Giresi et al. 2012, 2015, Jones et al. 2014). Based on the difficulty in identifying smoothhound species, M. canis and sinusmexicanus are presumed to have similar life history parameters, with females reaching maturity at 4.11 years and 75.09 cm FL and males reaching maturity at 3.28 years and 69.20 cm FL (Jones et al. 2014). M. canis primarily consumes large benthic crustaceans such as crabs (Gelsleichter et al. 1999, Churchill et al. 2015b).

S. cubensis, or the Cuban dogfish, is the most frequently-encountered squalid in the northern Gulf of Mexico (Jones et al. 2013). This relatively small species reaches a total length of 110 cm, although specimens caught in the northern Gulf of Mexico did not exceed 57 cm total length. They mature at 37.9 cm total length for males and 46.6 cm total length for females in the Gulf of Mexico (Jones et al. 2013), or at 44.5 cm total length for females in the Caribbean Sea (Tagliafico et al. 2019). They produce one to four embryos per litter via yolk sac viviparity (Jones et al. 2013). S. cubensis diets in the northern Gulf of Mexico are dominated by teleosts, but also include some squid (Churchill et al. 2015b).

S. clarkae, or Genie’s dogfish, is a recently-described dogfish shark that inhabits the Gulf of Mexico and western Atlantic Ocean (Pfleger et al. 2018). This species was formerly considered as a member of the Squalus mitsukurii species complex, along with many other closely related but distinct species distributed worldwide (Last et al. 2007, Daly-Engel et al. 2018, Pfleger et al. 2018). S. clarkae in the Gulf of Mexico have a diet dominated by teleosts and including squid, and stable isotope data indicates that their trophic niche broadens throughout ontogeny (Churchill et al. 2015b). An age and growth study of another member of the S. mitsukurii species complex, recently designated as S. Hawaiian’s (Daly-Engel et al. 2018) revealed that females reach maturity at age 15 and 64cm TL and males mature at age 8.5 and 47cm TL. S. cf. mitsukurii from the southwest Atlantic were mature at 51-55 cm TL for males and between 50 and 60 cm TL for females (Lucifora et al. 1999). There is no apparent seasonal pattern to mating and litters contain 3 to 10 embryos (Cotton et al. 2011).
C. uyato is a medium-bodied gulper shark with a worldwide distribution (Veríssimo et al. 2014). C. uyato from the Ionian Sea and originally reported as C. granulosus, exhibited low fecundity with one embryo per litter and a diet dominated by teleosts and, to a lesser extent, cephalopods (Megalofonou and Chatzispyrou 2006). In the Gulf of Mexico, C. uyato diets are dominated by teleost fishes and also include squid (Churchill et al. 2015b).

C. granulosus is a large species of gulper shark that with a wide geographic distribution, including the Gulf of Mexico as well as the Atlantic, Pacific and Indian Oceans. (White et al. 2013, Veríssimo et al. 2014). It can reach a total length of 166 cm (Bañón et al. 2008). Only one C. granulosus stomach was available for analysis in the Gulf of Mexico, and it contained an octopus (Churchill et al. 2015b).

Sharks were collected using bottom longlines as part of a fishery-independent survey in the northern Gulf of Mexico from 2012 through 2017 (Figure 3.1). The longlines consisted of an anchor, a chevron trap, fifty gangions with circle hooks, an eel trap, weight, temperature and depth recorder (TDR), and highflier with a radar reflector and strobe. Gangions comprised a variety of sizes of circle hooks and leader types: five 18/0 carbon fiber with stainless steel leaders, five 14/0 stainless steel with stainless steel leaders, ten 11/0 stainless steel with stainless steel leaders, ten 12/0 stainless steel with stainless steel leaders, and twenty 10/0 stainless steel with monofilament leaders. The three smaller hook sizes had short leaders while the 14/0 and 18/0 had a longer length of stainless steel leader attached to the hook and a length of monofilament attaching to the stainless steel leader on one end and to the snap on the other. Traps were baited with Atlantic menhaden (Brevoortia tyrnnanus) and hooks were baited with bonito (Euthynnus sp.) except for two 18/0 hooks, which were baited with Carcharhiniform shark. Longline survey methods are also reported by other authors (Churchill et al. 2015b, Prohaska et al. 2021).

Animals were brought on board the vessel as the longline gear was hauled in. Each animal was given a unique specimen number, photographed, identified, measured and weight. The sex of elasmobranchs was determined by looking for the presence (males) or absence (females) of claspers. Biological samples were collected including blood, a fin clip, bile, liver, muscle, parasites, full stomachs, reproductive tracts, aging structures (i.e., fin spines, vertebrae), brains, hearts, and rectal glands. Muscle samples approximately 2cm by 4cm were collected from below the first dorsal fin and frozen in individual zip-top plastic bags for mercury analysis.
Additional shark samples were obtained from Dauphin Island Sea Lab (Dauphin Island, Alabama) and the National Oceanic and Atmospheric Administration (NOAA) Southeast Fisheries Science Center. Reference samples were obtained from Virginia Institute of Marine Science (VIMS, Gloucester Point, Virginia), Mote Marine Laboratory and Aquarium (MMLA, Sarasota, Florida), and Cape Eleuthera Institute (CEI, Rock Sound, Eleuthera, Bahamas).

Shark muscle samples were prepared for mercury analysis by rinsing with deionized (DI) water to remove debris, patting with Kim wipes to remove excess water, and recording wet weight prior to lyophilization for 48 hours. The dry weight of each sample was recorded to determine the percent water after lyophilization, then dried muscle was ground to a fine powder using a ball mill grinder. Mercury contamination in shark muscle samples was measured by Cold Vapor Atomic Absorption Spectroscopy on a Tekran 2700 Mercury Analyzer at the National High Magnetic Field Laboratory (NHMFL, Tallahassee, Florida), using a protocol modified from EPA Method 1631 (Perrot et al. 2019). Mercury was extracted from muscle tissue by mixing with nitric acid (6M HNO₃) and heating in an oven at 70°C for 8 hours. Extracted samples were centrifuged at high speed for ten minutes, then the supernatant was diluted with DI water. Samples as well as extraction blanks, replicates, mercury standards and certified reference materials were derivatized using tetraethylborate (1% NaBE₄) allowing mercury speciation to be measured. Replication was performed at the levels of extraction and derivatization, and a replicate was analyzed for every fifteen samples. Mercury standards were used to create calibration curves for methylmercury (MeHg) and inorganic mercury (IHg), and total mercury was quantified by adding MeHg and IHg together. Quality control was assessed using percent recovery of Certified Reference Materials (DORM-3 Dogfish Muscle, DOLT-4 Dogfish Liver, ERM-CE-464 Tuna Muscle), MeHg and IHg spiked samples, and relative standard deviation (RSD) between replicates. Mercury contamination is reported within this chapter as methylmercury (MeHg) in ppm on a wet weight basis.

Differences in mercury contamination between sexes, species, and regions were investigated using analysis of covariance (ANCOVA) using shark length as a covariate. A significant interaction between length and the grouping variable (e.g., sex, species, or region) indicated a difference in slope between the two groups. A significant effect of group indicated a difference in intercept between the two groups. When the ANCOVA did not reveal a significant interaction, the interaction term was removed, and the effect of group (sex, species, or region)
was analyzed using one-way analysis of variance (ANOVA). Within a species, when there was not a significant interaction between shark sex and shark length and there was not a significant effect of sex, the sexes were pooled for analyses. Mercury contamination was compared between species within the Gulf of Mexico when there was not a significant interaction between shark length and species. Mercury contamination was also compared between disparate regions: 1) between the Gulf of Mexico and Eleuthera and 2) between the Gulf of Mexico and Norfolk Canyon, Virginia. These regional comparisons were made in cases where there was not a significant interaction between shark length and region. Fork length was used as the shark length covariate for most groups, but total length was used for regional comparisons of *C. granulosus* because it was the only measurement available for Virginia specimens. Data analyses were completed in R version 3.5.3 and using the *car* package.

### 3.3 Results

The portion of total mercury represented by MeHg ranged from 71 – 100%, with speciation less than 90% observed in Gulf of Mexico *C. uyato*, and *C. granulosus*, Norfolk Canyon *C. granulosus* and one Eleuthera *S. cubensis* (Table 3.1). MeHg increased linearly with shark length in most cases, but not always significantly and often with high variation (simple linear regressions, Fig. 3.2a-h). Significant relationships between MeHg and shark length were seen in Gulf of Mexico *M. sinusmexicanus* (Adj. R² = 0.17, p = 0.05; Fig. 3.2a), *S. cubensis* (Adj. R² = 0.35, p < 0.01; Fig. 3.2c), *S. clarkae* (Adj. R² = 0.22, p = 0.002; Fig. 3.2d) and *C. uyato* (Adj. R² = 0.54, p < 0.001; Fig. 3.2e), but no pattern was observed in *M. canis* (Fig. 3.2b) or *C. granulosus* (Fig. 3.2f). MeHg in Eleuthera *S. cubensis* increased significantly with FL (Adj. R² = 0.60, p < 0.001; Fig. 3.2g) and no pattern of MeHg with TL was observed in pre-spill *C. granulosus* from Norfolk Canyon (Fig. 3.2h). Not enough data points were available to analyze the relationship between length and MeHg in *M. canis* or *C. uyato* from Eleuthera.

Significant differences between sexes were observed in *S. cubensis*, *S. clarkae*, and *C. uyato* (Table 3.2). Males and females of both *Squalus* species had similar slopes but significantly different intercepts, but the intercepts for males and females of *C. uyato* could not be compared due to a significant interaction between sex and shark length. Sexes of all other species were pooled for interspecific comparisons within the Gulf of Mexico and regional comparisons.
Table 3.1. Summary of deep sea sharks analyzed for MeHg in the Gulf of Mexico, Eleuthera, and Norfolk Canyon, Virginia. Ranges, means and standard deviations are presented for shark length, methylmercury (MeHg) concentration, and %MeHg (the percent of total mercury represented as MeHg). Most shark lengths are fork lengths except CGRA from Virginia, which are total lengths. MeHg concentrations are given in ppm on a wet weight basis. MSIN = *Mustelus sinusmexicanus*, MCAN = *M. canis canis* (Gulf of Mexico) or *M. canis insularis* (Eleuthera), SCUB = *Squalus cubensis*, SCLA = *S. clarkae*, CUYA = *Centrophorus uyato*, CGRA = *C. granulosus*.

<table>
<thead>
<tr>
<th>Region</th>
<th>Species</th>
<th>n</th>
<th>Shark Length (cm)</th>
<th>MeHg (ppm, ww)</th>
<th>% MeHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Gulf of Mexico</td>
<td>MSIN</td>
<td>11/8</td>
<td>62.1 - 108</td>
<td>84.8 ± 11.9</td>
<td>0.18 - 1.44</td>
</tr>
<tr>
<td></td>
<td>MCAN</td>
<td>17/11</td>
<td>48.5 - 110</td>
<td>91.7 ± 15.5</td>
<td>0.62 - 4.63</td>
</tr>
<tr>
<td></td>
<td>SCUB</td>
<td>23/18</td>
<td>28.0 - 49.5</td>
<td>39.4 ± 6.3</td>
<td>0.37 - 4.26</td>
</tr>
<tr>
<td></td>
<td>SCLA</td>
<td>22/14</td>
<td>34.5 - 69.5</td>
<td>57.2 ± 8.4</td>
<td>0.57 - 7.77</td>
</tr>
<tr>
<td></td>
<td>CUYA</td>
<td>21/18</td>
<td>40 - 97.5</td>
<td>73.5 ± 16.7</td>
<td>0.35 - 5.17</td>
</tr>
<tr>
<td></td>
<td>CGRA</td>
<td>18/2</td>
<td>86 - 151</td>
<td>111.3 ± 21.1</td>
<td>1.47 - 4.76</td>
</tr>
<tr>
<td>Eleuthera</td>
<td>SCUB</td>
<td>18/4</td>
<td>34 - 66</td>
<td>52.5 ± 11.8</td>
<td>0.76 - 6.77</td>
</tr>
<tr>
<td>Virginia</td>
<td>CGRA</td>
<td>19/0</td>
<td>150 - 169</td>
<td>158 ± 6.3</td>
<td>0.77 - 3.38</td>
</tr>
</tbody>
</table>
Figure 3.2. Bioaccumulation of MeHg (ppm, wet weight) with shark fork length in deep sea sharks from the Gulf of Mexico (a-f), Eleuthera, Bahamas (g) and Norfolk Canyon, Virginia (h). Black symbols depict male sharks and gray symbols depict females. Sexes were pooled when there was not a significant interaction between sex and fork length (ANCOVA). A) *Mustelus sinusmexicanus* (y = 0.015x – 0.679, Adj. $R^2 = 0.169$, p = 0.05), b) *Mustelus canis* (y = 0.011x + 1.004, Adj. $R^2 = 0.003$, p = 0.35), c) *Squalus cubensis* males (y = 0.214x – 5.714, Adj. $R^2 = 0.343$, p < 0.01) and females (y = 0.132x – 3.595, Adj. $R^2 = 0.515$, p < 0.0001), d) *Squalus clarkae* males (y = 0.213x – 7.391, Adj. $R^2 = 0.345$, p = 0.02) and females (y = 0.107x – 3.136, Adj. $R^2 = 0.535$, p < 0.0001), e) *Centrophorus uyato* males (y = 0.090x – 3.690, Adj. $R^2 = 0.855$, p < 0.0001) and females (y = 0.043 – 0.888, Adj. $R^2 = 0.496$, p < 0.001), f) *Centrophorus granulosus* (y = 0.005x + 1.872, Adj. $R^2 = 0.043$, p = 0.65), g) Eleuthera *Squalus cubensis* (y = 0.122x – 2.999, Adj. $R^2 = 0.605$, p < 0.001), Virginia *Centrophorus granulosus* (y = 0.005x + 2.680, Adj. $R^2 = 0.06$, p = 0.857).
Figure 3.2 - continued
Figure 3.2 - continued
Figure 3.2 – continued
**M. sinusmexicanus** were able to be compared to *M. canis* (both *Mustelus* species inhabiting shallow coastal areas to 300 m depths), *C. uyato* females and *C. granulosus* (both *Centrophorus* species inhabiting 500 to 800 m depths). *M. sinusmexicanus* had a significantly lower intercept than each of these three species (Table 3.3) and had the lowest contamination of any species in this study. *M. canis* had both a similar slope and intercept to *C. granulosus* (Table 3.3). *C. uyato* females and *C. granulosus* had significant interactions with all other species and therefore their intercepts were not compared to any other species.

*S. cubensis* males and females were able to be compared to *S. clarkae* males and females (both species inhabiting 250 to 450 m depths). *S. cubensis* males and females had significantly different intercepts from *S. clarkae* females, but not from *S. clarkae* males (Table 3.3). *S. cubensis* and *S. clarkae* females were also able to be compared to *C. uyato* males, and differences in intercepts were significant (Table 3.3).

Regional comparisons were made between Gulf of Mexico *Squalus* species and Eleuthera *S. cubensis*. Both *Squalus* species from the Gulf of Mexico were included in this comparison because Eleuthera *S. cubensis* were more similar in size to Gulf of Mexico *S. clarkae* than they were to Gulf of Mexico *S. cubensis*. All *Squalus* groups had similar slopes, but Eleuthera *S. cubensis* had a significantly different intercept from Gulf of Mexico *S. clarkae* females (Table 3.4). A regional and temporal comparison between Gulf of Mexico and Virginia *C. granulosus* revealed that this species had similar slopes between the two regions and a difference in intercept that was not significant (*p = 0.07*; Table 3.4).

3.4 Discussion

Deep sea sharks within the Gulf of Mexico differed in their MeHg contamination, with *M. sinusmexicanus* having the lowest contamination and *S. clarkae* having the highest contamination. The low contamination observed in *M. sinusmexicanus* is similar to levels in pregnant female *M. higmani* from the Amazon coast of Brazil (less than 0.4 ppm; de Souza-Araujo et al., 2020) and in *M. henlei* off Mexico (less than 0.7 ppm; Medina-Morales et al., 2020), while *M. canis* contamination off Brazil was lower than both Gulf of Mexico *M. sinusmexicanus* and *M. canis* (de Pinho et al. 2002). *M. mustelus* from South Africa had contamination levels in between those measured for *M. sinusmexicanus* and *M. canis*, around 1 ppm (Bosch et al. 2016). Low MeHg contamination in *M. sinusmexicanus* could be a result of
low MeHg bioavailability in the region, strongly influenced by the Mississippi River plume and marine snow from the Deepwater Horizon oil spill, as proposed by Perrot et al. (2019).

High MeHg in *S. clarkae* could be driven by their predominantly piscivorous diet, but squid are also very common in the diet (Churchill et al. 2015b). Two other species, *S. cubensis* and *C. uyato* (reported as *C. cf. granulosus*) have been found to have similar diets to *S. clarkae* (Churchill et al. 2015b), yet they had lower MeHg contamination in this study. A closely-related member of the *S. mitsukurii* species complex had among the highest Hg concentrations of 16 deep sea chondrichthyans off Australia (3.74 ± 0.2 ppm, ww; Pethybridge et al., 2010).

Table 3.2. Differences in MeHg contamination (ppm, wet weight) between sexes for deep demersal sharks in the Gulf of Mexico, Eleuthera, Bahamas, and Norfolk Canyon, Virginia. P-values for slope and intercept are given, and values significant at an α-level of 0.05 are bolded. A significant interaction between sex and shark fork length (ANCOVA) indicated a significant difference between males and females in the slope of the relationship between MeHg contamination and shark fork length (p slope column). A significant effect of sex (ANOVA) indicated a significant difference in the intercept of the relationship between MeHg contamination and shark fork length (p intercept column). Effects of sex were not explored if there was a significant interaction between sex and shark length. Species are ordered from shallowest to deepest distribution: MSIN = *Mustelus sinusmexicanus*, MCAN = *M. canis canis*, SCUB = *Squalus cubensis*, SCLA = *S. clarkae*, CUYA = *Centrophorus uyato*, CGRA = *C. granulosus*

<table>
<thead>
<tr>
<th>Region</th>
<th>Species</th>
<th>p slope</th>
<th>p intercept</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gulf of Mexico:</td>
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<td>0.17</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>MCAN</td>
<td>0.50</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>SCUB</td>
<td>0.23</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td></td>
<td>SCLA</td>
<td>0.11</td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td></td>
<td>CUYA</td>
<td><strong>0.003</strong></td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>CGRA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Eleuthera:</td>
<td>SCUB</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Virginia:</td>
<td>CGRA</td>
<td>NA</td>
<td>all females</td>
</tr>
</tbody>
</table>
Table 3.3. Differences in MeHg contamination (ppm, wet weight) between deep demersal shark species in the Gulf of Mexico. P-values for slope and intercept are given (slope/intercept), and values significant at an α-level of 0.05 are bolded. A significant interaction between species and shark fork length (ANCOVA) indicated a significant difference in the slopes of the relationships between MeHg contamination and shark fork length. A significant effect of species (ANOVA) indicated a significant difference in the intercepts of the relationships between MeHg contamination and shark fork length. Effects of species were not explored if there was a significant interaction between species and shark fork length. Species are ordered from shallowest to deepest distribution: MSIN = *Mustelus sinusmexicanus*, MCAN = *M. canis canis*, SCUB = *Squalus cubensis*, SCLA = *S. clarkae*, CUYA = *Centrophorus uyato*, CGRA = *C. granulosus*

<table>
<thead>
<tr>
<th></th>
<th>MSIN</th>
<th>MCAN</th>
<th>SCUB males</th>
<th>SCUB females</th>
<th>SCLA males</th>
<th>SCLA females</th>
<th>CUYA males</th>
<th>CUYA females</th>
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<tbody>
<tr>
<td>MCAN</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>SCUB</td>
<td>&lt;0.001/NA</td>
<td>0.01/NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCUB</td>
<td>&lt;0.0001/NA</td>
<td>&lt;0.0001/NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCLA</td>
<td>0.001/NA</td>
<td>0.001/NA</td>
<td>0.99/0.07</td>
<td>0.25/0.91</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>SCLA</td>
<td>&lt;0.0001/NA</td>
<td>&lt;0.001/NA</td>
<td>0.11/0.02</td>
<td>0.47/0.03</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>CUYA</td>
<td>&lt;0.0001/NA</td>
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<td>0.11/&lt;0.0001</td>
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<tr>
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<td>0.01/NA</td>
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<td>0.01/NA</td>
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</tr>
<tr>
<td>CGRA</td>
<td>0.55/&lt;0.0001</td>
<td>0.70/0.40</td>
<td>&lt;0.01/NA</td>
<td>&lt;0.001/NA</td>
<td>&lt;0.01/NA</td>
<td>&lt;0.001/NA</td>
<td>&lt;0.001/NA</td>
<td>0.01/NA</td>
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Table 3.4. Differences in MeHg contamination (ppm, wet weight) between deep demersal shark species in the Gulf of Mexico and regions without oil spill exposure. Eleuthera, Bahamas samples were collected after the Gulf of Mexico’s 2010 Deepwater Horizon oil spill and Norfolk Canyon, Virginia samples were collected prior to the oil spill. P-values for slope and intercept are given (slope/intercept), and values significant at an α-level of 0.05 are bolded. A significant interaction between region and shark fork length (ANCOVA) indicated a significant difference in the slopes of the relationships between MeHg contamination and shark fork length. A significant effect of region (ANOVA) indicated a significant difference in the intercepts of the relationships between MeHg contamination and shark fork length. Species are ordered from shallowest to deepest distribution: SCUB = *Squalus cubensis*, SCLA = *S. clarkae*, CGRA = *C. granulosus*

<table>
<thead>
<tr>
<th></th>
<th>Gulf of Mexico <em>Squalus</em> sharks</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>SCUB males</td>
<td>SCUB females</td>
<td>SCLA males</td>
<td>SCLA females</td>
</tr>
<tr>
<td>Eleuthera <em>Squalus</em> sharks:</td>
<td>SCUB</td>
<td>0.27/0.24</td>
<td>0.81/0.61</td>
<td>0.19/0.62</td>
</tr>
<tr>
<td></td>
<td>CGRA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virginia <em>Centrophorus</em> sharks:</td>
<td>CGRA</td>
<td>0.76/0.07</td>
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<td></td>
</tr>
</tbody>
</table>
Squalus species similar in size to S. cubensis, such as S. albicaudus off Brazil (de Pinho et al. 2002, Hauser-Davis et al. 2020) and S. megalops off Australia (Pethybridge et al. 2010), overlapped much of the range of contamination observed in the Gulf of Mexico, but did not have mercury levels over 3 ppm. Similar in size to S. clarkae, S. acanthias from the Adriatic Sea had high mercury contamination, ranging up to 10 ppm (Storelli et al. 2001), but lower values were reported from Australia (Pethybridge et al. 2010) and the western Atlantic off Massachusetts, USA (Maynard and Baumann 2020). Gulf of Mexico S. clarkae also had generally higher mercury than S. mitsukurii from both Brazil (de Pinho et al. 2002) and Australia (Pethybridge et al. 2010). Gulf of Mexico C. uyato had higher mercury than the same species from the Macronesian Archipelago in the eastern Atlantic (Lozano-Bilbao et al. 2018). C. zeehaani from southeast Australia had similar mercury contamination as Gulf of Mexico C. uyato which overlapped with their size range (82-90 cm; Pethybridge et al., 2010). Mercury contamination of C. granulosus from the Gulf of Mexico were within the same range as this species from the eastern Mediterranean Sea, but that population also had sharks with higher contamination (up to 8.37 ppm) than the Gulf of Mexico sharks (Hornung et al. 1993).

M. canis has been shown to demonstrate an isotopic ontogenetic niche shift in which $\delta^{15}$N decreases with shark length (Churchill et al. 2015b). This ontogenetic shift could result in this species consuming lower trophic level and lower mercury prey as it grows, keeping contamination levels lower than S. clarkae despite shark size differences. Churchill et al. (2015) found C. uyato (reported as C. cf. granulosus) to have the highest $\delta^{15}$N values in the northern Gulf of Mexico deep sea shark assemblage, however this higher trophic level did not correspond to higher MeHg in this study.

Two pairs of congeners differed from each other significantly in their contamination levels, with M. canis having higher contamination than M. sinusmexicanus and S. clarkae having higher contamination than S. cubensis. In both cases, the congener with higher contamination ranges into deeper habitats, and in the case of the Squalus species, the congener with higher contamination grows to a larger size. Differences in MeHg contamination within pairs of congeners could be driven by size differences. The larger species could consume larger and potentially higher trophic level prey, which might have higher mercury contamination. Since size is essentially a proxy for age, larger and older animals have a longer amount of time to integrate more mercury into their muscle tissues. However, length at age data are not currently available.
for these species in the Gulf of Mexico, so comparing mercury in smaller and larger congeners at a given age is challenging. Pethybridge et al. (2010) found that Squalid sharks in shelf and upper slope habitats off Australia had higher mercury contamination than sharks in mid-slope regions, but further investigation revealed no effect of bathome (i.e., shelf, upper slope, mid-slope) on THg (Pethybridge et al. 2012). By contrast, deeper-foraging pelagic teleost fishes off Hawaii were found to have higher THg than those that foraged at shallower depths (Choy et al. 2009).

Differences between males and females were observed for both of the Squalus species and C. uyato. For C. granulosus, not enough male sharks were caught to investigate potential sex-based differences. In particular, the set of samples from Norfolk Canyon only contained mature, reproductively active females. Sex-mediated differences in chondrichthyan mercury bioaccumulation has been documented for other species. For example, differences in mercury contamination between male and female gonads, such as that reported for Galeus melastomus, make intuitive sense if females are offloading some of their mercury burden to their ova during vitellogenesis (Gaion et al. 2016). However, female Deania calcea off Portugal had significantly higher muscle mercury than males, which may have been because the females sampled were both larger (and therefore older) and more numerous than the males (Paiva et al. 2012).

Typical MeHg bioaccumulation patterns show an increase in contamination with animal size (Hueter et al. 1995, Adams and McMichael 1999, Gelsleichter and Walker 2010, Taylor et al. 2014, Gilbert et al. 2015), and the portion of THg represented by MeHg is usually higher than 95% in fishes (Bloom 1992). Generally, increasing MeHg with shark length was observed in deep sea sharks in this study with a few exceptions. No pattern of MeHg contamination with shark length was observed in M. canis or in C. granulosus from either the Gulf of Mexico or Virginia. It is important to note that these two species were caught at the longest body lengths of any in this dataset, and that smaller individuals are absent from the dataset so any differences in bioaccumulation rate at younger ages and smaller lengths are absent. Additionally, MeHg contamination levels in deep sea sharks were variable (see Fig. 3.2), as demonstrated by the coefficients of determination for the bioaccumulation curves. This variability and the absence in the lower end of the size range contribute to the apparent lack of a pattern in MeHg bioaccumulation in these species.

The observations of THg represented by less than 90% MeHg in both Centrophorus species and one individual S. cubensis in Eleuthera could be due to their dietary habits.
Consuming lower trophic level prey such as invertebrates could result in lower biomagnification of MeHg in these sharks (Walker 1976). It is also possible that these species reduce their body burden by demethylating MeHg (Wang et al. 2017, Le Croizier et al. 2020) or by offloading MeHg to ova or embryos (e.g., Adams and McMichael, 1999; de Souza-Araujo et al., 2020; Le Bourg et al., 2014; Lyons et al., 2017, 2013; van Hees and Ebert, 2017). Many of the C. granulosus with less than 90% MeHg were mature females and the S. cubensis was a mature female, but the C. uyato with low MeHg speciation comprised both males and females.

Regional comparisons showed significantly higher MeHg contamination in Eleuthera S. cubensis than in Gulf of Mexico female S. clarkae, but contamination was not significantly different from S. clarkae males or Gulf of Mexico S. cubensis. This result is complicated by the observation that Eleuthera S. cubensis are more similar in size to Gulf of Mexico S. clarkae than S. cubensis, and it is possible that the Eleuthera population comprises a mixture of more than one Squalus species. The slightly but not significantly higher MeHg observed in Gulf of Mexico C. granulosus compared to specimens from Norfolk Canyon, Virginia could potentially reflect an oil spill effect in the Gulf of Mexico, but no other evidence from this study supports that speculation. Alternatively, lower MeHg in Norfolk Canyon C. granulosus could be the result of maternal offloading to ova or embryos, as all of those specimens were mature pregnant females. Regional differences in mercury contamination have been observed in other shark species, for example, muscle mercury contamination was higher in Galeus melastomus from the Tyrrhenian Sea than from other regions of the Mediterranean Sea and the Atlantic Ocean (Gaion et al. 2016).

Although the C. granulosus sampled off Norfolk Canyon were larger than those sampled in the Gulf of Mexico, the Gulf of Mexico samples had slightly but not significantly higher mercury contamination. This is surprising given that mercury and other contaminants tend to increase with animal size, age, and trophic level. However, the limited size range of C. granulosus available for analysis do not demonstrate what the bioaccumulation curve might look like in this species in either region. The variability in MeHg contamination of sharks at this size could reflect individual differences in diet or perhaps mercury detoxification or maternal offloading, since all sharks from Norfolk Canyon were mature pregnant females.

Oil spill effects on mercury contamination have been investigated in other taxa. Lower levels of mercury were observed in tilefish (Lophilatilus chamaeleonticeps) near the DWH wellhead than at stations farther northeast of the wellhead. This could partly be due to the
increase in suspended particles in this area from marine snow after the oil spill, and from the nearby Mississippi River mouth, (Perrot et al. 2019) although it is surprising because the Mississippi River is a known source of mercury deposition in the Gulf of Mexico (Harris et al. 2012, Perrot et al. 2019).

In addition to the influx of oil that occurred after the Deepwater Horizon event, the Gulf of Mexico has many natural oil seeps (Beukelaer et al. 2003, MacDonald et al. 2015). If presence of oil as food for microorganisms that methylate mercury increases the bioavailability of MeHg in food webs, then the Gulf of Mexico could have higher baseline levels of MeHg than other regions lacking natural oil seeps. However, mercury at cold seeps was not found to be higher than at reference sites in the Gulf of Mexico (Brown et al. 2013). Mercury contamination in Gulf of Mexico red snapper (Lutjanus campechanus) varied regionally, but was not different based on whether fishes were caught on oil platforms or not (Zapp Sluis et al. 2013). Measurement of muscle mercury contamination up to 10 ppm in pelagic sharks and teleost fishes in the Gulf of Mexico from prior to the Deepwater Horizon oil spill (Cai et al. 2007) indicates that it was possible for these high levels of bioaccumulation and biomagnification to exist even without the influx of oil into the Gulf.

Further investigation of diet and trophic ecology, building upon work by Churchill et al. (Churchill et al. 2015b) may help to provide more context for understanding patterns of mercury contamination of deep sea sharks in the northern Gulf of Mexico. In an assemblage of deep sea sharks, trophic level measured as δ15N was a stronger predictor of THg than length (Pethybridge et al. 2012), highlighting the influence of diet on mercury contamination levels. Our understanding of the effects of oil spills on mercury contamination in mid to upper-level predators is complicated by multiple factors. First, pre-spill data on mercury in chondrichthyan in the Gulf of Mexico is lacking, necessitating the use of reference sites for regional and temporal comparisons. Second, deep sea sharks are long-lived and incorporate MeHg into their muscle tissue slowly, so it is unclear how quickly after an oil spill we should expect to see elevated MeHg contamination. Third, the consequences of high mercury contamination on the health and reproduction of chondrichthyan fishes in general are not well understood. This work represents the first analysis of muscle mercury contamination of these shark species in the northern Gulf of Mexico and shows that some species have elevated mercury contamination compared to other regions of the world, while others may have quite low contamination.
CHAPTER 4

EFFECTS OF LIPID AND UREA EXTRACTION ON LIGHT CARBON AND NITROGEN STABLE ISOTOPE AND MERCURY ANALYSIS IN COASTAL AND DEEP SEA TELEOSTS AND ELASMOBRANCHS

4.1 Introduction

Stable carbon and nitrogen isotopes ($\delta^{13}C$ and $\delta^{15}N$) measured in fish tissues are useful tracers of feeding habitat and trophic level, especially when measured in white muscle tissue, which has less variable $\delta^{13}C$ and $\delta^{15}N$ than other tissues (Pinnegar and Polunin 1999). However, storage in lipids in the muscle tissue can complicate stable isotope analysis. Lipid content is highly variable among animal tissues and can affect the interpretation of stable carbon isotope ratios, so extracting lipids or mathematically correcting for them is necessary (Post et al. 2007). Lipids contain more of the light isotope ($^{12}C$) than the heavy ($^{13}C$), thus variability in lipid content of tissues can lead to underestimates of $\delta^{13}C$ and misinterpretation of food web results (e.g., Post et al. 2007).

An additional challenge with elasmobranch fishes is that they retain the nitrogenous wastes urea and trimethylamine oxide (TMAO) in their tissues to assist with osmoregulation. These isotopically light nitrogenous wastes can deplete their $\delta^{15}N$ signatures, which can result in inaccurate interpretation of trophic level (Hussey et al. 2012). Urea can lower the C:N of muscle tissue, making a sample appear to be lower in lipid than it is (Carlisle et al. 2017).

Some studies have had success with using various species-specific lipid correction models, including for deep sea fishes, as long as lipid contents are not too high (e.g., C:N > 8; Hoffman and Sutton 2010, Li et al. 2016). However, these corrections have limited utility and should be used with caution (Li et al. 2016). Lipid extraction can remove some nitrogenous wastes from elasmobranch muscle, but urea extraction may also be necessary.

The confounding effects of lipids, urea and TMAO on muscle isotopic signatures underscore the importance of understanding how these substances affect $\delta^{13}C$ and $\delta^{15}N$ in a variety of species and a variety of habitats. Additionally, studies that use stable isotopes as tools to investigate trophic ecology are often paired with measurements of mercury contamination, since mercury bioaccumulates in organisms over time and biomagnifies in food webs. It is important to know the effects of muscle sample processing protocols on stable carbon and
nitrigen isotopes to accurately describe time-integrated diet, but it could also be useful to know if these protocols affect mercury analysis, because both stable isotope analysis and mercury analysis are performed on dried, ground tissue, and grinding tissue is labor- and time-intensive.

Investigating the effects of sample processing methods on light stable isotopes of coastal and deep sea teleost and elasmobranch fishes is necessary for accurate comparisons between fishes in coastal and deep sea environments in the northeastern Gulf of Mexico. Additionally, determining the effects of sample processing on mercury contamination may allow bulk sample processing to be streamlined for use for both food web studies and mercury biogeochemistry studies. Therefore, the primary objective of this study was to determine appropriate sample processing methods for both teleost and elasmobranch fishes in the Gulf of Mexico from coastal habitats to the deep sea. The secondary objective was to determine if the same sample processing methods used for stable isotope analysis affected mercury analysis.

4.2 Methods

Fishes were collected during fishery-independent bottom longline and trap surveys in the Gulf of Mexico (Fig. 4.1). The coastal teleost (Ariopsis felis) and elasmobranch (Rhizoprionodon terraenovae) were collected from Turkey Point Shoal and Dog Island Reef near the Florida State University Coastal and Marine lab, using methods described in Peterson et al. (2017). The deep sea teleost (Urophycis cirrata) and elasmobranch (Centrophorus uyato) were collected near DeSoto Canyon using methods described in Prohaska et al. (2021). Efforts to reduce bias associated with sampling location, date, and time of day included collecting fish of a given species from the same set. This was possible for all but R. terraenovae, which were collected from two different sets on the same day, in close proximity to one another (Fig. 1). Ten of each species were collected and lengths are given in Table 4.1.

Muscle samples were rinsed with deionized water, patted dry, refrozen, and lyophilized for 24-48 hours until dry. Dried muscle was ground to a fine powder using a ball mill grinder, then samples from individual fish were divided for treatment. Teleost samples were divided into two portions for control (bulk tissue) and lipid extracted treatments. Elasmobranch samples were divided into four portions for control (bulk tissue), lipid extraction, urea extraction, and sequential lipid and urea extraction treatments.
Lipids were extracted from teleost and elasmobranch muscle using a chloroform methanol protocol based on Folch et al. (1957), which can extract simple lipids as well as more complex lipids that are bound to proteins (Sweeting et al. 2006). Muscle was homogenized with a 2:1 chloroform: methanol solution by shaking with a model T91 shaker for 20 minutes at room temperature. The homogenate was centrifuged, and the supernatant removed, then the sample was washed with a 1:1 mix of methanol and water and vortexed for two minutes. The sample was centrifuged again, and the supernatant discarded. Urea was extracted from elasmobranch muscle by rinsing the tissue with DI water, vortexing for two minutes, then centrifuging and discarding the supernatant (Kim and Koch 2012). The lipid extraction procedure and urea extraction procedure were performed sequentially for the fourth, combined lipid and urea extraction treatment. After treatment, the samples were dried again for 48 hours, then ground with a ball mill grinder. Samples destined for stable isotope analysis were wrapped in tin cups, while those for mercury analysis were placed in borosilicate glass vials in preparation for mercury extractions.

Carbon and nitrogen stable isotope ratios, C:N ratio, %C, %N, and methylmercury and total mercury concentrations were determined for all treatments. Both stable isotope and mercury analyses were conducted at the Florida State University National High Magnetic Field Laboratory in 2013. Stable isotopes were analyzed using a CHNO elemental analyzer (Thermo Scientific, Waltham, MA, USA), coupled to a continuous flow Thermo Finnigan Delta Plus mass spectrometer. Carbon and Nitrogen calibration curves were constructed using standard materials (sucrose, phenylalanine, Vienna PeeDee Belemnite, and two urea concentrations). Isotope values are reported in standard δ notation, in which they are expressed as a ratio of the heavy to the light isotope of the element (X) being analyzed, and units are in parts per thousand (‰) difference from the reference standard:

\[ \delta X = \left( \frac{R_{\text{Sample}}}{R_{\text{Standard}}} - 1 \right) \times 1000 \]

Vienna PeeDee Belemnite (VPDB) and ambient Air were the reference standards for C and N, respectively. Duplicate samples were run to assess the analytical error of the instrument.
Mean differences between samples were 0.2 ‰ (SD = 0.1) for δ¹³C and 0.1 ‰ (SD = 0.1) for δ¹⁵N.

Mercury concentration and speciation were analyzed using cold vapor atomic absorption spectroscopy (EPA Method 1630) on a Tekran 2700 Mercury Analyzer (Tekran, Ontario, Canada). Approximately 2g of muscle was mixed with 5 mL of 6M HNO₃ in acid-cleaned glass vials and heated at 70 °C for 8 to 12 hours. The vials were allowed to cool and were then stored at 6-7 °C until derivatization. Samples analyzed on the Tekran 2700 were centrifuged and 1 mL of the supernatant was recovered and diluted with 10mL of ultrapure deionized water (or 5mL for embryos) to prepare for derivatization. Blanks were not diluted. Twenty µl of diluted sample was derivatized with 40 µl of 1% sodium tetraethylborate in 30 ml of ultrapure deionized water. Solutions of known monomethyl mercury (MeHg) and inorganic mercury (IHg) concentrations made from standards were used to generate a calibration curve for each analysis. Blanks and certified reference materials (Dogfish Muscle NRCC DORM-3, Dogfish liver NRCC DOLT-4) were run every 10 samples. Replication of samples and standards was performed at the levels of extraction and volatilization. The precision of the method was typically within 5% relative standard deviation and the limit of quantification was less than 0.05 ng/L MeHg and 0.2 ng/L IHg mercury. Total mercury (THg) was calculated by adding measured MeHg and IHg together. Mercury is reported in parts per million (ppm) on a dry weight basis.

Effects of tissue treatment on individual response variables of stable isotope analysis (δ¹³C, δ¹⁵N, C:N, %C and %N) and mercury contamination (MeHg, THg) were tested using a mixed modeling framework in which “species” was a between-subjects factor, “treatment” was a within-subjects factor, and individual fish id was incorporated into the error term. Teleosts were grouped separately from elasmobranchs for analysis because there were only two teleost treatments (CON = Control, LE = lipid extracted), while there were two additional elasmobranch treatments (U = urea extracted, ULE = lipid + urea extracted). Where there was a significant interaction between species and treatment, a one-way ANOVA was used to determine if treatment effects were significant for an individual species. Where there was a significant treatment effect, pair-wise comparisons with Bonferroni corrections were used to determine significance between treatments. Some individuals were excluded from analysis of tissue treatment effect on mercury contamination (i.e., 2 coastal elasmobranchs, 3 deep sea teleosts, 5 deep sea elasmobranchs) because unreliable results were obtained from the Tekran 2700 and the
statistical analysis required that all individuals be represented in the same number of treatments (see Table 4.1). Significance was determined using an $\alpha$-level of 0.05 and all analyses were conducted in R version 4.1.0.

Table 4.1. Sample sizes, lengths, and capture depths of coastal and deep sea teleosts and elasmobranchs used for stable isotope analysis (SIA) and mercury (Hg) analysis. AFEL is the coastal teleost \textit{Ariopsis felis}, RTER is the coastal elasmobranch \textit{Rhizoprionodon terraenovae}, UCIR is the deep sea teleost \textit{Urophycis cirrata} and CUYA is the deep sea elasmobranch \textit{Centrophorus uyato}.

<table>
<thead>
<tr>
<th></th>
<th>n (SIA)</th>
<th>n (Hg)</th>
<th>FL (cm)</th>
<th>Depth (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFEL</td>
<td>10</td>
<td>10</td>
<td>25 - 36</td>
<td>2.5</td>
</tr>
<tr>
<td>RTER</td>
<td>10</td>
<td>8</td>
<td>43 - 84</td>
<td>2.5</td>
</tr>
<tr>
<td>UCIR</td>
<td>10</td>
<td>7</td>
<td>30 - 41.5</td>
<td>500</td>
</tr>
<tr>
<td>CUYA</td>
<td>10</td>
<td>5</td>
<td>70 - 90</td>
<td>700</td>
</tr>
</tbody>
</table>

4.3 Results

Stable isotope and mercury measurement results for teleosts and elasmobranchs are summarized in Tables 4.2 and 4.3 (respectively). Coastal and deep sea teleosts had different mean $\delta^{13}$C and $\delta^{15}$N signatures. Coastal teleosts had $\delta^{13}$C around 11‰ while deep sea teleosts had $\delta^{13}$C around -18‰. $\delta^{15}$N was around -16‰ in the coastal teleost and 14‰ in the deep sea teleost. Mean carbon to nitrogen ratios ranged between 2.3 and 3.4 for both coastal and deep sea teleosts, while mean %C and %N ranged from 46 to 49 and 13 to 15, respectively. Mean MeHg and THg were 1.63 to 1.77 ppm (on a dry weight basis) in the coastal teleost and 0.26 to 0.37 in the deep sea teleost. Coastal and deep sea elasmobranchs had more similar $\delta^{13}$C and $\delta^{15}$N signatures to one another. Elasmobranchs had mean $\delta^{13}$C of around -17‰. Coastal elasmobranchs had mean $\delta^{15}$N of 12 to 13‰ while deep sea elasmobranchs had $\delta^{15}$N of 14 to 15‰. Mean carbon to nitrogen ratio ranged from 2.9 to 3.4, %C ranged from 45 to 49 and mean %N from 14 to 16. Mean MeHg and THg ranged from 4.55 to 5.18 ppm in the coastal elasmobranch and 11.53 to 15.91 ppm in the deep sea elasmobranch.
Figure 4.1. Map of sampling locations in the Gulf of Mexico where coastal and deep sea teleosts and elasmobranchs were collected.
Lipid extraction (LE) caused significant enrichment of \( \delta^{13} \)C compared to bulk tissue for both teleosts and the deep sea elasmobranch, with mean enrichment of 0.4 (\( p < 0.0001 \)) and 0.17\( \% \) (\( p = 0.03 \)) in the coastal and deep sea teleost, respectively, and 0.33\( \% \) in the deep sea elasmobranch. The mean \( \delta^{13} \)C enrichment of 0.12\( \% \) in the coastal elasmobranch was not significant (\( p = 0.15 \)). The mean enrichment of \( \delta^{13} \)C caused by urea extraction (U) or sequential lipid and urea extraction (ULE) compared to bulk tissue was not significant for any species (0.03 – 0.11\( \% \), \( p = 0.15 \) to 1). For the deep sea elasmobranch, mean \( \delta^{13} \)C was significantly higher in the lipid extraction treatment than the urea extraction treatment (\( p < 0.01 \)) and the sequential lipid and urea extraction treatment (\( p < 0.01 \)), and these two treatments were not significantly different from one another (\( p = 1 \); Fig. 4.2; Tables 4.4, 4.5).

All treatments resulted in significantly enriched \( \delta^{15} \)N compared to bulk tissue for all species. Lipid extraction caused enrichment of 0.33\( \% \) in the coastal teleost (\( p < 0.001 \)), 1\( \% \) in the coastal elasmobranch (\( p < 0.0001 \)), 0.62\( \% \) in the deep sea teleost (\( p < 0.001 \)) and 1.13\( \% \) in the deep sea elasmobranch (\( p < 0.0001 \)). Lipid extracted tissue had significantly different \( \delta^{15} \)N than urea extracted tissue for both the coastal and deep sea elasmobranch (\( p < 0.01 \) and \( p = 0.03 \), respectively). For both elasmobranchs, \( \delta^{15} \)N of lipid extracted tissue was not significantly different than sequential lipid and urea extracted tissue (\( p = 1 \)), but urea extracted tissue had significantly different \( \delta^{15} \)N than the sequential combined treatment for the coastal elasmobranch (\( p = 0.01 \); Fig. 4.3; Tables 4.4, 4.5).

Carbon to nitrogen ratio was slightly but not significantly lower (<0.1) in lipid extracted teleost tissue compared to bulk tissue (\( p = 0.09 \)), but the same treatment produced a significantly higher ratio (+0.04) in both elasmobranchs (\( p < 0.0001 \)). The deep sea elasmobranch had a significantly higher C:N when urea was extracted (+0.5; \( p < 0.01 \)), than with lipid extraction or the combined lipid and urea extraction treatments, which did not differ from each other (\( p = 0.75 \); Fig. 4.4; Tables 4.4, 4.5). Percent C was significantly higher than bulk tissue by 1.29 to 4.48% in all treatments, for all species (\( p < 0.0001 \); Fig. 4.5; Tables 4.4, 4.5). Opposite patterns for teleosts and elasmobranchs occurred for %N, in which measurements of treated tissue were significantly higher than bulk tissue for teleosts (+0.5 to 1.07%; \( p < 0.001 \)) and significantly lower than bulk tissue for elasmobranchs (-0.59 to -1.33%; \( p = 0.000 \), \( p = 0.03 \)). Treatments did not differ from one another in their effect on %N for either the coastal (0.14 < \( p < 1 \)) or deep sea (0.18 < \( p < 1 \)) elasmobranchs (Fig. 4.6; Tables 4.4, 4.5).
The differences in mean MeHg and THg for coastal and deep sea teleosts were significant (p < 0.001 for both), but the mean increases of 0.07 to 0.14 ppm with lipid extraction were small and not significant (Figs. 4.7, 4.8; Table 4.4). Coastal and deep sea elasmobranchs also had significantly different mean MeHg (p = 0.05) and THg (p = 0.03; Figs. 4.7, 4.8; Table 4.4). In the case of the coastal elasmobranch, all three treatments (L, U, ULE) resulted in small increases in MeHg or THg compared to bulk tissue (0.12 to 0.70 ppm). There was a larger effect of treatment on mean MeHg and THg in the deep sea elasmobranch (2.15 to 3.94 ppm), and the effect of treatment was significant (p = 0.05), but pairwise comparisons revealed no significant differences between treatments (Table 4.5).

4.4 Discussion

Extracting lipids from fish muscle generally results in the enrichment of δ^{13}C and δ^{15}N (Sweeting et al. 2006, Hoffman and Sutton 2010, Shipley et al. 2017), but the effect on δ^{13}C can depend on the lipid content of the muscle (Hoffman and Sutton 2010). This enrichment is sometimes small and not ecologically significant (Sotiropoulos et al. 2004), but some deep sea sharks have large lipid effects (e.g., Somniosus microcephalus; Shipley et al. 2017). Significant enrichment in δ^{13}C and δ^{15}N signatures with lipid and urea extraction were expected in sharks, which retain isotopically light lipids and urea within their tissues. While lipid and urea extraction caused significant enrichment in both δ^{13}C and δ^{15}N for the deep sea elasmobranch, as expected, the treatments caused significant enrichment only in δ^{15}N for the coastal elasmobranch. The lack of enrichment of δ^{13}C in treated R. terraenovae muscle compared to bulk tissue suggests that this species contains lower lipid content than the deep sea elasmobranch or either teleost. In fact, the Atlantic sharpnose shark has been described as a low lipid species (Lytle and Lytle 1994) and enrichment in δ^{13}C was observed to be small and not significant for this species in another study (Hussey et al. 2012). The enrichment of δ^{13}C in lipid extracted muscle of the deep sea elasmobranch and both teleosts demonstrates that the lipid extraction treatment successfully removed lipids from the tissues. The large effect of lipid extraction on δ^{13}C in the coastal teleost could indicate that this species carries a high lipid quantity in its muscle. Qualitatively, the coastal teleost had the highest C:N in bulk tissue, suggesting a higher lipid content. The hardhead catfish is a mouth-brooding species, so males could store lipids to prepare for mouthbrooding, though the specimens used in this study were not sexed. The lack of significant difference in
δ¹³C between urea extracted and sequentially lipid and urea extracted muscle in the deep sea elasmobranch indicates that these treatments were not sufficient to remove lipids. Given that lipid extraction successfully removed lipids, it is surprising that the sequential lipid and urea extraction treatment was not significantly different from bulk tissue. It would be beneficial for subsequent studies to analyze the lipid, protein and amino acid content of the extract for this species to determine the specific compounds that were extracted.

Table 4.2. Mean values and standard deviations of stable isotope analysis response variables (δ¹³C, δ¹⁵N, C:N, %C, %N) and measured mercury (MeHg and THg, reported as ppm on a dry weight basis) in muscle of coastal and deep sea teleosts. Two muscle samples were collected from each of ten individuals per species and subjected control (CON) and lipid extraction (LE) treatments. The coastal teleost was the hardhead catfish (Ariopsis felis) and the deep sea teleost was the Gulf hake (Urophycis cirrata). Some individuals were excluded from statistical analyses (n < 10) due to failure of mercury analysis for one or both treatments for that individual.

<table>
<thead>
<tr>
<th>Species</th>
<th>Parameter</th>
<th>n</th>
<th>CON mean (sd)</th>
<th>LE mean (sd)</th>
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<td>coastal teleost</td>
<td>δ¹³C</td>
<td>10</td>
<td>11.4 (0.6)</td>
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<td></td>
<td>δ¹⁵N</td>
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<td>-16.2 (0.7)</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>10</td>
<td>3.4 (0.2)</td>
<td>3.3 (0.04)</td>
</tr>
<tr>
<td></td>
<td>%C</td>
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<td>46.3 (2.02)</td>
<td>48.6 (1.8)</td>
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<td>deep sea teleost</td>
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<tr>
<td></td>
<td>δ¹⁵N</td>
<td>10</td>
<td>14.2 (0.8)</td>
<td>14.8 (0.7)</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>10</td>
<td>3.3 (0.04)</td>
<td>3.2 (0.02)</td>
</tr>
<tr>
<td></td>
<td>%C</td>
<td>10</td>
<td>47.6 (1.2)</td>
<td>48.9 (0.9)</td>
</tr>
<tr>
<td></td>
<td>%N</td>
<td>10</td>
<td>14.6 (0.3)</td>
<td>15.1 (0.3)</td>
</tr>
<tr>
<td></td>
<td>MeHg</td>
<td>7</td>
<td>0.26 (0.08)</td>
<td>0.33 (0.22)</td>
</tr>
<tr>
<td></td>
<td>THg</td>
<td>7</td>
<td>0.29 (0.06)</td>
<td>0.37 (0.21)</td>
</tr>
</tbody>
</table>
Table 4.3. Mean values and standard deviations of stable isotope analysis response variables ($\delta^{13}$C, $\delta^{15}$N, C:N, %C, %N) and measured mercury (MeHg and THg, reported as ppm on a dry weight basis) in muscle of coastal and deep sea elasmobranchs. Four muscle samples were collected from each of ten individuals per species and subjected control (CON), lipid extraction (LE), urea extraction (U), and sequential lipid and urea extraction (ULE) treatments. The coastal elasmobranch was the Atlantic sharpnose shark (*Rhizoprionodon terraenovae*) and the deep sea elasmobranch was the little gulper shark (*Centrophorus uyato*). Some individuals were excluded from statistical analyses (n < 10) due to failure of mercury analysis for one or more treatments for that individual.

<table>
<thead>
<tr>
<th>Species</th>
<th>Parameter</th>
<th>n</th>
<th>CON mean (sd)</th>
<th>LE mean (sd)</th>
<th>U mean (sd)</th>
<th>ULE mean (sd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>coastal elasmobranch</td>
<td>$\delta^{13}$C</td>
<td>10</td>
<td>-17.04 (0.3)</td>
<td>-16.9 (0.2)</td>
<td>-16.99 (0.2)</td>
<td>-17.03 (0.2)</td>
</tr>
<tr>
<td></td>
<td>$\delta^{15}$N</td>
<td>10</td>
<td>12.6 (0.6)</td>
<td>13.6 (0.7)</td>
<td>13.3 (0.8)</td>
<td>13.6 (0.7)</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>10</td>
<td>2.9 (0.1)</td>
<td>3.3 (0.03)</td>
<td>3.3 (0.05)</td>
<td>3.3 (0.03)</td>
</tr>
<tr>
<td></td>
<td>%C</td>
<td>10</td>
<td>45.00 (1.2)</td>
<td>49.1 (1.3)</td>
<td>49.5 (0.97)</td>
<td>48.4 (1.1)</td>
</tr>
<tr>
<td></td>
<td>%N</td>
<td>10</td>
<td>15.6 (0.3)</td>
<td>14.97 (0.4)</td>
<td>15.1 (0.2)</td>
<td>14.6 (0.4)</td>
</tr>
<tr>
<td></td>
<td>MeHg</td>
<td>8</td>
<td>4.55 (2.85)</td>
<td>5.13 (3.16)</td>
<td>4.95 (2.99)</td>
<td>5.05 (3.01)</td>
</tr>
<tr>
<td></td>
<td>THg</td>
<td>8</td>
<td>4.62 (2.86)</td>
<td>5.18 (3.16)</td>
<td>5.00 (2.99)</td>
<td>5.09 (3.00)</td>
</tr>
<tr>
<td>deep sea elasmobranch</td>
<td>$\delta^{13}$C</td>
<td>10</td>
<td>-17.2 (0.1)</td>
<td>-16.9 (0.2)</td>
<td>-17.2 (0.2)</td>
<td>-17.1 (0.2)</td>
</tr>
<tr>
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<td>$\delta^{15}$N</td>
<td>10</td>
<td>14.5 (0.5)</td>
<td>15.7 (0.3)</td>
<td>15.5 (0.3)</td>
<td>15.7 (0.4)</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>10</td>
<td>2.9 (0.1)</td>
<td>3.3 (0.03)</td>
<td>3.4 (0.05)</td>
<td>3.3 (0.04)</td>
</tr>
<tr>
<td></td>
<td>%C</td>
<td>10</td>
<td>46.04 (1.6)</td>
<td>49.0 (0.7)</td>
<td>49.6 (0.9)</td>
<td>48.9 (0.8)</td>
</tr>
<tr>
<td></td>
<td>%N</td>
<td>10</td>
<td>15.9 (0.5)</td>
<td>14.9 (0.2)</td>
<td>14.6 (0.3)</td>
<td>14.8 (0.4)</td>
</tr>
<tr>
<td></td>
<td>MeHg</td>
<td>5</td>
<td>11.53 (8.50)</td>
<td>14.54 (9.27)</td>
<td>14.26 (11.01)</td>
<td>12.11 (7.25)</td>
</tr>
<tr>
<td></td>
<td>THg</td>
<td>5</td>
<td>12.20 (9.04)</td>
<td>15.85 (10.30)</td>
<td>15.91 (12.41)</td>
<td>13.08 (7.65)</td>
</tr>
</tbody>
</table>
Table 4.4. Results of mixed modeling analysis considering species as a within-subjects effect and treatment as a between-subjects effect and considering the effect of individual (Step 1). When there was a significant interaction between species and treatment, the model was run on individual species to determine the effect of treatment within that species (Step 2). An α-level of 0.05 was used to determine significance and significant results are bolded.

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Interaction</th>
<th>Coastal Treatment</th>
<th>Deep Sea Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>p</td>
<td>F</td>
<td>p</td>
</tr>
<tr>
<td>Teleosts</td>
<td>δ¹³C</td>
<td>20140</td>
<td>&lt; 0.0001</td>
<td>48.06</td>
</tr>
<tr>
<td></td>
<td>δ¹⁵N</td>
<td>8789</td>
<td>&lt; 0.0001</td>
<td>20.84</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>8.64</td>
<td>&lt; 0.01</td>
<td>3.21</td>
</tr>
<tr>
<td></td>
<td>%C</td>
<td>2.519</td>
<td>0.13</td>
<td>13.486</td>
</tr>
<tr>
<td></td>
<td>%N</td>
<td>22.23</td>
<td>&lt; 0.001</td>
<td>22.9</td>
</tr>
<tr>
<td>MeHg</td>
<td></td>
<td>42.89</td>
<td>&lt; 0.001</td>
<td>2.951</td>
</tr>
<tr>
<td>Elasmobranchs</td>
<td></td>
<td>40.43</td>
<td>&lt; 0.001</td>
<td>2.91</td>
</tr>
<tr>
<td></td>
<td>δ¹³C</td>
<td>1.46</td>
<td>0.24</td>
<td>12.08</td>
</tr>
<tr>
<td></td>
<td>δ¹⁵N</td>
<td>73.4</td>
<td>&lt; 0.0001</td>
<td>252.81</td>
</tr>
<tr>
<td></td>
<td>C:N</td>
<td>6.09</td>
<td>0.02</td>
<td>282.09</td>
</tr>
<tr>
<td></td>
<td>%C</td>
<td>3.36</td>
<td>0.08</td>
<td>48.91</td>
</tr>
<tr>
<td></td>
<td>%N</td>
<td>0.01</td>
<td>0.92</td>
<td>44.01</td>
</tr>
<tr>
<td>MeHg</td>
<td></td>
<td>5.038</td>
<td>0.05</td>
<td>3.97</td>
</tr>
<tr>
<td>THg</td>
<td></td>
<td>5.86</td>
<td>0.03</td>
<td>4.16</td>
</tr>
</tbody>
</table>
Table 4.5. Results of pairwise comparisons for coastal and deep sea elasmobranchs to determine differences between bulk tissue or control (CON), lipid extracted muscle (LE), urea extracted muscle (U) and sequential lipid and urea extracted muscle (ULE). When there was a significant effect of treatment, pairwise comparisons were conducted to determine differences between treatments. The coastal elasmobranch was *Rhizoprionodon terraenovae* and the deep sea elasmobranch was *Centrophorus uyato*. An $\alpha$-level of 0.05 was used to determine significance and significant results are bolded. “NA” indicates that pairwise comparisons were not necessary because there was not a significant effect of treatment. %C was analyzed for both the coastal and deep sea elasmobranch together because there was not a significant interaction between species and treatment.

<table>
<thead>
<tr>
<th></th>
<th>coastal elasmobranch</th>
<th>deep sea elasmobranch</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\delta^{13}$C</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>$\delta^{15}$N</td>
<td>$&lt; 0.0001$</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>C:N</td>
<td>$&lt; 0.0001$</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>%C</td>
<td>$&lt; 0.0001$</td>
<td>$&lt; 0.0001$</td>
</tr>
<tr>
<td>%N</td>
<td>$&lt; 0.001$</td>
<td>$&lt; 0.001$</td>
</tr>
<tr>
<td>THg</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>
Figure 4.2. Mean difference in $\delta^{13}C$ between bulk tissue (control) and treatment for a coastal teleost (hardhead catfish), coastal elasmobranch (Atlantic sharpnose shark), deep sea teleost (Gulf hake), and deep sea elasmobranch (little gulper shark). Teleost muscle was unaltered (control) or subjected to lipid extraction (LE). Elasmobranch muscle was unaltered (control) or subjected to lipid extraction (LE), urea extraction (U), or sequential lipid and urea extraction (ULE). Bars above the x-axis indicate a mean enrichment in $\delta^{13}C$ for treated tissue compared to the control. Error bars represent plus and minus one standard deviation of the mean. Treatments that were significantly different from control are indicated with an asterisk. Letters indicate significance groups within a species, based on a Bonferroni adjusted p-value and an $\alpha$-level of 0.05.
Figure 4.3. Mean difference in $\delta^{15}N$ between bulk tissue (control) and treatment for a coastal teleost (hardhead catfish), coastal elasmobranch (Atlantic sharpnose shark), deep sea teleost (Gulf hake), and deep sea elasmobranch (little gulper shark). Teleost muscle was unaltered (control) or subjected to lipid extraction (LE). Elasmobranch muscle was unaltered (control) or subjected to lipid extraction (LE), urea extraction (U), or sequential lipid and urea extraction (ULE). Bars above the x-axis indicate a mean enrichment in $\delta^{15}N$ for treated tissue compared to the control. Error bars represent plus and minus one standard deviation of the mean. Treatments that were significantly different from control are indicated with an asterisk. Letters indicate significance groups within a species, based on a Bonferroni adjusted p-value and an $\alpha$-level of 0.05.
Figure 4.4. Mean difference in C:N between bulk tissue (control) and treatment for a coastal teleost (hardhead catfish), coastal elasmobranch (Atlantic sharpnose shark), deep sea teleost (Gulf hake), and deep sea elasmobranch (little gulper shark). Teleost muscle was unaltered (control) or subjected to lipid extraction (LE). Elasmobranch muscle was unaltered (control) or subjected to lipid extraction (LE), urea extraction (U), or sequential lipid and urea extraction (ULE). Bars above the x-axis indicate a mean increase in C:N for treated tissue compared to the control and bars below the x-axis indicate a mean decrease. Error bars represent plus and minus one standard deviation of the mean. Treatments that were significantly different from control are indicated with an asterisk. Letters indicate significance groups within a species, based on a Bonferroni adjusted p-value and an α-level of 0.05.
Figure 4.5. Mean difference in %C between bulk tissue (control) and treatment for a coastal teleost (hardhead catfish), coastal elasmobranch (Atlantic sharpnose shark), deep sea teleost (Gulf hake), and deep sea elasmobranch (little gulper shark). Teleost muscle was unaltered (control) or subjected to lipid extraction (LE). Elasmobranch muscle was unaltered (control) or subjected to lipid extraction (LE), urea extraction (U), or sequential lipid and urea extraction (ULE). Bars above the x-axis indicate a mean increase in %C for treated tissue compared to the control. Error bars represent plus and minus one standard deviation of the mean. Treatments that were significantly different from control are indicated with an asterisk. Letters indicate significance groups within a species, based on a Bonferroni adjusted p-value and an α-level of 0.05.
Figure 4.6. Mean difference in %N between bulk tissue (control) and treatment for a coastal teleost (hardhead catfish), coastal elasmobranch (Atlantic sharpnose shark), deep sea teleost (Gulf hake), and deep sea elasmobranch (little gulper shark). Teleost muscle was unaltered (control) or subjected to lipid extraction (LE). Elasmobranch muscle was unaltered (control) or subjected to lipid extraction (LE), urea extraction (U), or sequential lipid and urea extraction (ULE). Bars above the x-axis indicate a mean increase in %N for treated tissue compared to the control and bars below the x-axis indicate a mean decrease. Error bars represent plus and minus one standard deviation of the mean. Treatments that were significantly different from control are indicated with an asterisk. Letters indicate significance groups within a species, based on a Bonferroni adjusted p-value and an α-level of 0.05.
Figure 4.7. Mean difference in methylmercury (MeHg) between bulk tissue (control) and treatment for a coastal teleost (hardhead catfish), coastal elasmobranch (Atlantic sharpnose shark), deep sea teleost (Gulf hake), and deep sea elasmobranch (little gulper shark). Teleost muscle was unaltered (control) or subjected to lipid extraction (LE). Elasmobranch muscle was unaltered (control) or subjected to lipid extraction (LE), urea extraction (U), or sequential lipid and urea extraction (ULE). Bars above the x-axis indicate a mean increase in MeHg for treated tissue compared to the control. Error bars represent plus and minus one standard deviation of the mean. Treatments that were significantly different from control are indicated with an asterisk. Letters indicate significance groups within a species, based on a Bonferroni adjusted p-value and an \( \alpha \)-level of 0.05.
Figure 4.8. Mean difference in total mercury (THg) between bulk tissue (control) and treatment for a coastal teleost (hardhead catfish), coastal elasmobranch (Atlantic sharpnose shark), deep sea teleost (Gulf hake), and deep sea elasmobranch (little gulper shark). Teleost muscle was unaltered (control) or subjected to lipid extraction (LE). Elasmobranch muscle was unaltered (control) or subjected to lipid extraction (LE), urea extraction (U), or sequential lipid and urea extraction (ULE). Bars above the x-axis indicate a mean increase in THg for treated tissue compared to the control. Error bars represent plus and minus one standard deviation of the mean. Treatments that were significantly different from control are indicated with an asterisk. Letters indicate significance groups within a species, based on a Bonferroni adjusted p-value and an \( \alpha \)-level of 0.05.
Unlike $\delta^{13}C$, $\delta^{15}N$ was enriched compared to bulk tissue for all four species and all treatments. This result was expected. The enrichment in $\delta^{15}N$ in elasmobranchs suggest that all treatments successfully removed urea. The lack of significant difference between lipid extraction and urea extraction treatments in elasmobranchs suggests that the lipid extraction process was sufficient to also extract urea, so the additional DI water rinsing step for urea removal is not necessary for these species. However, the enrichment in $\delta^{15}N$, though significant, was less than one permille and therefore not likely to cause artificially high trophic level estimates. For analysis of shark muscle, urea extraction in tandem with lipid extraction is commonly recommended (e.g., Kim and Koch 2012, Li et al. 2016, Carlisle et al. 2017, Shipley et al. 2017). Although Kim and Koch (2012) did not observe significant differences between their lipid extracted treatment and their combined lipid and urea extracted treatment, they recommended both lipid and urea extraction for elasmobranch tissues. Studies have shown that chemical lipid extraction is necessary for accurate evaluation of both $\delta^{13}C$ and $\delta^{15}N$, and that mathematical lipid correction is not always appropriate (Shipley et al. 2017).

All treatments led to significantly higher C:N than bulk tissue for both the coastal and deep sea elasmobranch, bringing their ratios closer to 3.4, which is considered to be pure protein (Post et al. 2007). Typically, organisms with high lipid content are expected to have higher C:N ratios than pure protein, and lipid extraction is expected to decrease the ratio. However, species that retain both lipids and nitrogenous wastes in their muscle can display a lower C:N ratio that increases following lipid extraction (Carlisle et al. 2017). This phenomenon was observed in this study, and low C:N ratios of bulk tissue were likely due to the nitrogenous wastes in the muscle. There was no effect of treatment on C:N in teleosts. Treated muscle from all four species had significantly higher %C than did bulk tissue. In teleosts, treated muscle had significantly higher %N than bulk tissue, but in elasmobranchs, treated muscle had significantly lower %N than bulk tissue. There is some concern that chemical lipid extraction could remove compounds other than lipids from the tissue, such as membrane-bound proteins (Sweeting et al. 2006, Li et al. 2016).

Although MeHg and THg were higher in treated *C. uyato* muscle than bulk tissue, this effect was not significant, and there was very little difference in MeHg and THg between treated muscle and bulk tissue for the three other species. The absence of a treatment effect for MeHg suggests that sample preparation methods used for stable isotope analysis do not affect the results of mercury analysis, and therefore it would be possible to treat bulk samples the same way for
mercury and stable isotopes analyses. Mercury is bound to muscle tissue instead of fat tissue (Mason et al. 1995), and analysis of MeHg in separated lipid and protein fractions of Atlantic cod muscle showed that most MeHg was present in the protein fraction (Amlund et al. 2007). A decrease in MeHg compared to bulk tissue might suggest that proteins were lost in the lipid extraction or urea treatments, but this did not occur. Both the high levels of MeHg in sharks and the amount of variation between individuals (coefficient of variation 0.6 to 0.77) illustrate the importance of continuing to measure MeHg concentrations in tandem with feeding ecology studies using stomach contents and stable isotopes to better understand the relationships between trophic ecology and MeHg bioaccumulation from coastal to deep sea environments.

The results of this study indicate that accurate analysis of $\delta^{13}$C and $\delta^{15}$N in coastal and deep sea elasmobranch fishes in the Gulf of Mexico requires chemical lipid extraction, but that adding a urea extraction step does not significantly change results. Variability in lipid content in coastal and deep sea teleost fishes suggests that lipid extraction should also be carried out in those fishes as well. The absence of an effect of tissue treatment on MeHg and THg quantification for the coastal teleost and elasmobranch and the deep sea teleost suggests the potential for using leftover muscle that has been chemically treated to extract lipids and urea for mercury analysis and achieve accurate results.
CHAPTER 5

CONCLUSION

The challenge of understanding the complexity of mercury dynamics in sharks is compounded by several factors. Understanding how mercury bioaccumulates in a species requires the ability to sample a wide size range of that species. Thus far, those samples are not available for all species in question, particularly for deep sea sharks in the Gulf of Mexico. However, analysis of a wider size range of sandbar sharks in Hawaii than those previously reported did not reveal differences in rate of bioaccumulation. Most sharks analyzed for mercury contamination in this study demonstrated the typical biomagnification pattern of increasing contamination linearly with animal size and age (Hueter et al. 1995, Adams and McMichael 1999, Gelsleichter and Walker 2010, Taylor et al. 2014, Gilbert et al. 2015), and MeHg speciation of 95% or more, though there were some exceptions. The absence of a linear bioaccumulation pattern for some species was due to sampling a narrow size range of animals for that species, and possibly due to high variability among individuals. Individual variability in sharks that are otherwise similar in size and inhabit the same region may be caused by differences in trophic ecology (Walker 1976) or reproductive status (e.g., Adams and McMichael, 1999; de Souza-Araujo et al., 2020; Le Bourg et al., 2014; Lyons et al., 2017, 2013; van Hees and Ebert, 2017), or even differential ability to demethylate mercury (Wang et al. 2017, Le Croizier et al. 2020). Despite some individuals having a low portion of MeHg, most sharks conformed to the convention that most mercury is present as MeHg. Therefore, future studies could increase sample sizes and minimize effort by analyzing THg instead of both MeHg and THg. This may even be possible to do non-lethally, collecting only a small muscle biopsy.

Sharks carrying high levels of mercury contamination, such as sandbar sharks in Hawaii and deep sea sharks in the Gulf of Mexico and Virginia continue to grow, survive, and reproduce. Ehnert-Russo and Gelsleichter (2020) found that THg in the brain correlates with THg in R. terraenovae muscle but is present at levels too low to cause neurological damage. If there are sublethal effects of mercury contamination, it is unclear how they affect animal health or if they affect the health of embryos in utero.
Temporal dynamics of mercury contamination are challenging to address because long-term studies are lacking, and decadal comparisons such as the thirty year comparison of mercury in sandbar sharks in Hawaii involve essentially two data points. Given that sharks in general and deep-sea sharks in particular are long-lived and bioaccumulate mercury slowly, it is unclear over what time scale changes should be expected to appear. Mercury is accumulated in tissues from the diet rapidly, over a matter of days, but is eliminated much more slowly (Amlund et al. 2007), so chronic dietary mercury exposure could mask acute changes such as those hypothesized to occur resulting from bacterial community structure following the Deepwater Horizon oil spill unless effects are large and widespread in a population.

Temporal dynamics of mercury in deep sea sharks in the Gulf of Mexico cannot currently be directly addressed because there are no previous published values available for comparison. Regional comparisons of mercury in lieu of temporal comparisons are limited in their use, but still quite interesting. The similarity of Gulf of Mexico S. clarkae and Eleuthera S. cubensis in size and mercury contamination illustrate the role of size of the shark in predicting mercury contamination, although the influence of diet cannot be ruled out. Also interesting, and difficult to explain, is the pattern of lower mercury in the slightly smaller size range of Gulf of Mexico C. granulosus than in the slightly larger and all pregnant Virginia C. granulosus.

The lack of published studies on mercury in sharks and many other taxa in the deep regions of the Gulf of Mexico also precludes the ability to test hypotheses about the effects of natural oil seeps and the Deepwater Horizon oil spill on mercury biomagnification in the Gulf (but see Zapp Sluis et al. 2013, Perrot et al. 2019). Spatial dynamics are also difficult to address because the movement patterns of many species, particularly deep sea species, are unknown. Additionally other factors may mask potential oil spill effects, such as the Mississippi River effluent (Perrot et al. 2019).

Trophic ecology is both an explanatory and a compounding factor. Deep sea sharks in the Gulf of Mexico and sandbar sharks inhabiting shelf edge habitats in Hawaii have access to both mesopelagic and benthic food webs, as well as both lower trophic level prey such as invertebrates and higher trophic level prey such as fishes. Therefore, their mercury contamination could reflect consumption of prey with widely variable mercury contamination. Learning common prey of each species and then analyzing those prey species for mercury could be informative about differences between species and generating hypotheses about variation.
within species. Forthcoming stomach content and stable isotope analysis may help to provide context for the patterns observed in this study.

Similar to other studies, I found that chemical lipid extraction is necessary for accurate analysis of stable isotopes in coastal and deep sea teleost and elasmobranch fishes (Kim et al. 2012, Li et al. 2016, Carlisle et al. 2017, Shipley et al. 2017). However, there was no difference between lipid extracted treatments and treatments where both lipids and urea were extracted. This suggests that urea extraction is not necessary, although other researchers who obtained similar results with urea extraction concluded that urea extraction should always be performed on elasmobranch muscle tissue for accurate $\delta^{13}$C and $\delta^{15}$N analysis (Kim and Koch 2012, Carlisle et al. 2017). Another important factor to consider is comparison with other studies. There are limited data on trophic ecology of deep sea sharks in the Gulf of Mexico, therefore it is useful to be able to compare directly with the studies that do exist. For example, Churchill et al. (2015b) used stable isotope analysis to examine trophic ecology in an assemblage of deep sea sharks in the Gulf of Mexico, including five of the six species for which mercury was reported in Chapter 3, and for which further trophic ecology investigation is planned. Churchill et al. (2015b) used a chloroform and methanol solution to simultaneously extract both lipids and urea from shark muscle, rather than adding separate DI water rinsing steps for urea extraction. The results presented in Chapter 4 and the necessity of comparison with Churchill’s study both support eliminating the separate DI water urea extraction treatment.

The absence of an effect of tissue treatment on MeHg and THg quantification for the coastal teleost and elasmobranch and the deep sea teleost suggests that researchers could use leftover muscle that has been chemically treated to extract lipids and urea for mercury analysis and achieve accurate results. This could be useful in cases when there is chemically treated tissue remaining after weighing out quantities for stable isotope analysis, particularly if mercury analysis was not planned in advance. The marginally significant increase in mercury in the deep sea elasmobranch C. uyato is concerning, however, and researchers should proceed with caution and test a small number of samples compared to bulk tissue before proceeding with a large sample size.
APPENDIX A

ACUC ASSURANCE

June 4, 2021

The Graduate School
Florida State University

To Whom It May Concern:

Concerning the thesis/dissertation submitted to the Graduate School by:

Graduate Student:            Johanna Imhoff
Thesis/Dissertation Title:  Mercury contamination and its relation to trophic ecology and anthropogenic pollution in coastal and deep sea shark communities
Department:                 Biological Science
Major Professor:            R. Dean Grubbs

The graduate student named above has provided assurance to the FSU Animal Care and Use Committee that all animal procedures utilized in work resulting in this thesis/dissertation are described in FSU ACUC Protocol(s):

<table>
<thead>
<tr>
<th>Protocol Number</th>
<th>Title</th>
<th>Date ACUC Approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>1111</td>
<td>Life histories and trophic structure of coastal and deepwater fishes in the Gulf of Mexico and assessment of their exposure to petroleum-based pollutants</td>
<td>03/30/2011</td>
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<td>1412</td>
<td>Community structure, life histories, trophic structure and stress physiology of deepwater fishes in the Gulf of Mexico and assessment of their exposure to petroleum-based pollutants</td>
<td>02/24/2014</td>
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<tr>
<td>1708</td>
<td>Community structure, life histories, trophic structure and stress physiology of deepwater fishes in the Gulf of Mexico and assessment of their exposure to petroleum-based pollutants</td>
<td>02/27/2017</td>
</tr>
</tbody>
</table>
The Animal Care and Use Committee has confirmed that this student was included as a project member during the period covering their thesis/dissertation work. This institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance Number is D16-00491 (A3854-01).

Sincerely,

[Redacted]

ACUC Veterinarian
FSU Animal Care and Use Committee

cc: Johann Imhoff
    Dr. R. Dean Grubbs
LITERATURE CITED


Cai, Y., J. R. Rooker, G. A. Gill, and J. P. Turner. 2007. Bioaccumulation of mercury in pelagic...
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Mickle, A. 2016. Trophic ecology and bioaccumulation of mercury in the three hagfish (Myxinidae) species from the Gulf of Mexico. Florida State University.


BIOGRAPHICAL SKETCH

Johanna Leigh Imhoff was born and raised in New Bridgeville, PA. She graduated from the University of North Carolina Wilmington (UNCW) with a BS in Marine Biology in 2004. During her undergraduate years, she volunteered at the Bimini Biological Field Station, and that experience led her to develop her MS thesis project, “Evaluation of ultrasonic accelerometry as a technique for monitoring foraging in juvenile lemon sharks (Negaprion brevirostris). After graduating with a MS in Marine Biology from UNCW, she worked as a field technician at UNCW Center for Marine Science, conducting a seine net survey of fishes in coastal and estuarine habitats and identifying surf zone invertebrates from sediment cores. In 2008, she joined the Florida Program for Shark Research at the Florida Museum of Natural History (FLMNH) as a field technician. In this position, and later as a research biologist, she studied shark pupping and nursery area in the eastern Gulf of Mexico, movements and habitat use of batoids, bull sharks and alligators in the Indian River Lagoon System, and movements and habitat use of the endangered smalltooth sawfish in Florida Bay. She joined the lab of R. Dean Grubbs at Florida State University Coastal and Marine Lab and the FSU Department of Biological Science in 2011 to pursue her PhD. Her research focused on trophic ecology of deep sea sharks in the Gulf of Mexico and mercury contamination in sharks. In 2020, she accepted a position as faculty coordinator of general biology for nonmajors in the Department of Biological Science at FSU. She intends to pursue a career in teaching, maintains broad interests in marine fish ecology and deep sea biology, and will always be a field biologist at heart.