A COMPARISON OF MERCURY LOCALIZATION, SPECIATION, AND HISTOLOGY IN MULTIPLE FISH SPECIES FROM CADDO LAKE, A FRESH WATER WETLAND

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This work explores the metabolism of mercury in liver and spleen tissue of fish from a methylmercury contaminated wetland. Wild-caught bass, catfish, bowfin and gar were collected. Macrophage centers, which are both reactive and primary germinal centers in various fish tissues, were hypothesized to be the cause of demethylation of methylmercury in fish tissue. Macrophage centers are differentially expressed in fish tissue based on phylogenetic lineage, and are found primarily in the livers of preteleostean fish and in the spleen of teleostean fish. Histology of liver and spleen was examined in both control and wild-caught fish for pathology, size and number of macrophage centers, and for localization of mercury. Total mercury was estimated in the muscle tissue of all fish by direct mercury analysis. Selenium and mercury concentrations were examined in the livers of wild-caught fish by liquid introduction inductively coupled plasma mass spectrometry (ICP-MS). Total mercury was localized in histologic sections by laser ablation ICP-MS (LA-ICP-MS). Mercury speciation was determined for inorganic and methylmercury in liver and spleen of fish by bas chromatography-cold vapor atomic fluorescence spectroscopy (GC-CVAFS).

Macrophage center tissue distribution was found to be consistent with the literature, with a predominance of centers in preteleostean liver and in spleens of teleostean fish. Little evidence histopathology was found in the livers or spleens of fish examined, but differences in morphology of macrophage centers and liver tissue across species are noted. The sole sign of liver pathology noted was increased hepatic hemosiderosis in fish with high proportions of liver...
inorganic mercury. Inorganic mercury was found to predominate in the livers of all fish but bass. Organic mercury was found to predominate in the spleens of all fish. Mercury was found to accumulate in macrophage centers, but concentrations of mercury in this compartment were found to vary less in relation to total mercury than hepatocyte mercury. No association was found between selenium content and inorganic mercury proportions. Overall, findings from this study to not support a primary role for macrophage centers in the demethylation of methylmercury in fish tissues.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapters</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACKNOWLEDGEMENTS</strong></td>
<td>iii</td>
</tr>
<tr>
<td><strong>LIST OF TABLES</strong></td>
<td>vi</td>
</tr>
<tr>
<td><strong>LIST OF ILLUSTRATIONS</strong></td>
<td>vii</td>
</tr>
<tr>
<td><strong>Chapters</strong></td>
<td></td>
</tr>
<tr>
<td>1. <strong>GENERAL INTRODUCTION</strong></td>
<td>1</td>
</tr>
<tr>
<td>1.1 Overview of Mercury Metabolism in Animal Tissues</td>
<td>3</td>
</tr>
<tr>
<td>1.2 Melanomacrophages</td>
<td>6</td>
</tr>
<tr>
<td>1.3 Conclusion</td>
<td>12</td>
</tr>
<tr>
<td>2. <strong>HISTOLOGY AND HISTOPATHOLOGY OF FISH EXPOSED TO MERCURY IN CADDO LAKE</strong></td>
<td>18</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>18</td>
</tr>
<tr>
<td>2.2 Materials and Methods</td>
<td>20</td>
</tr>
<tr>
<td>2.2.1 Fish Collection and Tissue Processing</td>
<td>20</td>
</tr>
<tr>
<td>2.2.2 Tissue Mercury Analysis</td>
<td>21</td>
</tr>
<tr>
<td>2.2.3 Histologic Examination</td>
<td>24</td>
</tr>
<tr>
<td>2.2.4 Image Analysis</td>
<td>26</td>
</tr>
<tr>
<td>2.3 Results</td>
<td>26</td>
</tr>
<tr>
<td>2.3.1 Muscle Total Mercury Analysis</td>
<td>26</td>
</tr>
<tr>
<td>2.3.2 Liver Total Mercury by ICP-MS</td>
<td>27</td>
</tr>
<tr>
<td>2.3.3 Histologic Analysis</td>
<td>28</td>
</tr>
<tr>
<td>2.3.4 Relationship between Macrophage Center Area and Mercury...</td>
<td>51</td>
</tr>
<tr>
<td>2.4 Discussion</td>
<td>53</td>
</tr>
<tr>
<td>3. <strong>THE ROLE OF MACROPHAGE CENTERS IN MERCURY METABOLISM OF FISH:</strong></td>
<td>60</td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>60</td>
</tr>
<tr>
<td>3.2 Materials and Methods</td>
<td>62</td>
</tr>
</tbody>
</table>
3.2.1 Fish Tissue Collection
3.2.2 Preparation of Histologic Slides
3.2.3 Image Analysis
3.2.4 Laser Ablation ICP-MS Analysis

3.3 Results
3.3.1 Image Analysis
3.3.2 Mercury Localization by ICP-MS
3.3.3 Liver Total Mercury

3.4 Discussion

4. MERCURY SPECIATION AND SELENIUM TO MERCURY RATIO OF TELEOSTEAN AND PRETELEOSTEAN FISH FROM CADDO LAKE
4.1 Introduction
4.2 Materials and Methods
4.2.1 Fish Collection and Processing
4.2.2 Determination of Tissue Mercury
4.3 Results
4.3.1 Mercury Speciation in Liver and Spleen Tissues
4.3.2 Total Mercury and Selenium Analysis in Liver
4.4 Discussion

5. GENERAL DISCUSSION

REFERENCES
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
</tr>
<tr>
<td>2.1</td>
</tr>
<tr>
<td>2.2</td>
</tr>
<tr>
<td>2.3</td>
</tr>
<tr>
<td>2.4</td>
</tr>
<tr>
<td>3.1</td>
</tr>
<tr>
<td>4.1</td>
</tr>
<tr>
<td>4.2</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Illustration</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Total mercury in fish muscle from Caddo Lake Texas (Mean+SD)</td>
<td>11</td>
</tr>
<tr>
<td>2.1</td>
<td>Melanomacrophages in bass liver and spleen</td>
<td>30</td>
</tr>
<tr>
<td>2.2</td>
<td>Catfish liver histology</td>
<td>32</td>
</tr>
<tr>
<td>2.3</td>
<td>Catfish spleen histology</td>
<td>33</td>
</tr>
<tr>
<td>2.4</td>
<td>Gar liver and spleen histology</td>
<td>35</td>
</tr>
<tr>
<td>2.5</td>
<td>Bowfin histology: liver and spleen</td>
<td>37</td>
</tr>
<tr>
<td>2.6</td>
<td>Caddo catfish H&amp;E and melanin bleach of liver macrophage centers</td>
<td>40</td>
</tr>
<tr>
<td>2.7</td>
<td>Caddo gar and bowfin H&amp;E and melanin bleach of liver macrophage centers</td>
<td>41</td>
</tr>
<tr>
<td>2.8</td>
<td>PAS staining of livers of Caddo fish and positive control</td>
<td>43</td>
</tr>
<tr>
<td>2.9</td>
<td>PAS staining of bowfin and bass liver macrophage centers</td>
<td>44</td>
</tr>
<tr>
<td>2.10</td>
<td>Prussian blue of positive control and bass liver macrophages</td>
<td>47</td>
</tr>
<tr>
<td>2.11</td>
<td>Prussian blue of catfish liver macrophage centers and catfish hepatic hemosiderosis</td>
<td>48</td>
</tr>
<tr>
<td>2.12</td>
<td>Prussian blue stain of bowfin liver macrophages and hepatic hemosiderosis</td>
<td>49</td>
</tr>
<tr>
<td>2.13</td>
<td>Association between total liver mercury concentration and macrophage center area in Caddo fish (R² values for linear regression)</td>
<td>52</td>
</tr>
<tr>
<td>3.1</td>
<td>Example of mercury signal in Caddo gar MC liver</td>
<td>66</td>
</tr>
<tr>
<td>3.2</td>
<td>Average number of macrophage centers in livers and spleens of Caddo fish (n=5 for each group) (Mean + SD)</td>
<td>69</td>
</tr>
<tr>
<td>3.3</td>
<td>Percentage areas of macrophage centers in liver and spleen of Caddo fish (n=5 for all groups) (Mean + SD)</td>
<td>70</td>
</tr>
<tr>
<td>3.4</td>
<td>Ratio of macrophage mercury to adjacent hepatocyte mercury in bowfin test by LAICP-MS (Mean + SD)</td>
<td>72</td>
</tr>
<tr>
<td>3.5</td>
<td>Comparison of bass macrophage center and adjacent hepatocyte total mercury (Mean + SD)</td>
<td>74</td>
</tr>
</tbody>
</table>
3.6 Comparison of catfish macrophage center and adjacent hepatocyte total mercury (Mean + SD) .................................................................................................................................. 75
3.7 Comparison of bowfin macrophage center and adjacent hepatocyte total mercury (Mean + SD) .................................................................................................................................. 75
3.8 Comparison of gar macrophage center and adjacent hepatocyte total mercury (Mean + SD) ..................................................................................................................................... 76
3.9 Mercury signal in macrophage centers of fish from Caddo Lake (Mean + SD) ................ 77
3.10 Mercury signal in hepatocytes of fish from Caddo Lake ................................................. 77
3.11 Comparison of macrophage to hepatocyte total mercury in Caddo fish livers (Mean + SD) ........................................................................................................................................... 78
3.12 Total liver mercury estimated by liquid introduction ICP-MS (Mean + SD) ................. 79
3.13 Total mercury relationship to localization ratio ................................................................. 79
4.1 Liver total mercury (Mean + SD, n =3 for all groups) ......................................................... 92
4.2 Liver methylmercury (Mean + SD, n=3 for all groups) ......................................................... 93
4.3 Liver percent of total mercury as methylmercury (Mean + SD, n=3 for all groups) .......... 94
4.4 Spleen total mercury (Mean + SD, n=3 for all groups) ........................................................ 95
4.5 Spleen methylmercury (Mean + SD, n=3 for all groups) ....................................................... 96
4.6 Spleen percent of total mercury as methylmercury (Mean + SD, n=3 for all groups) ..... 97
4.7 Fish liver mercury (Mean + SD, n) ..................................................................................... 98
4.8 Fish liver selenium (Mean + SD, n) .................................................................................... 98
4.9 Ratio of selenium to mercury (Mean + SD, n) ................................................................. 99
4.10 Association between selenium and mercury levels ....................................................... 100
4.11 Association of gar liver selenium and total mercury ...................................................... 101
5.1 Association of muscle to liver ratio and percent of liver inorganic mercury in bass, gar, and catfish .......................................................................................................................... 117
CHAPTER A

GENERAL INTRODUCTION

Mercury is a widespread contaminant that impacts human health and ecosystems. It is found in both inorganic and organic compounds, is environmentally persistent, and biomagnifies along the food chain (NRC, 2000). Estimates of annual mercury releases suggest that up to two-thirds of all mercury deposition is the result of human activities such as the burning of fossil fuels and is especially associated with the burning of coal (ATSDR, 1999).

Mercury released from fossil fuel combustion exists in the atmosphere as metallic mercury (Hg0) and, due to its half-life of 1-2 years, can travel great distances before it becomes oxidized, associates with water droplets or particulates, and is deposited on the earth’s surface. The rate of atmospheric deposition of mercury is likely to increase in the future as the demand for, and use of, fossil fuels increases among developing nations. Deposition of atmospherically released mercuric mercury can contaminate lakes and streams where microorganisms in the water and soil convert it to methylmercury (Morel, Karaepiel, & Amyot, 1998; Ullrich, Tanton, & Abdrashitova, 2001).

EPA estimates that over 90% of mercury in edible fish tissue is in the form of methylmercury based on a Mercury Study Report to Congress (USEPA, 1997), and a total mercury routinely used as a surrogate for methylmercury in fish tissues and seafood. USEPA Water Quality Criteria are based on fish tissue concentrations since this route is expected to define the dominant human exposure pathway; other routes of exposure are estimated to be negligible (Borum, Manibusan, Schoeny, & Winchester, 2001; NRC, 2000). Methylmercury is a much more bioavailable from oral exposures than are other forms of mercury and at A...
In humans, mercury accumulates in the brain and kidney where it can cause permanent damage. As of special concern are children and pregnant women. Children born to exposed mothers can have effects ranging from small reductions in IQ at low chronic exposures, to delays in reaching developmental milestones and mental retardation at higher chronic doses (ATSDR, 1999). The RfD of 0.0001 mg/kg/day for methylmercury is based on human epidemiological studies in which developmental and neuropsychological impairment was observed for maternal daily intakes of 0.857-1.472 μg/kg/day and was derived using benchmark dose methodology (IRIS, 2002).

In fish, mercury is thought to accumulate in kidney, muscle, and liver resulting in signs of necrosis with inflammatory infiltrate (Mela et al., 2007b), apoptosis with upregulation of apoptosis-signaling pathway genes, and lesions in Disse's space of the liver (Klaper et al., 2008). Methylmercury exposure in fish has been shown to impair coordination and swimming activity (Crump & Trudeau, 2009) which can reduce species fitness by reducing successful hunting, evasion of predation, and mating behaviors (Sandheinrich & Miller, 2006). Environmentally relevant concentrations of mercury have been shown to impair reproduction in fish and are thought to act through induction of apoptosis in steroidogenic cells and/or through disruption of the hypothalamic-pituitary-gonadal axis (Crump & Trudeau, 2009; Drevnick et al., 2008; Drevnick & Sandheinrich, 2003; Drevnick, Sandheinrich, & Oris, 2006; Vieira, Gravato, Soares, Morgado, & Guilhermino, 2009).
Biomagnification of methylmercury in fish translates to greater risk to organisms that feed at higher levels of the food chain. Mercury concentrations in birds have been found to be highest in those species that eat fish and other birds. In mammals, the highest concentrations of mercury in tissues have been found in marine pinnipeds and other piscivorous species (Eisler, 1987). Among fur-bearing mammals, those that feed on fish have higher levels of mercury than herbivorous species (Eisler, 1987). The distribution of mercury species in fish tissues, and thus its bioavailability, directly affects the risk to species of higher trophic levels and can significantly impact whole ecosystem health.

Emerging evidence suggests that demethylation of methylmercury may be occurring in some animal species, most notably species of birds, mammals, and fish. At this not known how demethylation of methylmercury may affect the toxicokinetics and toxicodynamics of organic mercury. Some evidence exists for the sequestration of mercury in the livers of animals (Eagles-Smith, Ackerman, Yee, & Adelsbach, 2009; Palmisano, Cardellicchio, & Zambonin, 1995), and this may protect other organs, such as brain and gonads, from mercury mediated damage. In light of this evidence, assumptions about forms of mercury in fish tissue and current ecological and human health risk assessment practices are in question. Continued contamination of aquatic systems by mercury makes the development of realistic estimates for risk to ecosystem and human health from fish consumption imperative.

1.1 Overview of Mercury Metabolism in Animal Tissues

Orally absorbed methylmercury is first transferred to the blood where it is associated with the red blood cell. From the blood, mercury moves rapidly to the viscera and is slowly transported out of the viscera to the muscle tissue, where long-term storage occurs, probably in a way that protects other organs, such as brain and gonads, from mercury-mediated damage.
as mercurial amino acids incorporated into proteins (Leaner & Mason, 2004; McCloskey, Schultz, & Newman, 1998; Schultz & Newman, 1997). High concentrations of inorganic mercury have been found in liver and kidney of fish and are thought to be organs that are active in the metabolism of organic mercury to inorganic mercury (Kasper et al., 2009). The elimination half-life of methylmercury from tissues has been shown to be much higher than that of inorganic mercury (Kehrig et al., 2009; M. Nigro, Campana, Anzellotta, & Ferrara, 2002; Vahter et al., 1995) and the mobility of the pool of inorganic mercury from tissues is likely to be very low. Demethylation of methylmercury has been shown to occur in many if not all species, but is very slow in most species (Yasutake & Hirayama, 2001). Orally absorbed methylmercury is first transferred to the blood where it is associated with the red blood cell. From the blood, mercury moves rapidly to the viscera and is slowly transported out of the viscera to the muscle tissue, where long-term storage occurs, probably as mercurial amino acids incorporated into proteins (Leaner & Mason, 2004; McCloskey et al., 1998; Schultz & Newman, 1997). In most species, methylmercury reacts with glutathione in the liver and is transported into the bile canaliculus and out into the large intestine. Glutathione conjugated methylmercury can be absorbed in the enterohepatic circulation or conjugated by bacteria in the gut. Mercury demethylated in the gut by bacteria is very poorly reabsorbed and virtually all of this is lost to the feces. Almost all mercury found in feces is inorganic (Engström et al., 2008).

It is widely accepted that selenium has a protective effect against mercury toxicity, but the underlying mechanisms for this protective effect are not known. Different studies have found selenium exposure to increase mercury elimination but others to be without effect.

4A
Recently, high selenium diets have been found to decrease assimilation efficiency in fish orally exposed to inorganic mercury, but not organic mercury (Dang & Wang, 2011). Administration of methylmercury and selenium has been found to decrease biliary mercury elimination (Urano, Amura, & Naganuma, 1997). Increased levels of mercury have also been associated with increased selenium in mammalian and bird tissues, and selenium:mercury ratios of 1:1 have been specifically linked to increased demethylation (Eagles-Smith, et al., 2009; J. Yang, Kunito, Tanabe, & Miyazaki, 2007; Yoneda & Suzuki, 1997). Ratios of mercury and selenium with selenium in molar excess have been associated with those individuals below the threshold for demethylation, while 1:1 or even mercury excess have been associated with demethylation (Eagles-Smith, et al., 2009). However, it has been suggested that this 1:1 ratio is more appropriately associated with selenium: inorganic mercury than total mercury. Detoxification is thought to culminate in the formation of complexes of inorganic mercury bound with selenoproteins or selenium salts in crystals (Khan & Wang, 2009; J. Yang, et al., 2007). Selenoproteins include the important antioxidant protein glutathione peroxidase which is upregulated in oxidative stress (Reeves & Hoffmann, 2009). Mercury demethylating enzymes similar to those found in bacteria have not been found in vertebrates. Demethylation of mercury in animals has been thought to occur through a free radical mechanism (Gailer et al., 2000). Methymercury has been found to be demethylated by reactive oxygen species (specifically superoxide) in liver Kupffer cells, lymph nodes, and in macrophage-rich parts of spleen (Havarinasab, Björn, Nielsen, & Hultman, 2007). The respiratory burst is an important immune function of phagocytic cells that produces superoxide to destroy foreign cells. Ethyl- and methylmercury have been shown to be degraded to inorganic mercury in
human, rat, and rabbit polymorphonuclear leukocytes and ethylmercury are degraded in guinea pig macrophages and human monocytes (Suda, Totoki, Ichida, & Takahashi, 1992). Blockade of the reticuloendothelial system in the rat has been shown to inhibit biotransformation of methylmercury to inorganic mercury (Suda & Takahashi, 1990). This leads to the possibility that a large proportion of mercury demethylation in tissues may be due to the action of macrophages and other phagocytes. Additional support for the involvement of macrophages in the demethylation of methylmercury is lent by mechanistic data. Mercury is known to increase intercellular levels of calcium and this effect leads to the well-documented mercury-mediated apoptosis and necrosis (S. H. Kim & Sharma, 2004). Increased intercellular calcium can lead not only to deregulation of the electron transport system and production of oxidative and pro-apoptotic signals in the cell, but is known to mediate the respiratory burst in macrophages and other phagocytic cells (Hotchkiss, Bowling, Karl, Osborne, & Flye, 1997; Ales & Forman, 2002; S. H. Kim & Sharma, 2004). Exposure of human neutrophils to mercury has been shown to induce the respiratory burst (Jansson & Harms-Ringdahl, 1993) and fail to do so at subtoxic doses (Freitas, Aima, Porto, & Fernandes, 2010). The lack of respiratory burst in phagocytic cells at low dose may at least partially explain the observation of a dose threshold for mercury demethylation (Eagles-Smith, et al., 2009).

1.2 Melanomacrophages

Melanomacrophages have been used as biomarkers of general pathology (Hartley, Thiyagarajah, & Treinies, 1996; Wolke, 1992) but are also thought to occur in certain tissues as non-pathologic terminal centers of fixed macrophages (Agius, 1980; Agius & Roberts, A
In fish, melanomacrophages appear to follow an evolutionary pattern in distribution and degree of organization with a trend from random distribution and low levels of organization in Condrichthyes and Agantha, to highly organized structures in the liver of the primitive bony fish, and finally well-organized structures in the spleens and kidney of the Teleosts (Agius, 1980).

Very little is known about the function and normal structure of melanomacrophages in fish. Some sources claim that melanomacrophages are not phagocytic (Koppang, Fischer, Satoh, & Airillo, 2007), while other studies show uptake of colloidal carbon that seems to indicate active phagocytosis (Adedeji & Kakulu, 2011). The literature suggests that melanomacrophages produce respiratory burst, and this is supported by the finding of hemosiderin, lipofuscin, and ceroid in melanomacrophage centers (MMCs) (Wolke, 1992). In Caddo Lake, a freshwater wetland in Texas, increasing concentrations of hepatic mercury have been observed to correlate with increasing numbers of melanomacrophages and increased tissue pathology in the form of darkening of gross liver (B. Barst et al., 2011).

Caddo Lake is a freshwater reservoir that spans the Louisiana and Texas border, and it is reported to have among the highest levels of mercury in fish in the state of Texas (TCEQ, 2004). The Texas side of Caddo Lake is primarily shallow, forested wetland while the Louisiana side is more open water habitat. Mercury levels in fish are significantly higher on the wetland side of the lake than the open water side of the lake (Chumchal MM, Drenner RW, Fry B, Hambright KD, & LW, 2008). The top fish predators in this ecosystem include the spotted gar (Lepisosteus oculatus) and largemouth bass (Micropterus salmoides). Largemouth bass belong to the Teleostei, or modern fish, while spotted gar belong to a more primitive preteleostean group.
Recent mercury speciation data from livers of spotted gar and largemouth bass from Caddo Lake show a pattern of the predominance of inorganic mercury in the liver of preteleostean gar (98% of total mercury), while in teleostean bass, methylmercury was found to dominate (74% of total mercury) (M. M. Chumchalam et al., 2011). Spotted gar also show much higher total levels of mercury (30,171 ± 12,377 ng/g) in liver tissue than do largemouth bass (977 ± 154 ng/g) (M. M. Chumchal, et al., 2011). Melanomacrophage centers (MMCs) are found mostly in the livers of gar while those in bass occur mostly in spleen and kidney (CAgius, 1980).

Extreme differences in mercury form and concentration in the livers of spotted gar and largemouth bass may result from (1) prehepatic, (2) hepatic, and (3) posthepatic differences in mercury toxicokinetics. Potential prehepatic mechanisms include a high oral exposure of spotted gar to inorganic mercury over that to largemouth bass. Methylmercury exposure in fish is primarily through diet and feeding behaviors and trophic level are primary determinants of exposure. A largemouth bass and a spotted gar from Caddo Lake have been shown to occupy similar trophic levels through the use of stable nitrogen isotopic ratios (Chumchal MM & Hambright KD, 2009). One primary determinant of mercury concentration in fish is trophic level, with higher trophic levels correlating with higher $\delta^{15}$N (Cabana & Rasmussen, 1994), which itself correlates with mercury content of fish tissues (Boudou & Ribeyre, 1997; K. Kidd, Messlein, Fudge, & Hallard, 1995).

Species feeding at the same trophic level may eat different prey items. Some studies have also shown that the nature of the base of the food chain from which organisms are feeding affects mercury concentration. This can be estimated through measuring of stable isotopes of carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N). High $\delta^{13}$C correlates with feeding from a food chain that is higher in trophic level.
chain-based in phytoplankton, while high $\delta^{13}C$ indicates a carbon source in periphyton (France, 1995; Hecky & Hesslein, 1995). Phytoplankton-based food webs have associated with a higher mercury concentration when compared with the same trophic level species that feed on an A periphyton-based communities (Gorski, Cleckner, Hurley, Sierszen, & Armstrong, 2003; K.A. Kidd, Bootsma, Hesslein, Aylecockhart, & Hecky, 2003; Power, Klein, Guiguer, & Kwan, 2002). A Work at Caddo Lake has failed to show stable carbon isotopic ratios as significant determinants of mercury concentrations in fish tissues (Chumchal MM & Hambright KD, 2009).

The current study is primarily concerned with mercury speciation. Spotted gar and largemouth bass both occupy the top of the food chain in Caddo Lake, as evidenced by a stable nitrogen ratio. Trophic transfer rates of inorganic and organic mercury for whole experimental food chains from primary producers to terminal consumers have been shown to be quite different, with 1.3% for inorganic mercury and 87% for MeHg. Even lower trophic level transfers have been shown to favor transfer of methyl mercury, with 6% of inorganic mercury transferred between contaminated algae and Daphnia magna and 58% of MeHg being transferred under the same conditions. This suggests that the form of mercury to which both bass and gar can be expected to be exposed from dietary sources will be predominantly methylmercury (Boudou & Ribeyre, 1997). Similar trophic level predicts that the fraction of organic and inorganic mercury from dietary sources should be the same for both gar and bass. This makes the hepatic explanation for increased gar inorganic mercury levels unlikely.

Absorption of inorganic mercury from the gut following excretion of methylmercury-glutathione conjugates from the liver as a possible posthepatic mechanism of increased liver inorganic mercury. An A this proposed mechanism, excretion of methylmercury-glutathione A
conjugates occur normally, bacteria in the gut convert organic mercury to inorganic mercury, and bacteria in the gut convert organic mercury to inorganic mercury. For this mechanism to be plausible, reabsorption of inorganic mercury would need to be enhanced beyond the reported 7% efficiency (USEPA, 1987, 1997). There is no known evidence for such a mechanism and no theoretical support for such a mechanism.

Hepatic mechanisms appear to be the best candidates to explain the observed differences in liver mercury speciation and concentration between spotted gar and largemouth bass in Caddo Lake. Animal tissues are thought to demethylate methylmercury at a slow rate and enrichment of inorganic mercury may occur through selective binding of inorganic mercury to selenoproteins. Excessive sequestration of mercury in the gar liver tissue could account for high proportions of inorganic mercury and retention of high concentrations of total mercury in spotted gar liver over largemouth bass liver.

Methylmercury excretion from the body is through glutathione conjugation and excretion into the bile. Decreased rate of conjugation for methylmercury, decreased levels of hepatic glutathione, or decreased transport of conjugated methylmercury out of the hepatocyte and into the bile, could all increase methylmercury levels in the liver and through slow demethylation, inorganic mercury could build up in hepatic tissue over time. Decreased hepatic excretion of methylmercury would be expected to result in increased levels of total mercury in muscle tissue over time, since muscle is a major long-term storage depot for organic mercury that enters the systemic circulation from the liver. However, muscle tissue from
spotted gar and largemouth bass sampled from Caddo Lake show no significant differences in total mercury concentration within habitats (Figure 1.1; data from Chumchal et al. 2011). The finding of indistinguishable concentrations of total mercury in muscle in largemouth bass and spotted gar but liver concentrations that vary by a factor of thirty is not consistent with the pattern expected in the hepatic mechanism of reduced methylmercury secretion.

Another potential explanation for the increased proportion of inorganic mercury and the higher overall level of total mercury in the livers of spotted gar is differences in metabolism between the two species. Histologic analysis of the tissues of gar and bass from Caddo Lake show large differences in the types of cells in residence. Spotted gar show significant brown...
black centers that are associated with perivascular spaces and surrounded by normal liver tissue. The centers are not found in any of the bass sampled from the lake but can constitute up to 30% of the surface of histologic liver sections in the spotted gar (unpublished data). These brown-black masses have been identified through differential staining as melanomacrophage centers. The association of free radical formation in the melanomacrophage centers, demethylation of mercury in macrophage-rich tissues of mammals, accumulation of mercury in melanomacrophage centers in the spotted gar from Caddo Lake, and the observation of enhanced ratios of inorganic mercury in the liver of preteleostean gar over the more "advanced" teleostean bass may all point to evolutionary differences in the location of macrophage hematopoietic centers leading to differences in tissue demethylation of mercury.

1.3 Conclusion

The purpose of environmental risk assessment is to predict the probability of significant changes in the health of individual humans or populations in ecosystems. Mercury contamination of aquatic systems is unlikely to improve in the near future, and with continued input from developing nations into the global pool, is in fact likely to worsen. The metabolism of mercury in animal tissues from organic to inorganic forms has implications for the fate of mercury in foodwebs as well as toxicity to organisms. Knowledge of the mechanism of this metabolic activity gives us the ability to predict the extent to which populations of organisms within specific ecosystems may metabolize methylmercury allowing greater predictive power for the assessment of potential impacts from mercury contamination.
The purpose of this research was to identify possible links between observations of large differences in mercury speciation in organs of fish from different species and the presence of those phagocytes. In particular, the phagocytes of interest were suspected to be melanomacrophages which follow a phylogenetic distribution in tissue of residence and tend to be found in the liver of primitive fish, and in spleen and kidney of modern fish. Findings of the involvement of this cell type as a predictor of the extent of mercury metabolism may also support the role of other, similarly acting, immune cells such as the granulocytes in mercury metabolism. This work posits five hypotheses:

Hypothesis I: Methylmercury demethylation is much more active in the liver of preteleostean fish than teleostean fish.

Hypothesis II: Methylmercury demethylation is much more active in the spleen of teleostean fish than in preteleostean fish.

Hypothesis III: The organic:inorganic mercury ratio is higher in tissues and fish with high abundance of tissue macrophages.

Hypothesis IV: Tissue mercury levels are higher in areas of MMCs and deposits than in surrounding parenchyma.

Hypothesis V: Increased levels of histopathology will be found.

These hypotheses led to the design of this research which is detailed in Objectives I, II, and III.

Objective I: Determine the Phylogenetic and Tissue Distribution of Mercury Demethylation
Hypothesis I: Methylmercury metabolism is much more active in the liver of preteleostean fish than teleostean fish.

Hypothesis II: Methylmercury metabolism is much more active in the spleen and kidneys of teleostean fish than in preteleostean fish.

Approach: Sampling of preteleostean and teleostean fish species was carried out at Caddo Lake, Texas. As noted above, according to the literature, tissue MMCs are most prevalent in the livers of preteleostean fish, while those same hematopoietic centers are localized in the liver and spleen of teleostean fish. Caddo Lake was chosen due to its level of mercury contamination, familiarity with the lake and working relationship with the Caddo Lake Institute, preliminary data associated with populations from this body of water, the presence of diverse fish species spanning the phylogenetic groups, and the characteristics of local fishery use that potentially impact human health. Caddo Lake has many preteleostean fish which include lampreys (Agnatha), paddlefish (Chondrostei), several species of gar (preteleostean Neopterygii), and bowfin (preteleostean Neopterygii). At least 14 families of teleosts have also been identified from the lake, including the primitive teleostean freshwater eels (Hubbs 2002). Paddlefish, bowfin, and gar have been found to have large numbers of MMCs in their livers with few in spleen and kidney.

Targeted sampling of fish species representing teleostean (largemouth bass and channel catfish) and preteleostean (spotted gar and bowfin) fish was carried out (Table A.1). Mercury concentrations and speciation were determined in each of the sampled tissues using a combination of direct mercury analysis (DMA) for muscle tissue estimates, liquid digestion ICP/MS for estimates of mercury and selenium concentrations in liver, and gas chromatography A.
cold vapor atomic fluorescence spectroscopy (GC/CV/AFS) for mercury speciation in liver and spleen tissues. Histologic sections were prepared and analyzed, and melanomacrophage center number and area were estimated through image analysis, and special stains were used to identify MMC content.

Table A.1A

**Fish Taxonomy, Phylogenetic Group, Tissues, and Analyses for Proposed Sampling**

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Genus and Species</th>
<th>Tissues for Mercury Determination (Speciation and Total Mercury)</th>
<th>Tissue for Macrophage center counts and Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preteleostean Fish</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spotted Gar</td>
<td><em>Lepisosteus oculatus</em></td>
<td>Liver, Spleen</td>
<td>Liver, Spleen</td>
</tr>
<tr>
<td>Bowfin</td>
<td><em>Amia rostrata</em></td>
<td>Liver, Spleen</td>
<td>Liver, Spleen</td>
</tr>
<tr>
<td><strong>Teleostean Fish</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Channel Catfish</td>
<td><em>Ictalurus punctatus</em></td>
<td>Liver, Spleen</td>
<td>Liver, Spleen</td>
</tr>
<tr>
<td>Largemouth Bass</td>
<td><em>Micropterus salmoides</em></td>
<td>Liver, Spleen</td>
<td>Liver, Spleen</td>
</tr>
</tbody>
</table>

Outcome: High inorganic:organic ratios for mercury in tissues possessing high abundance of MMCs support the role of macrophages in the demethylation of mercury. High levels of mercury deposition in macrophage centers and melanin-lipofuscin oxidative lesions support a role for macrophages in mercury demethylation and the role of an oxidative mechanism.
Objective I: Determine the association of methylmercury demethylation to the relative abundance of tissue macrophages.

Hypothesis I: The organic:inorganic mercury ratio is higher in tissue and fish with a high abundance of tissue macrophages.

Hypothesis II: Tissue mercury levels are higher in areas of MMCs and deposits than in surrounding parenchyma.

Approach: Histologic sections were stained with H&E and MMCs in liver and spleen were counted and compared with mercury speciation data to determine if a correlation existed between high organic:inorganic mercury ratio and high abundance of MMCs. Slides were taken for relative total mercury imaging by LA-ICP-MS and area sizes of normal parenchyma and melanoMMCAs were analyzed and statistically compared by ANOVA and imaged.

Outcome: High inorganic:organic ratios for mercury in tissues possessing a high abundance of MMCs support the role of macrophages in the demethylation of mercury. High levels of mercury deposition in macrophage centers and melanin-lipofuscin oxidative lesions support a role for macrophages in mercury demethylation and the role of an oxidative mechanism.

Objective III: Determine the protective nature of mercury demethylation.

Hypothesis V: In the tissue of active methylmercury demethylation increased levels of histopathology will be found.

Approach: As was discussed earlier in the introduction, exposure to mercury can cause apoptosis and/or necrosis in the tissues in which it accumulates (Mela et al., 2007a). Necrosis and apoptosis can be seen as extreme points along a continuum of the path to cell death. Necrosis is attended by inflammatory infiltrate and necrosis can be visualized by the formation of A...
of apoptotic bodies (Cotran, Kumar, & Collins, 1999). Histologic sections of liver and were analyzed for pathology, with special attention to the development of melanin-lipofuscin deposits, apoptotic bodies, inflammatory infiltrate, and other lesions.

Outcome:

The development of a greater abundance of histopathologic signs in tissues with high levels of inorganic mercury is considered supportive of an association between accumulation of inorganic mercury in tissue and tissue damage.

Results of these experiments are detailed in the following chapters. Chapters 2-4 are written as primary research articles for submission for publication. Chapter 6 provides an overall conclusion and synthesis of each of the research chapters.
CHAPTER 2

HISTOLOGY AND HISTOPATHOLOGY OF FISH EXPOSED TO MERCURY IN CADDOTH E AKEA

2.1 Introduction

Mercury is a widespread contaminant that impacts the health of human populations and ecosystems. It is found in both inorganic and organic compounds, is environmentally persistent, and biomagnifies in the food chain (NRC, 2000). In fish, mercury is thought to accumulate in kidney, muscle, and liver, resulting in signs of necrosis with inflammatory infiltrate (Mela, et al., 2007b), apoptosis with upregulation of apoptotic signaling pathway genes, and lesions in Disse’s space of the liver (Klaper, et al., 2008). Methylmercury exposure in fish has been shown to impair coordination and swimming activity (Crump & Trudeau, 2009), which can reduce species fitness by reducing successful hunting, evasion of predation, and mating behaviors. Environmentally relevant concentrations of mercury have been shown to impair reproduction in fish and are thought to act through induction of apoptosis in steroidogenic cells and/or through disruption of the hypothalamic-pituitary-gonadal axis (Crump & Trudeau, 2009; Drevnick, et al., 2008; Drevnick & Sandheinrich, 2003; Drevnick, et al., 2006; Vieira, et al., 2009).

Caddo Lake is a freshwater reservoir that spans the Louisiana and Texas border, and is reported to have among the highest levels of mercury in fish in the state of Texas (TCEQ, 2004). The Texas side of Caddo Lake is primarily shallow forested wetland while the Louisiana side is a more open water habitat. The mercury levels in fish are significantly higher on the wetland side of the lake than the open water side of the lake (Chumchal MM, et al., 2008). The top predators in this ecosystem include spotted gar and largemouth bass (M. M. Chumchal, et al., 2011).
Melanomacrophages, fixed tissue macrophages of amphibians and fish, have been used as biomarkers of general pathology (Hartley, et al., 1996; Volke, 1992) but are also thought to occur in certain tissues as non-pathologic germinal centers of fixed macrophages (Agius, 1980; Agius & Roberts, 2003a). Melanomacrophage centers (MMCs) (the melanomacrophages of fish) appear to follow an evolutionary pattern in distribution and degree of organization with a trend from random distribution and low levels of organization in Condrichthyes and Agantha, to highly organized structures in the liver of the primitive bony fish, and finally to well-organized structures in the spleens and kidney of the Teleosts (Agius, 1980).

Macrophage centers have been shown to increase in the livers of fish with high body burdens of mercury, as has liver color and lipofuscin content. In northern pike from Isle Royale, increases in lipofuscin content and liver color were observed with increasing mercury concentration (Drevnick, et al., 2008). Increases in macrophage centers in spleen and liver have also been observed in Pike from the Oder River in Germany with increasing muscle mercury (Meinelt, Krüger, Pietrock, Osten, & Steinberg, 1997), and independently of age in trout in the U.S. (Schwindt, Fournie, Anders, Schreck, & Kent, 2008). An Devil's Swamp Louisiana, Increased MMCs and increased hepatic Iron were seen in Gar liver from Tunica Swamp Gar controls. Gar muscle tissues from Tunica Swamp Gar were found to be much lower in several contaminants, including mercury (Hg 5.8 ng/g), than in Devil's Swamp Gar (426.6 ng/g) (Hartley, et al., 1996). In Caddo Lake increasing concentrations of hepatic mercury have been observed to correlate with increasing levels of melanomacrophages in spotted Gar and increased tissue pathology in the form of darkening of gross liver (B. Barst, et al., 2011).
The goal of this study was to conduct a histological examination of macrophage centers in Caddo Lake fish species known to be high in total mercury concentrations. Species were selected to include both primitive preteleosts (spotted gar and bowfin) and modern teleosts (largemouth bass and channel catfish) to provide an evolutionary comparison based on macrophage center distribution. Low mercury fish were obtained from other sources to act as a histological baseline. This study reports strong correlations between MMC size/number and total mercury concentrations in each of the four species. These data further support the role of macrophages in hepatic mercury accumulation.

2.2 Materials and Methods

2.2.1 Fish Collection and Tissue Processing

Fish were collected in accordance with Texas Parks and Wildlife Scientific Research Permit No. SPR’0311’092 and the University of North Texas Institutional Animal Care and Use Committee Protocol #11005. Fish were collected from the wetland habitat at the Western edge of Caddo Lake, near Uncertain, Texas. The specific fish collection methods used were electro-shocking from a boat, gill netting, and line fishing. The target species for this research were largemouth bass (Micropterus salmoides), channel catfish (Ictalurus punctatus), spotted gar (Lepisosteus oculatus), and bowfin (Amia rostrata). Collected fish were kept alive in aerated water until sacrificed.

Histologic baseline samples of largemouth bass were taken from a pond (stock tank) at a ranch in West Texas (32°25’14.29"N, 98°59’35.47"W). The ranch is located near Cisco, Texas and has been used as agricultural land for raising small herds of cattle for over 70 years. No
industry exists near the ranch and in the surrounding region. This body of water was chosen as the source of low mercury exposure because of ease of use, low cost of obtaining fish, and expected low levels of tissue mercury.

Histologic baseline samples for alligator gar and channel catfish were taken as donations from the Private John Allen National Fish Hatchery in Tupelo, MS.

Fish were sacrificed by placing them in tricaine solution (MS222) until dead. Tissue samples were taken for liver and spleen histology (n = 5 individuals per species from baseline and Caddo fish groups), muscle mercury analysis (n = 5 baseline fish and Caddo fish per species), and liver mercury analysis (n = 5 Caddo fish per species). Biopsies of spleen and liver tissue were immediately taken for histology and were placed in 20-ml histology vials pre-filled with 10-ml 10% buffered formalin (Life Science Products HW1103). The remaining liver and spleen were placed in labeled quart Ziploc® plastic bags (SC Johnson) and placed on ice for later mercury analysis. However, due to histologic results and small spleen tissue sizes, spleen mercury levels were not analyzed in this study. Samples of muscle for mercury analysis were similarly taken from all fish.

2.2.2 Tissue Mercury Analysis

Batches of scintillation vials stored samples were removed from storage and freeze dried for 72 hours in a lyophilizer (Labconco FreeZone 6-Liter Console System 7753027) at −56°C and a pressure of 3.5 pascals (3.45 x 10⁻⁵ atmospheres). Dried samples were ground in the scintillation vial using glass stir rods. Stir rods were washed three times between each sample in deionized water and wiped clean before moving to the next sample. Ground samples were stored at −80°C until mercury content analysis was carried out.
2.2.2.1 Muscle Total Mercury Content by Direct Mercury Analyzer

Lyophilized and ground samples were weighed and run on a DMA-80 Direct Mercury Analyzer (Milestone Inc). To each run of samples was added certified reference material every 10 samples. Certified reference materials (CRM) used included Mess-3, TORT-2, DOLT-3, and DORM-3 (National Research Council Canada). All recoveries for CRM were from 97 to 106% of the reported certified values. Duplicate values were from 80-99% of one another.

2.2.2.2 Liver Total Mercury Analysis by ICP-MS

ICP-MS was chosen as the analytical method for total liver mercury due to the ability of this method to quantify multiple elements simultaneously. Selenium was quantified as well as mercury in all samples (data not shown, see Chapter A). Mercury concentrations were determined by acid digestion of dried, powdered samples and liquid introduction to an Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Digestions were carried out with a modification and adaptation of the method used by Ashoka (Ashoka, Peake, Bremner, Hageman, & Reid, 2009), in which a domestic microwave oven was used in closed vessel digestion with nitric acid and hydrogen peroxide. All samples and standards were made up, digested, and analyzed in an molded clarified homopolymer polypropylene screw cap digestion vessels (Environmental Express SC475), which allowed sample preparation and analysis in one vessel and minimized contamination or loss through vessel transfer. Lyophilized and ground samples were weighed and placed in screw cap digestion vessels and 1 ml 70% nitric acid (Fischer Scientific, Trace Metal Grade) and 500 µl 50% hydrogen peroxide (Fischer Scientific, certified, stabilized) were added. A domestic style 700 watt microwave (Maytag A)
Digestions were carried out at 50% power for 1 minute followed by a 5 minute rest period. This microwave and rest cycle was carried out three times on each sample. After digestion and cooling, internal standard and gold solution were added to digestate and the volume of the samples was brought up to 50 ml with MiliQ water. All standards and dilutions were made up in 18 MΩ MiliQ water.

A multi-element standard containing bismuth, holmium, indium, lithium, terbium, and yttrium was used to determine proper dilution of digestate (Ricca Chemical CLISS-1) by monitoring the Terbium content during analysis. The final concentration of the internal standard was 50 ppb in each solution. Gold standard was also placed in all solutions to reduce carryover of mercury (Ricca Chemical MSAU1KN100). Gold was also added to ICP wash solution (2% (v/v) trace metal grade 70% nitric acid) and in both wash solution and analytical solutions the concentration of gold was kept at 100 ppb. Single element mercury (MSHG1KN100) and a selenium (MSSE1KN100) standard were obtained to produce an external standard curve. To all solutions 100 ppb gold standard and 50 ppb internal standard were added after digestion and cooling and before dilution of digestate.

All analyses were conducted using a Varian 820 ICP-MS with SPS3 Sample Preparation System autosampler. Isotopic mass data for Mercury 202 were collected. Five estimates of each measurement were made; percent relative standard deviations and mean counts per second at each measured mass were reported by Varian software. Development of standard curves, calculation of solution and tissue concentrations, and variability of internal standards were performed on exported data using Microsoft 2007 Excel software.
2.2.3 Histologic Examination

Histologic samples were processed to make slides, embedded, sectioned, stained, read, and photographed at Dermatopathology Associates in Jackson, MS. Reference fish were used for histologic comparison to Caddo Lake fish to evaluate possible pathologic processes and were necessary as a point of departure for histologically unremarkable tissue in comparison with mercury-exposed fish. This was particularly important to evaluate the preteleostean fish whose tissues are poorly characterized in the literature.

2.2.3.1 Tissue Processing and Embedding

Tissue samples were grossed and placed in properly labeled cassettes. Tissue was processed overnight in an Excelsior Advanced Tissue processor (Thermo Shandon A78410100). The reagents used in processing the tissue were 10% neutral buffered formalin (Leica 3800598), reagent alcohol (Leica 3803686), Sub-X, an aliphatic hydrocarbon xylene substitute (Leica A 3803670), and blue ribbon paraffin (Leica 8801360). All reagents addition was made at ambient temperature, except for paraffin, which was held at 61°C. The stir agitation function was held at an intermediate setting.

2.2.3.2 Sample Sectioning and Staining

Hematoxylin and eosin staining: Slides stained for routine histologic examination and a light microscopy analysis were sectioned on a microtome to 4 μm, mounted on glass slides (Leica Surgepath glass slides 8800375), and stained with H&E using a Shandon Varistain Gemini Slide Stainer (Thermo Shandon A78000014) and an Leica CV5030 automated glass coverslipper (Thermo Shandon).
Melanin bleaching: Sections (4 μm) were mounted on glass microscope slides (Leica A Surgepath glass slides 880375). Slides were prepared as sequential sections for melanin bleaching followed by H&E staining and sections for H&E staining alone. This allowed for a comparison of the same structures with and without melanin bleaching treatment. One individual was chosen randomly from each group for melanin bleaching of liver tissue. Melanin bleaching was performed using potassium permanganate 0.25% (Polyscientific S2273-8oz) followed by decoloration with 5% aqueous oxalic acid (S2199-8oz) and slides were subsequently stained with H&E in the Varistain Gemini Slide Stainer as above.

Periodic Acid-Schiff staining: One individual from each group (liver and spleen) was randomly chosen for PAS staining to confirm H&E analysis of vacuolation and melanomacrophage PAS positivity. Staining was carried out using a commercial kit (Sigma Aldrich PAS Kit 395B) by the standard procedure. Staining was carried out as per the instruction in the kit with the exception that deparaffination of the slides and drying were carried out in the Varistain Gemini Stainer. Slides were overslipped using the automatic coversliper. Sections (4 μm) were mounted on charged slides (Fischer Brand Probe on Plus 22' 230' 900). A PAS positive control was used with each run of PAS staining. This control consisted of human skin tissue of an individual with a dermal fungal infection.

Prussian blue staining: Initially on staining was carried out on liver and spleen from one randomly chosen individual from each group, but due to the deposition of large amounts of iron in hepatocytes of certain fish and the possible relation of iron deposits to oxidative processes in tissue, samples from each individual and group were stained and inspected. Iron staining was carried out with a Prussian blue reaction with a Pararosaniline counterstain using a

25A
26A

commercial kit (Sigma Aldrich Iron Stain Kit HT20). Staining was carried out as per the instruction. An exception that deparaffinization of the slides and drying were carried out in the Varistain Genmini Stainer. Slides were overslipped using the automatic coversliper. Sections (4 μm) were mounted on charged slides as above. A positive control of human spleen was prepared with each run of Prussian blue staining.

2.2.4 Image Analysis

Slide imaging was carried out using a Motic Instruments Microscope Camera (Moticam 2000SP10’0382) on an Olympus BX40 microscope. Image analysis of MMCs was performed on TIF images treated through Motic 2000 software. An automated analysis for MMCs count and percent area of MMCs in relation to the total captured field was carried out using ImageJ (NIH, http://rsbweb.nih.gov/ij/), an area Java-based image processing and analysis software. Digital TIF images were converted to binary images in ImageJ and the size and number of MMCs were estimated through the Analyze Particles function in the software. Size of captured field was adjusted based on the image, circularity was kept in the all-inclusive range from 0 to 1, outlines were shown for comparison with H&E stain, and results were displayed and summarized.

2.3 Results

2.3.1 Muscle Total Mercury Analysis

Reference fish muscle tissue show lower concentrations of mercury than Caddo lake fish of the same species (one-tailed test: Bass p = 0.0063, Catfish p = 0.001, Gar p < 0.0001) (Table 2.1). Wet weight tissue concentrations of 200 ng/g and lower in fish muscle have been estimated to be protective of the health of juvenile and adult fish (Dillon, Beckvar, & Kern, A
2010). The tissue residue criterion for the edible portion of fish is 300 ng/g wet weight based on a total fish and shellfish consumption weighted at 17 g fish/day. This estimate is based on the methylmercury RfD of 0.0001 mg methylmercury/kg/day (Borum, et al., 2001). All reference fish in this study are well below the EPA tissue criterion for human health and literature-derived estimated fish protective levels and even the maximum measured values of all reference concentrations. All Caddo fish groups have muscle tissue concentrations higher than the muscle tissue concentration expected to be without health effects. Average muscle tissue concentrations for Caddo Lake fish are all above the EPA criterion, except for catfish, and all maximum values for Caddo fish exceed the EPA tissue criterion. These results support the use of these reference fish for histologic comparison.

Table 2.1A

<table>
<thead>
<tr>
<th>Fish Group</th>
<th>Mean Total Mercury in Muscle (ng/g dry weight +SD)</th>
<th>Mean Total Mercury in Muscle (ng/g wet weight +SD)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bass Reference A</td>
<td>687.3 ± 246.7A</td>
<td>137.5 ± 49A</td>
</tr>
<tr>
<td>Bass Caddo</td>
<td>3631 ± 229A</td>
<td>726.2 ± 445A</td>
</tr>
<tr>
<td>Catfish Reference A</td>
<td>84.1 ± 51.68A</td>
<td>16.8 ± 10A</td>
</tr>
<tr>
<td>Catfish Caddo</td>
<td>1143 ± 601.8A</td>
<td>228.5 ± 120A</td>
</tr>
<tr>
<td>Bowfin Caddo</td>
<td>5001 ± 2792A</td>
<td>1000 ± 58A</td>
</tr>
<tr>
<td>Gar Reference A</td>
<td>58.14 ± 4A</td>
<td>11.63 ± 0.7A</td>
</tr>
<tr>
<td>Gar Caddo</td>
<td>2200 ± 947A</td>
<td>440 ± 90A</td>
</tr>
</tbody>
</table>

*Estimated as 1/5 the dry weight concentration.

4.3.2 Liver Total Mercury by ICP-MSA

Mercury concentrations were found to be similar in liver for Caddo fish analyzed in this study and no significant differences were found between species (Kruskal-Wallis Test, p = 0.51). This lack of significance is largely due to the high levels of variation between individuals within a...
species. This variability is an artifact that two of the five gar livers were very high in mercury and may suggest the existence of a subpopulation (table 2.2).

Table 2.2A

Analytical Results for ICP-MS Estimates of Liver Mercury in Caddo Fish (Mean, SD)

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Mean Concentration of Mercury in Liver tissue (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BassA</td>
<td>3317A±041A</td>
</tr>
<tr>
<td>CatfishA</td>
<td>2435A±440A</td>
</tr>
<tr>
<td>BowfinA</td>
<td>2289A±342A</td>
</tr>
<tr>
<td>GarA</td>
<td>16421A±8570A</td>
</tr>
</tbody>
</table>

2.3.3 Histologic Analysis

2.3.3.1 Hematoxylin and Eosin Analysis

Largemouth bass: Largemouth bass from both reference and Caddo populations were infected with a parasitic worm. Almost all individuals examined. Parasites were large and were found in both spleen and liver tissue. Worm larvae were found in vascular spaces and in some cases were associated with clot formation and occlusion of vascular lumina. The livers of bass reference and Caddo populations showed occasional large, macrophage centers with uniform golden-brown cytoplasm that was agranular and whose color was not as dark as that expected for melanin (Figure 2.1). In general, spleens showed a much higher abundance of macrophage centers than liver. Both populations but were, as in the liver, consistently large and pale. No apparent melanin was seen in macrophages in either population. Melanin bleaching, Prussian blue staining, and PAS staining were used to identify the brown material. An A
macrophage centers, which was provisionally identified as bile, lipofuscin, and/or iron. A histologic examination of liver and spleen were otherwise unremarkable (Figure 2.1). A
Figure 2.1. Melanomacrophages in bass liver and spleen.

Bass H&E, Panel A: Reference bass liver 400x, Panel B: Caddo bass liver 400x, Panel C: Reference bass spleen 400x, Panel D: Caddo bass spleen 400x. MC: Macrophage center, Hep: Hepatocytes, Sp: Spleen parenchyma (lymphocytes, red cells).
Catfish: Both reference and Caddo fish showed extensive pancreatic tissue embedded in normal liver tissue (hepatopancreas). Hepatocytes of reference fish showed moderate vacuolation in comparison with those of Caddo populations, which may be due to enhanced glycogen storage in the well-fed state. PAS staining was performed on liver to identify this vacuolar material as glycogen. Hepatocytes of catfish from Caddo had cytoplasmic granules of brown material which were provisionally identified as iron, perhaps more likely bile. Later Prussian blue staining confirmed iron deposition in hepatocytes of catfish. Hepatic cytoplasmic granules were not seen in reference fish (Figure 2.2).

Macrophage centers in livers of both reference and Caddo populations are occasional, almost universally associated with pancreatic tissue, and are more prevalent based on the amount of pancreatic tissue in the section being examined. Macrophage centers in livers of both reference and Caddo populations were characterized by pale, golden brown, agranular cytoplasm. No obvious dark granular material characteristic of melanin was seen in liver macrophage centers.

Catfish spleen showed lymphocyte and red blood cells as the predominant cell types with large macrophage centers. The cytoplasm of macrophage centers in spleens of reference catfish were agranular and pale golden brown, while those in Caddo fish were similar, except that they also showed stippling of granular black material characteristic of melanin. In general, the splenic macrophage centers of Caddo fish were darker than those of reference catfish, and were in some cases quite dark. No increase in macrophage centers was obvious in Caddo fish over the reference population (Figure 2.3).
Figure 2.2. Catfish liver histology.

Catfish liver H&E, Panel A: Reference 100x, Panel B: Reference 400x, Panel C: Caddo 100x, Panel D: Caddo 400x, MC: Macrophage center, Bv: Blood vessel, Hep: Hepatocytes, P: Pancreatic tissue.
Figure 2.3. Catfish spleen histology
A: Reference 400x, B: Caddo 400x
*Spotted Gar:* Gar hepatocytes from reference fish were uniformly highly vacuolated with very little visible cytoplasm. This is in contrast with Caddo gar populations, which showed condensed cytoplasm with little vacuolation. Caddo and reference livers showed perivascular cuffs of hematopoiesis populated with lymphocytes, eosinophilic granulocytes, plasma cells, and hematopoietic precursors (blasts). Reference gar showed smaller, more prominent aggregates of macrophages than those seen in bass or catfish, and the cytoplasm of these macrophages was filled with dark, black, granular material. The macrophage centers of gar from the Caddo population were very prevalent with intensely dark, granular inclusions in their cytoplasm, and were sometimes completely obscured by the dark material. Some Caddo fish showed very large numbers of macrophage centers that commanded large portions of the surface area of liver sections (Figure 2.4). A

The spleens of gar from both Caddo and reference populations showed signs of germinal activity that was much more prominent than in another species. The reference fish showed very high levels of hematopoietic activity with frequent mitosis, hematopoietic precursors (blasts), and prominent nucleoli. The background cellularity of the spleen was composed of lymphocytes and red blood cells with scattered macrophages, and small, infrequent islands of liver cells. Macrophages in both reference and Caddo populations were very dark with granular black material (Figure 2.4). A
Figure 2.4. Gar Liver and Spleen Histology

Panel A: Reference Liver 100x, Panel B: Caddo Liver 100x, Panel C: Reference Spleen 400x, Panel D: Caddo Spleen 400x
Bowfin: No reference fish were available for bowfin and all descriptions are of Caddo fish. Hepatocytes were unremarkable in most fish, but some showed granular deposits of golden brown material, and, in one case, increased vacuolation. All livers showed periportal lymphocytes, which were considered non-inflammatory and unremarkable. In these fish, melanomacrophage centers were very prominent, if slightly less in number than an gar, and like gar contained large amounts of black granular material. Unlike other fish, melanomacrophage centers did not seem to be predominantly perivascular and were scattered throughout the liver. One bowfin (Bowfin 4) showed a large well-circumscribed area with very low abundance of macrophages, normal appearing hepatocytes, and an absence of biliary structures. This well-circumscribed lesion could be appreciated grossly as a round area of pale liver tissue.

Spleens of bowfin showed isolated, scant, dark macrophages in a background of lymphocytes and red blood cells. Some (bowfin 4) showed granular deposits scattered in the parenchyma of the spleen, and had granular deposits of black material in vascular endothelial cells (Figure 2.5).
Figure 2.5. Bowfin histology: Liver and Spleen

Panel A: Bowfin Liver 100x, Panel B: Bowfin Spleen 400x
2.3.3.2 Incidental Findings

Some observations were made during the course of the H&E analysis of slides that were considered abnormal, but not related to mercury exposure. One of these was the parasitic infection of virtually all bass in the study, both reference and Caddo fish. Another finding was a pale staining mass in the liver of one bowfin, which was examined both microscopically and grossly. This mass was circular, large, and well-circumscribed and showed a lack of biliary structures. This mass is consistent with biliary adenoma and was considered to be unrelated to mercury exposure.

2.3.3.3 Special Stain Analysis

Macrophage centers were found to be heterogeneous in composition, containing aron, melanin, and glycogen. Melanin was confirmed to be in abundance in the macrophage centers of gar and bowfin, while catfish and bass were likewise confirmed to have low contributions of melanin. According to the literature, melanomacrophages were found to be PAS positive, which was best appreciated in bass and catfish, since macrophage centers in these species were less obscured by melanin. An A bowfin, macrophage centers were also observed to be PAS positive, since they were almost darkly pigmented Ahan Agar. Melanin and aron were major constituents of melanomacrophages A bowfin.

Hepatocyte aron deposits were somewhat variable within species, but large deposits were found in all bowfin, and were found in some Caddo Ake catfish. Aron deposition in A hepatocytes was not seen in bass A gar. Based on these results, further examination of hepatic A iron was carried out (Hepatic Aron Scoring Aection, Ahis Chapter). A
2.3.3.4 Melanin Bleaching

Melanin is often a prominent component of fish and amphibian macrophages, which has led to the term melanomacrophages to describe them (Wolke, 1992). In the current study, initial inspection of macrophage centers in liver and spleen of both reference and Caddo fish showed species-specific difference in the expression of macrophage pigments. Gar and bowfin macrophages were very dark with a pigment that appeared to be melanin, while bass and catfish appeared golden brown with no apparent melanin.

Melanin bleaching confirmed that melanin was a major component of cytoplasmic pigment of melanomacrophages in spotted gar and bowfin (Figure 2.7), but was a minor component in bass and catfish (Figure 2.6). Melanin bleaching followed by H&E stain showed a characteristic change in color toward blue in subsequent H&E stain; this effect is well known and expected with this procedure. Largemouth bass and channel catfish (Figure 2.6) liver sample showed little change in macrophage center characteristics with and without bleaching. The dark material in gar and bowfin liver showed almost complete removal with bleaching (Figure A 2.7).
Figure 2.6. Caddo catfish H&E and melanin bleach of liver macrophage centers.

Panel A: Catfish Liver H&E, Panel B: Catfish Melanin Bleach followed by H&E, Panel C: Catfish Liver H&E, Panel D: Catfish Melanin Bleach followed by H&E. MC: macrophage, MCMB: macrophage melanin bleach. All fields 400x magnification.
Figure 0.7: Caddo Gar and Bowfin H&E and Melanin Bleach of Liver Macrophage Centers

2.3.3.5 Periodic Acid Schiff Stain

Periodic Acid Schiff (PAS) is a staining method that stains glycogen in tissues by oxidizing the glucose residues in the molecule to produce aldehydes that react with the Schiff reagent to produce a purple color. A high degree of liver cell vacuolation was found in reference fish in this study. Vacuolation of hepatocytes can be due to pathologic processes or, in some animals, to energy storage in the well-fed state. PAS staining shows that the prominent vacuolation in the livers of reference fish is associated with glycogen storage. Reference channel catfish and alligator gar were found to have extensive vacuolation which was provisionally attributed to extensive glycogen storage in the well-fed state. PAS staining confirms that this vacuolation is associated with glycogen store and is not considered to result from any pathologic process (Figure 2.8).

Macrophage centers in fish have been shown to contain large amounts of ceroid material, which has been found to be PAS-positive (Brusle, Anadon, Datta, & Dutta, 1996). Ceroid, or lipofuscin, is an age pigment that is produced in cells from oxidatively modified proteins and lipids. It forms a polymeric cellular inclusion that is difficult to digest and builds up in the cell over time (Terman & Brunk, 2004). PAS staining in Caddo fish confirms PAS positivity of macrophage centers and further shows significant deposits of lipofuscin in fish macrophage centers (Figure 2.9). Macrophage centers are focal points of oxidative activity and this finding is expected.

A

A

A
Figure 2.9. PAS staining of livers of Caddo fish and positive control.
Panel A: PAS Positive Control, human fungal infection in skin, 100x magnification.
Panel B: Reference gar liver, 100x magnification.
Panel C: Reference catfish 100x magnification.
Panel D: Caddo catfish 100x magnification.
Figure 2.10. PAS staining of bowfin and bass liver macrophage centers.

Panel A: Bowfin macrophage center showing PAS positivity, 400x magnification.
Panel B: Bass macrophage center showing PAS positivity, 400x magnification.
2.3.3.6 Prussian Blue Aron Stain A

Hemosiderin occurs as yellow to brown intercellular granules and as a waste product of iron metabolism. Hemosiderin is a heterogeneous mixture of iron oxides and perhaps ferritins, but iron is present as oxidized iron III (Richter, 1978). Hemosiderin is a normal pigment in macrophages, which often phagocytize senescent red blood cells, but an abnormal, generalized overabundance of iron deposits is called hemosiderosis. Hepatic iron anemia of a chronic disease (Batts, 2007). Hepatic hemosiderosis has been observed in fish exposed to mixed pollutants, including mercury, for example in the Mississippi basin (Thiyagarajah, Hartley, Abdelghani, 1998) and with methylmercury exposure in frogs (Chang, Reuhl, & Dudley, 1974). A second reaction called Turnbull's blue also produces the Prussian blue pigment, but it uses ferricyanide and preferentially stains ferrous iron (iron II). The Prussian blue reaction is most often used, perhaps because of the biological importance of the formation of oxidized iron precipitates in the cell (Sheehan & Hrapchak, 1980).

The Prussian blue reaction exposes tissue sections to acid solutions and ferrocyanides which react with ferric iron (iron III) resulting in the formation of a bright blue pigment which is called Prussian blue. The reaction is very sensitive and can reveal even single granules of iron. A second reaction called Turnbull’s blue also produces the Prussian blue pigment, but it uses ferricyanide and preferentially stains ferrous iron. The Prussian blue reaction is most often used, perhaps because of the biological importance of the formation of oxidized iron precipitates in the cell (Sheehan & Hrapchak, 1980).

Prussian blue staining showed iron to be a large proportion of the pigment of macrophages in bass (Figure 2.10) and catfish (Figure 2.11), and was also a component of bowfin and gar (Figure 2.12, panel B). A
showed marked hepatocellular hemosiderosis. Hepatocellular hemosiderosis, as a pathologic condition in these fish, was scored and has been analyzed further in this study.
**Figure 2.11.**

Panel A: Iron Stain Positive Control, human spleen
Panel B: Caddo Bass macrophage Center, Prussian Blue 100x
Panel C: Caddo Bass macrophage Center, Prussian Blue 400x
Figure 2.12. Prussian blue of catfish liver macrophage centers and catfish hepatic hemosiderosis.

Panel A: Caddo Catfish macrophage Center, Prussian Blue, 10x
Panel B: Caddo Catfish macrophage Center, Prussian Blue, 40x
Panel C: Caddo Catfish Hepatocytes, showing hepatic hemosiderosis, 40x
Figure 2.13. Prussian blue stain of bowfin liver macrophages and hepatic hemosiderosis.
Panel A: Gar liver, 400x, Panel B: Bowfin liver 400x magnification.
2.3.3.8 Hepatic Iron Scoring

Hepatic iron deposition was scored in all Caddo fish and controls since the deposition of iron in liver is generally a pathologic process and as often a result of oxidative processes. Variation in iron deposition was suspected in initial H&E staining and in subsequent iron stains so a complete workup and scoring was performed to quantify the observed differences. The scoring method defines scant as occasional trace iron, followed by one plus to four pluses as to hepatic iron content. One plus is occasional but well defined iron particles in hepatocytes. Four pluses is very widespread (almost total organ involvement) and heavy hemosiderin deposits in hepatocytes.

Control fish were found to have generally low levels of hepatic hemosiderin deposits. Caddo bass were found to be similar in iron deposition with reference fish, but catfish, bowfin and gar were found to have high levels of iron deposition in their livers (Table 2.3).

<table>
<thead>
<tr>
<th>Fish ID</th>
<th>Control Catfish</th>
<th>Control Bass</th>
<th>Control Gar</th>
<th>Caddo Catfish</th>
<th>Caddo Bass</th>
<th>Caddo Bowfin</th>
<th>Caddo Gar</th>
</tr>
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<tbody>
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<td>+A</td>
<td>0A</td>
<td>+++A</td>
<td>++A</td>
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</tr>
<tr>
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<td>0A</td>
<td>+A</td>
<td>0A</td>
<td>+++A</td>
<td>0A</td>
<td>+++A</td>
<td>ScantA</td>
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<tr>
<td>3A</td>
<td>0A</td>
<td>++A</td>
<td>0A</td>
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<td>0A</td>
<td>+++A</td>
<td>+A</td>
<td>+++A</td>
<td>+A</td>
</tr>
</tbody>
</table>

Hepatic Hemosiderin Score for Caddo Fish
2.3.3.9 Image Analysis

Image analysis of macrophage centers shows expected tissue tropism based on the literature, with predominance of macrophage centers in livers of preteleostean fish and spleens of teleosts. The percent area shows better concordance than simple macrophage center counts. This is related to the histologic finding of larger, less frequent MMCs in teleostean fish and smaller, abundant centers in preteleostean fish (Table 2.4).

Table 2.3

Number and Total Size of Macrophage Centers in Liver of Caddo Fish (Mean ± SD)

<table>
<thead>
<tr>
<th>Species</th>
<th>Count</th>
<th>Area Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bass</td>
<td>1.60(1.34)A</td>
<td>0.37(0.43)A</td>
</tr>
<tr>
<td>Catfish</td>
<td>0.8(0.67)A</td>
<td>0.27(0.21)A</td>
</tr>
<tr>
<td>Bowfin</td>
<td>123.4(35.25)A</td>
<td>6.0(2.26)A</td>
</tr>
<tr>
<td>Gar</td>
<td>106.2(8.27)A</td>
<td>9.14(7.50)A</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bass</td>
<td>14.7(6.35)A</td>
<td>5.38(6.21)A</td>
</tr>
<tr>
<td>Catfish</td>
<td>7.5(6.35)A</td>
<td>2.43(1.83)A</td>
</tr>
<tr>
<td>Bowfin</td>
<td>2.4(1.29)A</td>
<td>0.12(0.03)A</td>
</tr>
<tr>
<td>Gar</td>
<td>2.1(1.48)A</td>
<td>0.23(0.10)A</td>
</tr>
</tbody>
</table>

2.3.4 Relationship between Macrophage Center Area and Mercury

Earlier research suggested that macrophage center number increased with increasing mercury concentration. Comparisons were made of estimated macrophage area with mercury concentrations from ICP-MS analysis (Figure 2.13).

A
Evidence was found for an association between total liver mercury concentration and relative liver macrophage center area in Caddo fish (R² values for linear regression).

Higher concentrations of liver total mercury were associated with increases in macrophage center area in all fish examined (figure 2.13). The association was found to be strongest in bass (Pearson correlation R² = 0.9833, p = 0.0026) and bowfin (Pearson correlation R² = 0.9681, p = 0.0068). Correlations of associations between mercury concentration and macrophage center area are lower in gar (Pearson correlation R² = 0.7841, p = 0.116) and catfish (Pearson R² = 0.818, p = 0.091) than in bass and bowfin. An individual in each of these groups reduces correlation, when these individuals are removed from analysis the Pearson correlation coefficients for gar become 0.9984 (p = 0.0016) and 0.985 (p = 0.015) for a catfish. The strength of association in bass and bowfin is coupled with the suggestion of a similar A

52A
association in catfish, and a very low measurement for catfish macrophage area. An average the lowest estimate gives confidence that apparent outliers in catfish and bass can be removed from the data set for analysis. The measurement of area of macrophage centers as based an an average of two fields from H&E sections from each individual, and some variation in estimation is expected.

2.4 Discussion

Macrophage centers in fish and amphibian tissues have been termed melanomacrophages based on their accumulation of pigments, yellow to black in color, rather than specifically on the melanin molecule (Roberts, 1975). This is an historic definition of the term melanin, rather than one currently used that only recognizes a set of closely related pigment molecules as melanins (Agius & Roberts, 2003a). Results from the current study have identified the types of pigments commonly associated with melanomacrophages: melanin, hemosiderin, and lipofuscin/ceriod, and have shown them to often co-occur within the same cell.

In mammals, melanin production is confined to neuroectodermally derived tissues, but in amphibians and fish melanogenesis can also occur in hematopoietic tissue. Recently, fish cells from a leukocyte cell line (CD83+) were shown to express dopachrome tautomerase/tyrosine-related protein 2 (Haugarvoll, Thorsen, Laane, Huang, & Koppang, 2006). This demonstrates that leukocytes in fish are capable of producing melanin. Tyrosinase, the key enzyme in melanin synthesis, has been shown to play an integral role in encapsulation and phagocytosis in invertebrates, as produced with infestation or inflammation, and as recently been shown to increase in immunologic challenge in fish (Wang et al., 2009).
In this study, species-specific differences in pigmentation of macrophage centers of the liver were found. Caddo gar and reference gar have macrophages that are characterized by very abundant melanin, while those of catfish and bass have high levels of hemosiderin, but very little melanin. All macrophage centers were found to be PAS positive and to contain large amounts of lipofuscin. The literature often reports differences in melanin content of macrophage centers. These differences have been variously characterized as independent of phylogenetic relationship and as highly variable between species and individuals (Wolke, 1992) and as remarkably constant within organs by species (C Agius, 1980).

Spleenic iron in melanomacrophage centers has been linked to hematopoiesis and hemoglobin synthesis, while that of liver and kidney has been suggested to occur with hemolytic crisis (Wolke, 1992). In the current study, high levels of iron were found in the macrophage centers of the liver both in wild-caught and reference fish. The finding of hepatic hemosiderosis in some Caddo fish leads us to suggest that some iron deposits in MMCs may be due to oxidative processes in hepatocytes rather than being confined to hemolysis or iron overload.

Differences were also found in the location and size of macrophage centers in this study. In the gar and bass, macrophage centers were found to be perivascular, while those in bowfin were more randomly distributed, and finally, those in the catfish were almost uniformly confined to pancreatic tissue. Sporadic studies have noted differences in the appearance of macrophages by species, tissue of residence, and in different physiological conditions within the same species, but few, if any, have systematically reviewed these differences across species from one body of water with reference tissues.
Morphological differences of melanomacrophage centers have been noted by species, organ and physiologic condition in the literature, however most of this work has been done in teleosts. One seminal study comparing various phylogenetic groups of fish found increasing organization of these structures from diffuse, rudimentary structures in Chondrichthyes to well-organized structures of all Osteichthyes, except for salmonids (Agius, 1980). Morphologic comparison of MMCs is mostly confined to broad generalizations about organization of the structures in the literature. No pathological processes, other than hepatic hemosiderosis were observed. Vacuolation was common among reference fish, but PAS staining identified this as likely due to glycogen deposition in the well-fed state. Observation of differences in normal hepatic tissues of the examined species included the finding of perivascular germinal centers in gar (both Caddo and reference) that were absent in other species, and the high degree of inclusion of pancreatic tissue in catfish liver. Incidental findings included parasitic worm infection in Caddo and reference bass. Tumor was found in bowfin liver. Little evidence was found in this study for gross histopathology related to mercury in the spleen or liver of fish from Caddo Lake. Evidence was found for extensive hepatic hemosiderosis in catfish, gar, and bowfin, but not bass. This hemosiderosis may be due to oxidative processes occurring in hepatocytes that result in the deposition of oxidized iron (iron III). Mercury is known to cause tissue damage through the formation of reactive oxygen species and this may have led to the oxidation and precipitation of iron in the cell. Such extensive oxidative stress in the liver may lead to reductions in species fitness, as more cellular resources are used.
to protect from, and repair, oxidative damage. Chronic wounding could also lead to increased levels of neoplasia, such as that seen in one bowfin from this study. Evidence was found for the reactivity of macrophage centers to increasing levels of mercury. This has been found in earlier studies in our laboratory and in the literature (Hartley, et al., 1996; Mela, et al., 2007a; Schwindt, et al., 2008). This increase in macrophage center mass may be due to increased cell turnover secondary to mercury exposure or may result from a more direct response to mercury itself. Macrophages are cells of the primary immune system that clear cellular debris in necrosis, so increases in macrophage center mass may be secondary to increased necrosis in hepatic parenchymal cells and the need to clear cellular debris and reduce inflammatory response, which can produce further damage to tissues. Alternatively, the increase in MMC mass may be due to direct effects of mercury on macrophages. These effects may increase intercellular calcium, promotion of cellular division, and hypertrophy of the structures.

Macrophage centers were found to vary greatly in presentation between species of fish, but were not found to show large differences between reference fish and Caddo fish. Variation in histologic presentation of macrophage centers has been noted by species (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b). Few studies have been carried out comparing histologic presentation of macrophage centers across fish species, and within a species by site of collection (Agius, 1980; Agius & Roberts, 2003b).
Few publications address the specific liver or spleen histologic findings of mercury exposure at defined tissue concentrations. The population health-based level of 200 ng/g (Dillon, et al., 2010) derived as a no-effect estimate is based on percent hatching success, spawning success, and severe developmental anomaly mixed endpoints. There is, of course, no reason to expect liver or spleen lesions, visible by light microscopy at this tissue concentration. Experimental evidence in the neotropical fish Hoplias malabaricus from a 70-day feeding methylmercury study showed histologic effects at liver tissue concentrations of 1,069 ng/g of exposed fish but not in the controls which had mercury concentrations of 601 ng/g (both in wet weight). The exposed group showed inflammatory infiltrate, necrotic lesions, and increased melanomacrophage centers by light microscopy. Further ultrastructural analysis confirmed necrosis, cytoskeletal disarray, and severe damage in Disse’s space (Mela, et al., 2007a). Histopathology in the form of hepatic necrosis, interstitial inflammation, reduced glycogen storage, and bile duct hyperplasia was likewise seen in spotted seatrout from south Florida in comparison with Indian River Lagoon control fish. Liver concentrations of mercury in south Florida fish were 630 ng/g, while those of controls were 320 ng/g (both in wet weight) (Adams et al., 2010). Comparison of dry weight values with these wet weight concentrations in liver may be carried out using a factor of five conversion. Histopathology was seen at an estimated equivalent liver concentration of 5345 ng/g dry weight in H. malabaricus and 3150 ng/g dry weight in spotted seatrout using this conversion. Other feeding studies found in the literature were the result of exposures to inorganic mercury which may or may not mimic high trophic level exposures of fish in the field.
Average liver concentrations in the Caddo fish studied here were lower than those found in the literature to be associated with hepatopathologic lesions, but were higher than those expected to be associated with effects on reproduction and development. However, high concentrations in individual gar reached concentrations in excess of 37,000 ng/g (data not shown). Liver concentrations exceeded the 3150 ng/g level in two bass, one catfish, one bowfin, and four of the gar. These mercury concentrations reflect total mercury and do not take into account possible effects of mercury speciation or the possible attenuating effects of secondary factors, such as selenium levels. This lack of histopathology may be a result of species-specific differences in sensitivity to mercury in specific tissues, which could include mechanisms involving mercury metabolism with binding and activation of mercury species. Evidence was found for increasing macrophage center number with increasing mercury concentration (as was reported in Melina, Randi et al., 2007) and increased hepatohemosiderin deposits in all fish species but bass. A

Continued characterization of macrophage center cytoplasmic inclusions and morphology by species would be of particular aid in future attempts to develop biomarkers. Morphologic differences across species make subtle differences in macrophage cytoplasmic content, which may be indicative of species-specific processes. Characterization of species and exposure-related differences in morphology may increase melanomacrophage centers as a biomarker of pathologic processes.

This study is the first to show morphologic differences in melanomacrophage centers between species from wild-caught fish with reference to farm-raised fish. Large species-specific differences were found that could not be explained by exposures in the wild. This is the first...
study is the first to suggest the development of hepatic hemosiderosis in fish with high proportions of inorganic mercury, suggesting an oxidative process in the liver resulting in demethylation. Finally, the current study shows that liver melanomacrophage centers increase in area with increasing hepatic mercury concentrations, and is the first to report this across fish species.
CHAPTER 3
THE ROLE OF MACROPHAGE CENTERS AN MERCURY METABOLISM OF FISH: EVIDENCE FROM A LIVER AND SPLEEN

3.1 Introduction

Mercury is a widespread contaminant that impacts human populations and ecosystems. It is found in both inorganic and organic compounds, is environmentally persistent, and biomagnifies along the food chain (NRC, 2000). In fish, mercury is thought to accumulate in kidney, muscle, and liver resulting in signs of necrosis with inflammatory infiltrate (Mela, et al., 2007a), apoptosis with upregulation of apoptotic signaling pathway genes, and lesions in Disse's space of the liver (Klaper, et al., 2008). Methylmercury exposure in fish has been shown to impair coordination and swimming activity (Crump & Trudeau, 2009) which can reduce species fitness by reducing successful hunting, evasion of predation, and mating behaviors. Environmentally relevant concentrations of mercury have been shown to impair reproduction in fish and are thought to act through induction of apoptosis in steroidogenic cells and/or through disruption of the hypothalamic-pituitary-gonadal axis (Crump & Trudeau, 2009; Drevnick, et al., 2008; Drevnick & Sandheinrich, 2003; Drevnick, et al., 2006; Vieira, et al., 2009).

Mercury demethylating enzymes similar to those found in bacteria have not been found in vertebrates. Demethylation of mercury in animals is thought to occur through a free radical mechanism (Gailer, et al., 2000). Methylmercury has been found to be demethylated by reactive oxygen species (specifically superoxide) in liver Kupffer cells, lymph nodes, and in macrophage-rich parts of the spleen (Havarinasab, et al., 2007). The respiratory burst is an important
immune function of phagocytic cells that produce superoxide to destroy foreign cells. Ethyl and methylmercury have been shown to be degraded to inorganic mercury in human, rat, and rabbit polymorphonuclear leukocytes and ethylmercury as degraded in guinea pig macrophages and human monocytes (Suda, et al., 1992). Blockade of the reticuloendothelial system in the rat has been shown to inhibit biotransformation of methylmercury to inorganic mercury (Suda & Takahashi, 1990). This leads to the possibility that a large proportion of mercury demethylation in tissues may be due to the action of macrophages and other phagocytes.

Melanomacrophages, fixed tissue macrophages of amphibians and fish, have been used as biomarkers of general pathology (Hartley, et al., 1996; Volke, 1992) but are also thought to occur in certain tissues as non-pathologic germinal centers of fixed macrophages (Agius, 1980; Agius & Roberts, 2003a). In Caddo Lake, a freshwater wetland in Texas, increasing concentrations of hepatic mercury have been observed to correlate with increasing levels of melanomacrophages and increased tissue pathology in the form of darkening of gross liver. Mercury has also been found to accumulate in macrophage centers which may indicate an active role for these structures in demethylation of methylmercury (B. Barst, et al., 2011). Melanomacrophage centers (MMCs) in the macrophage of fish) appear to follow an evolutionary pattern in distribution and degree of organization with a trend from random distribution and low levels of organization in Condrichthyes and Agantha, to highly organized structures in the liver of the primitive bony fish, and finally to well-organized structures in the spleens and kidney of the Teleosts (Agius, 1980).

Caddo Lake is a freshwater reservoir that spans the Louisiana and Texas border, and as a reported to have among the highest levels of mercury in fish in the state of Texas (TCEQ, 2004).
The Texas side of Caddo Lake is primarily shallow, forested wetland, while the Louisiana side is open water habitat. Mercury levels in fish are significantly higher on the wetland side of the lake than the open water side of the lake (Chumchal MM, et al., 2008). Top predators in this ecosystem include spotted gar and largemouth bass (M. M. Chumchal, et al., 2011).

In earlier studies (B. Barst, et al., 2011) (Chapter Two) a relationship was found between a macrophage center area and mercury concentration in largemouth bass, channel catfish, spotted gar, and bowfin from Caddo Lake. This study examines (1) the distribution of a macrophage centers in tissue in relation to literature tissue tropisms based on phylogeny, (2) explore the localization of mercury in liver tissue by LA-ICP-MS, and (3) examine relationships between MMCs and mercury metabolism.

3.2 Materials and Methods

3.2.1 Fish Tissue Collection

Fish were collected in accordance with Texas Parks and Wildlife Scientific Research Permit No. SPR 0311092 and the University of North Texas Institutional Animal Care and Use Committee Protocol #11005. Fish were collected from the wetland habitat at the western edge of Caddo Lake, near the city of Uncertain, Texas. The specific fish collection methods used were electro-shocking from a boat, gill netting, and line fishing. The target species for this research were largemouth bass (Micropterus salmoides), channel catfish (Ictalurus punctatus), a spotted gar (Lepisosteus oculatus), and bowfin (Amia rostrata). Collected fish were kept alive in aerated water until sacrifice.

Fish were sacrificed by placing them in a ricane solution (MS222) until dead. For those fish that were used for histologic analysis (n = 5 per species), biopsies of spleen and liver tissue
were immediately placed in 20 ml histology vials pre-filled with 10% buffered formalin (Leica 3800598). Tissue samples were placed in labeled quart Ziploc® plastic bags (SC Johnson) and placed on ice for later mercury analysis. Batches of scintillation vial stored samples were removed from storage and freeze dried for 72 hours in a lyophilizer (Labconco FreeZone 6 Liter Console System 7753027) at -56°C and a pressure of 3.5 pascals (3.45 x 10^-5 atmospheres). Dried samples were ground in the scintillation vial using glass stir rods. Stir rods were washed three times between each sample in deionized water and wiped clean before moving to the next sample. Ground samples were stored at -80°C until mercury content analysis was carried out.

3.2.2 Preparation of Histologic Slides

Tissue samples were grossed and placed in properly labeled cassettes. Tissue was processed overnight in an Excelsior Advanced Tissue processor (Thermo Shandon A78410100). The reagents used in processing the tissue were 10% neutral buffered formalin (Leica 3800598), reagent alcohol (Leica 3803686), Sub-X, an aliphatic hydrocarbon xylene substitute (Leica A 3803670), and blue ribbon paraffin (Leica A 801360). All reagents addition was made at ambient temperature, except for paraffin, which was held at 61°C. The agitation function was held at the intermediate setting.

Slides stained for light microscopy image analysis were sectioned on a microtome to 4 µm, mounted on glass slides (Leica Surgepath glass slides 3800375), and stained with H&E using an Shandon Varistain Gemini Slide Stainer (Thermo Shandon A78000014) and an Leica CV 5030 automated glass coversliper (Thermo Shandon). Slides for ICP-MS imaging were cut at 20 µm thickness and placed on charged microscope slides (Fischer Brand Probe on Plus 22-230-5030)
Slides for LA-ICP-MS were stained with Prussian blue without counterstain but were left uncoverslipped and were placed in plastic slide containers until laser ablation to protect the surfaces of the slides.

3.2.3 Image Analysis

Photography and Image Analysis: Slide imaging was carried out using a Motic Instruments Microscope Camera (Moticam 2000 SP10-0382) on an Olympus Bx40 microscope. Image analysis of MMCS was performed on TIF images created through the Motic 2000 software on H&E stained slides. Processing of images for MMC count and percent area of MMC in relation to the total captured field was carried out using Image J (NIH, http://rsbweb.nih.gov/ij/), a free Java based image processing and analysis software. Digital TIF images were converted to binary images in Image J and the size and number of MMCS were estimated through the Analyze Particles function in the software. Size of captured field was adjusted based on the image, circularity was kept in the all-inclusive range from 0-10, outlines were shown for comparison with H&E stain, and results were displayed and summarized.

3.2.4 Laser Ablation ICP-MS Analysis

Relative mercury concentrations were analyzed in macrophage centers from microscope slides produced from three randomly chosen individuals per species using laser ablation inductively coupled plasma mass spectrometry. Microscope slides stained with Prussian blue without counterstain to visualize macrophage centers. Slides were placed into the chamber of a 213 nm Nd:YAG (neodymium-doped yttrium aluminum garnet) laser source (New Wave Research, Fremont, CA) and laser ablated material was introduced into a Varian ICP-MS coupled to the laser source to monitor samples. Attempts were also made to...
measure relative concentrations of selenium (78Se isotope), but interferences made noise levels too high to produce adequate signal for analysis. A CCD camera allowed visualization of macrophage centers on the slide and direction of the laser to areas of interest. A CCD camera allowed visualization of macrophage centers on the slide and direction of the laser to areas of interest. A

Areas of MC and adjacent liver parenchyma were compared for mercury content and A

the total number of counts of MC and liver tissue was computed for each spot by subtracting A

the average noise from each estimate of the number of counts while the laser fired. A

The estimated number of counts was reported by the ICP software every 0.44 seconds. During the first 10 seconds the laser was warming up and the estimates of counts per second during this A

period were detector noise. After this period the laser was firing and the average counts per second A

of the noise phase of each sampling period was subtracted from the each data point during the A

firing of the laser. Negative net count values were treated as zero, and the counts per second A

during the firing of the laser were summed for each sampling event as the relative measure of A

mercury content for the ablated tissue (Figure 3.1). A

A
3.2.5 Total Mercury Analysis in Liver by ICP-MS

Mercury concentrations were determined by acid digestion and liquid introduction to an Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Digestions were carried out with a modification and adaptation of the method used by Ashoka (Ashoka et al., 2009), in which a domestic microwave oven was used in closed vessel digestion with nitric acid and hydrogen peroxide. All samples and standards were made up, digested, and analyzed in an molded clarified homopolymer polypropylene screw cap digestion vessels (Environmental Express SC475), which allowed sample preparation and analysis in one vessel and minimized contamination or loss through vessel transfer. Lyophilized and ground samples were weighed and placed in screw cap digestion vessels and to each tube 1 ml 70% nitric acid (Fischer Scientific, Trace Metal Grade) and 500 μl 50% hydrogen peroxide (Fischer Scientific, certified, stabilized) were added. A domestic style 700 watt microwave (Maytag Magic Chef, Model # MCM770W) was used in all digestions. Digestions were carried out at 50% power for AA
minute followed by a 5-minute rest period; this microwave and rest cycle was carried out three times on each sample. After digestion and cooling, an internal standard and gold solution were added to the digestate. The volume of the samples was brought up to 50 ml with MiliQ water. All standards and dilutions were made up in 18 MΩ MiliQ water. An multi-element standard containing bismuth, holmium, andium, lithium, and yttrium was used to determine proper dilution of the digestate (Ricca Chemical CLISS-1). By monitoring the Terbium content during analysis, the final concentration of the internal standard was 50 ppb in each solution. Gold standard was also placed in all solutions to reduce carryover of mercury (Ricca Chemical MSAU1KN-100). Gold was also added to the wash solution (2% [v/v] trace metal grade 70% nitric acid) and in both the wash solution and analytical solutions. A concentration of gold was kept at 100 ppb. Single element mercury (MSHG1KN100) and selenium (MSSE1KN100) standards were obtained to produce an external standard curve. To all solutions, 100 ppb gold standard and 50 ppb internal standard were added after digestion and cooling and before dilution of the digestate. All analyses were conducted using a Varian 820 ICP-MS with SPS3 Sample Preparation System autosampler. Isotopic mass data for Mercury 202 were collected. Five estimates of each measurement were made; percent relative standard deviations and mean counts per second at each measured mass were reported by Varian Software. Development of standard curves, calculation of solution and tissue concentrations, and variability in internal standards were performed on exported data using Microsoft 2007 Excel software. A
3.3 Results

3.3.1 Image Analysis

Table 3.1A

*Number and Total Size of Macrophage Centers in Liver and Spleen (Mean ± SD)*

<table>
<thead>
<tr>
<th>Species</th>
<th>Count</th>
<th>Area Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bass</td>
<td>1.60 ± 1.34</td>
<td>0.37 ± 0.43</td>
</tr>
<tr>
<td>Catfish</td>
<td>0.8 ± 0.67</td>
<td>0.27 ± 0.21</td>
</tr>
<tr>
<td>Bowfin</td>
<td>123.4 ± 35.25</td>
<td>6.0 ± 2.26</td>
</tr>
<tr>
<td>Gar</td>
<td>106.2 ± 8.27</td>
<td>9.14 ± 7.50</td>
</tr>
<tr>
<td><strong>Spleen</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bass</td>
<td>14.7 ± 6.35</td>
<td>5.38 ± 6.21</td>
</tr>
<tr>
<td>Catfish</td>
<td>7.5 ± 6.35</td>
<td>2.43 ± 1.83</td>
</tr>
<tr>
<td>Bowfin</td>
<td>2.4 ± 1.29</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td>Gar</td>
<td>2.1 ± 1.48</td>
<td>0.23 ± 0.10</td>
</tr>
</tbody>
</table>

The highest liver counts of macrophage center numbers were found in gar and bowfin (Kruskal-Wallis, Dunn’s post hoc test, p < 0.05), while those of bass and catfish were very low, and in some fields none were counted (Table 3.1, Figure 3.2). In spleen, bass and catfish macrophage numbers were higher than gar and bowfin, but the difference was less pronounced than that observed in the liver and failed to achieve significance (Kruskal-Wallis, Dunn’s post hoc test) (Table 3.1, Figure 3.2).
Average number of macrophage centers in livers and spleens of Caddo fish (n=5 for each group) (Mean ± SD).

When macrophage center areas are compared, the morphologic differences noted during H&E analysis of tissues become apparent. Catfish and bass macrophage centers were found to be less numerous but significantly larger than those in gar and bowfin. This difference is reflected in the fact that spleen macrophage center areas show a greater difference in comparison with bowfin and gar spleen than was apparent when comparing numbers of macrophages in his issue (Table 2.1, Figure 3.3). Bowfin liver macrophage center number was found to be significantly higher than those in the livers of catfish or bass (Kruskal-Wallis, p=0.0015, Dunn’s post hoc test), but no other differences were found to be significant. An Ahe spleen Ahe number of macrophage centers was found to be Ahe.

Figure 3.2. Average number of macrophage centers in livers and spleens of A. Caddo fish (n=5 for each group) (Mean ± SD).
significantly higher in bass spleen than gar or bowfin (Kruskal-Wallis, $p=0.0032$, Dunn’s post hoc test). No other differences were found to be significant in the spleen.

Gar and bowfin liver $MC$ total relative size is greater than bass and catfish, and that of bass and catfish are greater than bowfin and gar in spleen. Gar liver macrophage centers area was found to significantly higher than bass or catfish liver areas (Kruskal-Wallis $p=0.00240$, Dunn’s post hoc test), no significance was found for any other differences in area. In the spleen, bass and catfish macrophage center areas were found to be significantly larger than bowfin (Kruskal-Wallis, $p=0.0014$, Dunn’s post hoc test), but no other difference achieved significance.

Figure 3.3. Percentage areas of macrophage centers in liver and spleen of Caddo fish (n=5 for all groups) (Mean ± SD)
3.3.2 Mercury Localization by ICP-MS

3.3.2.1 Preliminary Testing

During histologic evaluation, it was found that only gar and bowfin have significant melanin in macrophage centers, but that all macrophage centers contain iron deposits. Earlier data showed that H&E staining did not affect mercury determination (data not shown). However, centers of bass and catfish are very difficult to visualize with H&E stain under the CCD microscope of the ICP, so a similar study was used to determine the effect of Prussian blue staining on the determination of mercury in tissue. Two slides of bowfin were made, one Prussian blue stained and one H&E stained, and laser ablation was performed on both slides at 9 pairs of macrophage centers and adjacent hepatic cells. No statistical significance was found for differences between Prussian blue stained (ratio of macrophage center to hepatocyte counts 1.3±1.3) and H&E slides (1.23±0.87, p=0.896, t-test, Figure 3.4). However, signals were relatively low and visualization in Prussian blue stained slides with counterstain was difficult; modifications were made to slide preparation in Caddo fish sample preparation. Difficulty in producing raster patterns of the same uniform size in non-adjacent pairs also lead to the use of spots in the determination of mercury content in Caddo fish.
3.3.2 Caddo Fish Liver Sample Analysis

In order to increase Hg signal, thicker slides were prepared for LA‐ICP‐MS than those that were used in the preliminary testing (20 μm) and a series of spots rather than raster patterns was used. As was mentioned in the preliminary testing section, the problem of comparisons across macrophage–hepatocyte pairs was reduced by changing from raster patterns, which are hard to make uniform to dot patterns of a specific size. An 80 μm spot diameter was determined to produce adequate signal for sampling of MC and hepatocyte areas while still being within the range of sizes of macrophage centers.

Due to the thickness of the slides, the staining time for Prussian blue was reduced to about half the time recommended in the kit (Sigma Aldrich Iron Stain Kit AlT20) and the Pararosaniline counterstain was not used. It was found during the preliminary testing that...
Pararosaniline counterstain was difficult to differentiate from Russian blue on the CCD camera and, since the goal of this section of the research was to investigate relative ratios of mercury in macrophage centers and surrounding tissues, recognition of these macrophage centers from other tissues was of paramount importance. The other tissues around the liver were found to stain less intensely than macrophage centers and so relevant structures could be easily visualized.

Pairs of macrophage and adjacent hepatocyte pairs were measured for each individual and the same three individuals that had been selected for determination of mercury speciation were used for this analysis (Bass 1, 4, 5; Catfish 1, 2, 3; and Gar 2, 3, 5; Bowfin 1, 4, 5). Seven pairs of macrophage centers and hepatocyte areas were selected from each species (bass, gar, and bowfin) except for catfish, for which even pairs were sampled for fish 1, six for fish 2, and four for fish 3. Catfish have few, small macrophage centers in liver and in some cases less than seven were found for sampling. For each pair of macrophage–hepatocyte areas a ratio for relative mercury concentration was calculated. The estimates of each of these fish per species are thus averages of seven measurements of macrophage counts, mercury counts, and ratios of macrophage to hepatocyte mercury.

Mercury concentration were found to be significantly higher in bass (one-tailed t-test, p=0.0185) (Figure 3.5), catfish (one-tailed t-test, p=0.0002) (Figure 3.6) and bowfin (one-tailed t-test, p=0.0081) (Figure 3.7), but not in gar (one-tailed t-test, p=0.175) (Figure 3.8). Catfish pancreatic tissue was also sampled for mercury, but levels in this tissue were not high enough to distinguish from noise using the same conditions of slide preparation and laser settings as those used to determine macrophage and hepatocyte mercury.

A
Figure 3.5. Comparison of bass macrophage center and adjacent hepatocyte total mercury (Mean±SD).
Figure 3.6. Comparison of catfish macrophage center and adjacent hepatocyte total mercury (Mean±SD).

Figure 3.7. Comparison of bowfin macrophage center and adjacent hepatocyte total mercury (Mean±SD).
Figure 3.8. Comparison of gar macrophage center and adjacent hepatocyte total mercury (Mean ± SD).

Neither macrophage center relative mercury counts (Figure 3.9, ANOVA, p = 0.157) nor hepatocyte mercury counts were found to be significantly different across groups, with the exception of comparisons of hepatocyte counts in gar and catfish (Figure 3.10, ANOVA, p = 0.046).
Figure 3.9. Mercury signal in macrophage centers of fish from Caddo Lake (Mean ± SD).

Figure 3.10. Mercury signal in hepatocytes of fish from Caddo Lake (Mean ± SD).
However, when proportions of macrophage center to hepatocyte mercury were compared, catfish were found to be significantly higher than any other group studied (ANOVA, p < 0.0001, followed by Tukey's test) (Figure 3.11).

![Figure 3.11. Comparison of macrophage to hepatocyte total mercury in Caddo fish livers (Mean ± SD)](image)

Mercury accounts from hepatocyte sampling by LA-ICP-MS were found to be highly correlated with liver total mercury by ICP-MS liquid digestion (Pearson correlation $r^2 = 0.91$, p < 0.001).

3.3.3 Liver Total Mercury

Total mercury concentrations in liver were determined by liquid introduction to ICP-MS. Mercury concentrations were highest in gar (11262 ± 14508), followed by bass (3515 ± 2997), bowfin (1887 ± 1293) and catfish (1884 ± 1181) having similar average concentrations (figure 3.12). When total liver mercury was compared to the ratio of mercury signal in macrophages and hepatocytes, a suggestion of decreasing ratio with increasing total mercury was found (Figure 3.13).
Figure 3.12. Total liver mercury estimated by liquid introduction ACP-MS (Mean ± SD).

Figure 3.13. Total mercury relationship to localization ratio (catfish, bass, gar, n=3/group).
3.4 Discussion

In this study, macrophage centers follow expected literature distributions across species (Agius, 1980) with high abundance in the livers of preteleostean gar and bowfin and much lower abundances in the livers of the teleostean catfish and bass. The abundance in spleens likewise was predicted in the literature, with high abundance in the spleens of teleostean fish and lower levels in the spleens of preteleosts. Tissue tropisms of macrophage centers were thus found to be consistent with the literature by phylogenetic group. The distribution is consistent with the pattern of organic to inorganic mercury suggested in earlier studies in Caddo Lake for spotted gar and largemouth bass (M. M. Chumchal, et al., 2011) under the hypothesis that macrophage centers are the primary metabolizers of methylmercury in fish tissues. Establishment of the prevalence of melanomacrophage centers in tissue is not only important to confirm literature observations, and to suggest the tissues in which one expects to find mercury metabolism in a fish species, but also important to an individual fish since MMCs can be a primary germinal centers or may increase in number in pathologic conditions (Agius, 1980; Agius & Roberts, 2003a). One might expect mercury metabolism in preteleostean livers, for example, under conditions that favor melanomacrophage proliferation in that tissue.

LA-ICP-MS has been used to localize elements in animal tissues (Becker & Rubakhin, 2010; Becker, Zoriy, Dobrowolska, & Matusch, 2007; Becker, Zoriy, Dressler, & Wu, 2008) and has been applied especially to brain research. Application of this technology to tissues derived from environmental sources has also been carried out, although to a lesser extent than anache medical sciences. AA-ICP-MS has been used to analyze elements from animal tissues of shellfish and order to monitor environmental changes over a time (Bellotto & Miekeley, 2000; Raith, Perkins, ...
In this study, LA-ICP-MS examination of macrophage centers found consistently higher levels of mercury than adjacent hepatocytes in all fish species, although this difference failed to achieve significance in the gar. In earlier studies from Caddo Lake, macrophage centers in gar were found to be higher than adjacent liver tissue (B. Barst, et al., 2011), but in the current study the general pattern is one of the ratio decreasing as total liver mercury increases. This suggests that increasing mercury is increasingly portioned into the hepatocyte compartment and that macrophage centers are slower to increase in mercury as total liver mercury increases. Catfish, with the lowest liver mercury concentrations, have the highest proportion of macrophage to hepatocyte mercury levels, while gar and bass have higher levels of mercury and lower ratios of macrophage to hepatocyte mercury. The lowest ratios were found in gar with the highest levels of total mercury. Bowfin do not fit this pattern, and are found to have relatively low ratios of macrophage mercury to hepatocyte mercury, but in these fish the concentration of mercury in macrophage centers seems to be lower than that experienced in other fish. These findings suggest that the more dynamic pool of mercury with increasing mercury concentrations may be in liver cells, and that macrophage centers may be much less responsive to changing liver mercury concentrations. Few animal studies have sought to localize mercury in relation to subcellular structures in environmentally derived liver tissue (Anan, Kunito, Sakai, & Tanabe, 2002; Bastrup & Danscher, 1987; Akemoto, et al., 2004; Rawson, A
localization between cell types from environmental liver samples (Augier, Benkoël, Brisse, Chamlian, & Park, 1993; MNigro, 2009; Wagemann, Trebacz, Boila, & Lockhart, 2000). Only one study found in the literature derived relative abundances between cell types within the liver of fish (B. D. Barst, et al., 2011). To our knowledge, this is the first study to relate relative mercury concentrations of macrophages and hepatocytes across species, so further research will be required to confirm this finding.

Some species of fish livers contain numerous rests of pancreatic cells leading to the term hepatopancreas to describe the combined liver/pancreatic organ (Di Giulio & Hinton, 2008). In the current study this extensive presentation of pancreatic tissue was found in the liver of the catfish. Mercury concentrations were found to be beneath detection for pancreatic tissues in catfish despite high levels of mercury in macrophage centers that are almost universally embedded in pancreatic tissue in this species. This shows the specificity of mercury accumulation into macrophage centers. Due to the lack of mercury concentration in pancreatic tissues in catfish, this organ is not likely to participate in the metabolism of mercury in a fish liver.

Many metals have been shown to accumulate in melanomacrophage centers, including mercury and selenium (B. D. Barst, et al., 2011; Christensen, Morgensen, & Rungby, 1988; MNigro, 2009), cadmium (Grasseschi, Ramaswamy, Levine, Klaassen, & Wesselius, 2003), and Aron (C. Agius, 1979; Wolke, 1992). An this study, mercury was found to accumulate in a greater extent than in other cells of the liver. Accumulation of inorganic mercury occurs in the livers of animals and can become the predominant form of mercury in a liver with chronic exposure (Yasutake & Hiroshima, 2001). Subcellular localization of A
mercury in rat liver slices exposed to methylmercury by injection show compartmentalization in hepatocyte mitochondria, which can become uncoupled with mercury exposure and produce superoxide anion (Dare, Zhivotovsky, Yuan, Ceccatelli, 2001; Konigsberg, Lopez; Diazguerrero, Bucio, Gutierrez Ruiz, 2001; Yasutake, Hirayama, 2001). Superoxide anion has been shown to be an important mediator of demethylation of methyl mercury (Yasutake, Hirayama, 2001). Some evidence was found for greater mercury localization in hepatocytes with increasing liver total mercury, which may suggest a greater role for hepatocytes in mercury metabolism than that of macrophage centers.

This study suggests an accumulation of mercury in macrophage centers and shows that these structures have tropisms for tissues suggested in the literature based on phylogeny. The current study is the first to suggest two pools of mercury accumulation in the liver, with hepatocytes being the more dynamic pool for mercury than melanomacrophages.
CHAPTER 4

MERCURY SPECIATION AND SELENIUM TO MERCURY RATIO OF TELEOSTEAN AND PRETELEOSTEAN FISH FROM CADDOLA/AAKEA

4.1 Introduction

Mercury, a widespread contaminant that affects the health of human populations and ecosystems, is found in both inorganic and organic compounds, is environmentally persistent, and biomagnifies along food chains (NRC, 2000). In fish, mercury is thought to accumulate in kidney, muscle, and liver resulting in signs of necrosis with inflammatory infiltrate (Mela, et al., 2007a), apoptosis with upregulation of apoptotic signaling pathway genes, and lesions in Disse’s space of the liver (Klaper, et al., 2008). Methylmercury exposure in fish has been shown to impair coordination and swimming activity (Crump & Trudeau, 2009) which can reduce species fitness by reducing successful hunting, evasion of predation, and mating behaviors. Environmentally relevant concentrations of mercury have been shown to impair reproduction in fish and are thought to act through induction of apoptosis in steroidogenic cells and/or through disruption of the hypothalamic-pituitary-gonadal axis (Crump & Trudeau, 2009; Drevnick, et al., 2008; Drevnick & Sandheinrich, 2003; Drevnick, et al., 2006; Vieira, et al., 2009).

Caddo Lake, a freshwater reservoir that spans the Louisiana and Texas border, and as a reported to have among the highest levels of mercury in fish in the state of Texas (TCEQ, 2004). The Texas side of Caddo Lake is primarily shallow forested wetland while the Louisiana side is more open water habitat. The mercury levels in fish are significantly higher on the wetland side.
Recent evidence from Caddo Lake has shown marked differences in liver mercury speciation in largemouth bass and spotted gar (M.M. Chumchal, et al., 2011). Mercury in livers from spotted gar in Caddo Lake have very low proportions of methylmercury, while in bass almost all of the mercury in the liver is in the methyl form. This difference in predominant form of mercury may be due to more extensive demethylation in the liver of gar.

Mercury demethylation is known to occur in the tissues of animals and has been hypothesized to occur through at least two mechanisms. One proposed mechanism is thought to involve the generation of reactive oxygen species and associated with tissues containing large numbers of phagocytic leukocytes that are capable of producing oxidative burst (Havarinasab, et al., 2007). Melanomacrophages, a form of fixed tissue macrophage, as found to be differentially expressed in different tissues of fish in a phylogenetically consistent pattern (Agius, 1980). Another proposed pathway involves ratios of selenium and mercury that have been linked to mercury demethylation in birds and mammals, and selenium and selenoproteins have been suggested to be directly related to the mechanism of demethylation but this mechanism has not been fully described (Eagles-Smith, et al., 2009; A.A Yang, et al., 2007; Yoneda & Suzuki, 1997).

Melanomacrophage centers (MMCs) in the melanomacrophages of fish) appear to follow an evolutionary pattern in distribution and degree of organization with a trend from random distribution and low levels of organization in Condrichthyes and Agantha, to highly organized structures in the liver of the primitive bony fish, and finally to well-organized structures in the
spleens and kidney of the Teleosts (CAgius, 1980). The presence or absence of ACh enters, A capable of carrying out oxidative burst activity, may explain the differences in accumulation of inorganic mercury. The presence or absence of such centers, capable of carrying out oxidative burst activity, may explain the differences in accumulation of inorganic mercury in the spotted gar and largemouth bass from Caddo Lake. Selenium has long been associated with protection from mercury toxicity and evidence for an association of increased inorganic mercury concentrations and selenium to mercury ratios has been suggested in mammals and birds (Eagles-Smith, et al., 2009; A. Yang, et al., 2007; A. Yoneda & Suzuki, 1997). This association has been hypothesized to be related to the mechanism of demethylation and evidence of a threshold for mercury demethylation based on total mercury concentrations and selenium to mercury ratios have been derived. Selenium to mercury ratios equal to or greater than one have been associated with a lack of demethylation activity while ratios of less than one have been associated with demethylation in liver of birds and marine mammals (Eagles-Smith, et al., 2009; A. Yang, et al., 2007). Thresholds of total mercury concentration for demethylation of methylmercury have been proposed to be 8.5 µg/g in birds (Eagles-Smith, et al., 2009), 20–30 µg/g in Dall porpoises (A. Yang, et al., 2007), and 100 µg/g in stripped dolphins (Palmisano, et al., 1995).

This study analyzes mercury species and total mercury in the liver and spleens of preteleostean and teleostean fish from Caddo Lake and investigates selenium to mercury ratios and concentrations of mercury in livers of Caddo Lake fish and spleens of teleostean and preteleostean fish. The expected finding of high proportions of inorganic mercury in livers of preteleostean and teleostean fish and a predominance of organic mercury in spleens of preteleostean and teleostean fish would be considered evidence for a primary role of...
melanomacrophage centers in the demethylation of methylmercury in fish tissue. Selenium to mercury ratios are also explored as an alternative mechanism for demethylation.

4.2 Materials and Methods

4.2.1 Fish Collection and Processing

Fish were collected in accordance with Texas Parks and Wildlife Scientific Research Permit No. SPR-0311-092 and the University of North Texas Institutional Animal Care and Use Committee Protocol #11005. Fish were collected from the wetland habitat in the western edge of Caddo Lake, near the city of Uncertain, Texas. The specific fish collection methods used were electro-shocking from a boat, gill netting, and line fishing. The target species for this research were largemouth bass (Micropterus salmoides), channel catfish (Ictalurus punctatus), spotted gar (Lepisosteus oculatus), and bowfin (Amia rostrata). Collected fish were kept alive in aerated water until sacrifice.

Fish were sacrificed by placing them in MS222 solution until dead. Liver and spleen were placed in labeled quart Ziploc® plastic bags (SC Johnson) and placed an ice for later analysis. Batches of scintillation vial stored samples were removed from storage and freeze-dried for 24 hours using a lyophilizer (Labconco FreeZone 6-Liter Console System 753027) at 56°C and a pressure of 3.5 pascals (3.45 x 10^5 atmospheres). Dried samples were ground in the scintillation vial using glass stir rods. Stir rods were washed three times between each sample in deionized water and wiped clean before moving to the next sample. Ground samples were stored at -80°C until mercury content analysis was carried out.
4.2.2 Determination of Tissue Mercury

4.2.2.1 Mercury Speciation by CVAFS

Samples for mercury speciation were weighed and digested in 7ml of 4.57N nitric acid in a 60°C water bath for 12 hours. Digestates were separated into two subsamples: one for detection of monomethyl mercury and one for determination of total mercury. Speciation digestates were analyzed for monomethyl mercury with a mercury speciation analyzer (MSA) by a derivation with sodium tetraethylborate (NaTEB) and detection with flow-injection gas chromatographic atomic fluorescence spectrometry. The mercury species analyzer produces ethylmethyl mercury and diethyl mercury by NaTEB derivatization of the aqueous sample. The MSA then strips the derivatized methyl mercury from the aqueous solution through gas-liquid separation with a nitrogen carrier gas, preconcentrates the sample on Tenax cartridges and thermally releases ethylated mercury species into an argon carrier gas. The concentrated sample is isothermally separated on a 30x3.2 mm column packed with Chromosorb WAW-DMSC coated with 15% OV-3, and finally pyrolytically decomposes the ethylated mercury species and detects the resulting mercury vapor with cold vapor atomic fluorescence spectrometry (Tseng, Hammerschmidt, & Fitzgerald, 2004). The total mercury portion of the digestate was treated with BrCl for 12 hours to oxidize mercury, followed by addition of stannous chloride as a reductant at least one hour prior to mercury determination by dual Au amalgamation CVAFS (Hammerschmidt & Fitzgerald, 2006).

Due to the cost of mercury speciation analysis, three of the individuals for each species were chosen randomly for mercury speciation analysis.
4.2.2.2 Total Mercury and Selenium by ICP-MS

Mercury and selenium concentrations were determined in liver tissue by acid digestion and liquid introduction to an Inductively Coupled Plasma Mass Spectrometer (ICP-MS). Liver subsamples of the three fish used for estimation of mercury speciation were analyzed for total mercury and selenium along with other samples in this portion of the research. Digestions were carried out with modification and adaptation of the method used by Ashoka (Ashoka, et al., 2009), in which a domestic microwave oven was used in closed vessel digestion with nitric acid and hydrogen peroxide. All samples and standards were made, digested, and analyzed in molded clarified homopolymer polypropylene screw cap digestion vessels (Environmental Express SC475), which allowed sample preparation and analysis in one vessel and minimized contamination or loss through vessel transfer. Lyophilized and ground samples were weighed and placed in screw cap digestion vessels and to each tube 1 ml 70% nitric acid (Fischer Scientific, Trace Metal Grade) and 500 \( \mu \)l 50% hydrogen peroxide (Fischer Scientific, certified, stabilized) were added. A domestic style 700 watt microwave (Maytag Magic Chef, Model number MCM770W) oven was used in all digestions. Digestions were carried out at 50% power for 1 minute followed by a 5 minute rest period; this microwave and rest cycle was carried out three times on each sample. After digestion and cooling, internal standard and gold solution were added to digestate and the volume of the samples was brought up to 50 ml with MiliQ water. All standards and dilutions were made up in 18 M\( \Omega \) MiliQ water.

4.2.2.3 Quality Control Samples for Speciation Method

In the mercury speciation method certified reference materials (DORM-3, TORT-2, DOLT-3 from National Research Council Canada) were run for confirmation of recovery, relative accuracy, and precision.
percent differences between duplicates were calculated as estimates of analytical precision, and relative standard deviations of replicate triplicate readings of the same digestate were computed for estimation of procedural precision. Calibration curves were included with each batch run, and correlation coefficients were greater than 0.9995. Methylmercury percent recovery in DORM-3 and TORT-2 were 94.1 and 102%, respectively. Total mercury recovery was 101% and for TORT-2 was 106%.

4.2.2.4 Quality Control Samples for Total Mercury/Selenium Method

In the total mercury/total selenium method, a multi-element standard was used to determine proper dilution of digestate (Ricca Chemical CLISS-1) by monitoring the Terbium content during analysis. The final concentration of internal standard was 50 ppb in each solution. Gold standard was also placed in all solutions to reduce carryover of mercury (Ricca Chemical MSAU1KN-100). Gold was also added to ICP wash solution (2% (v/v) trace metal grade 70% nitric acid) and in both wash solution and analytical solutions the concentration of gold was kept at 100 ppb. Single element mercury (MSHG1KN100) and selenium (MSSE1KN100) standards were obtained to produce an external standard curve. To all solutions 100 ppb gold standard and 50 ppb internal standard were added after digestion and cooling and before a dilution of digestate.

All analyses were conducted using a Varian 820 ICP-MS with SPS3 Sample Preparation System autosampler. Isotopic mass data for Mercury 202 were collected. Five estimates of each measurement were made; percent relative standard deviations and mean counts per second at each measured mass were reported by Varian software. Development of standard curves,
calculation of solution and tissue concentrations, and variability. An internal standards were performed on exported data using Microsoft Excel software.

Certified reference materials (DOLT-3 and DORM-3) were used to verify the accuracy of the method. These samples were run in duplicate. Pre-digestion and post-digestion spiked samples were used to test recovery and possible analyte loss during the digestion process. All spike and duplicate recoveries were between 86 and 124%.

4.3 Results

4.3.1 Mercury Speciation in Liver and Spleen Tissues

Liver raw data show high levels of variability between individuals and across species. Terms of total mercury, methylmercury, and the percent of liver mercury burden as methylmercury. Total liver mercury was highest in gar, while methylmercury was found to be highest in bass. Bass were also found to have high percentages of total liver mercury as methylmercury. Spleen concentrations of mercury species were also variable, but were general, much higher than liver tissue for any species except bass (Table 4.1).

Table 4.1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Methylmercury (ng/g dw)</th>
<th>Total Mercury (ng/g dw)</th>
<th>Percentage of Total Mercury as Methylmercury</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bass</td>
<td>1943 (1049) A</td>
<td>3650 (2688) A</td>
<td>59 (13) A</td>
</tr>
<tr>
<td>Catfish</td>
<td>499 (113) A</td>
<td>2927 (1182) A</td>
<td>18 (5) A</td>
</tr>
<tr>
<td>Gar</td>
<td>1108 (533) A</td>
<td>23090 (17832) A</td>
<td>8 (6) A</td>
</tr>
<tr>
<td>Bowfin</td>
<td>632 (408) A</td>
<td>3260 (2026) A</td>
<td>19 (6) A</td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bass</td>
<td>731 (731) A</td>
<td>1190 (1197) A</td>
<td>64 (8) A</td>
</tr>
<tr>
<td>Catfish</td>
<td>204 (62) A</td>
<td>279 (93) A</td>
<td>75 (20) A</td>
</tr>
</tbody>
</table>

Caddo Tissue Total Mercury and Methylmercury Content (Mean ± SD, n=3 all groups)
Total mercury concentrations in fish livers were found to be similar in all species except the gar, which was at least six times higher than the other species tested. Despite the large difference in gar total liver mercury in comparison with other species, differences failed to achieve statistical significance due to small sample size and one gar that had about two times less liver mercury than the other individuals analyzed (One-way ANOVA, \( p = 0.067 \)). The high level of liver mercury in gar is consistent with the literature regarding bass in Caddo Lake (Chumchal et al., 2008) (Figure 4.1).

**Figure 4.1.** Liver total mercury (Mean ± SD, n = 3 for all groups)

Methylmercury values were similarly variable, with being the highest concentrations in bass at 1943 (±1049) ng/g dw, followed by gar with 1108 (±532.6), bowfin with 532.0 (±408.2),
and catfish with 99.3 (± 12.9) ng/g dw. Differences in methylmercury content failed to achieve statistical significance (One-way ANOVA, p = 0.0799) (Figure 4.2).

The percentage of liver mercury burden as methylmercury was found to be different among species, and was found to be significantly higher in the bass over other tested fish species (ANOVA followed by Tukey’s Test, p = 0.0003). No other differences were found to be significant between species (Figure 4.3).
Figure 4.3. Liver percent of total mercury as methylmercury (Mean ± SD, n=3 for all groups).

Total spleen mercury was quite variable across individuals and was found to be highest in bass (1190 ± 1197), followed by gar (1017 ± 647.3), bowfin (489.7 ± 465.0) and catfish (278.2 ± 92.9) ng/g dw (Figure 4.4). No significant differences were found between species for total mercury in spleen.
Estimates of methylmercury concentrations in spleen tissue showed the same variability and pattern as those for total mercury with bass and gar showing the highest concentrations: 730.7 (±730.8) and 23.3 (±451.4) ng/g dw, respectively. Bowfin 81.7 (±879.3) and gar 203.7 (±6.27) ng/g dw showed lower concentrations (Figure 4.5). No significant differences were found for methylmercury concentrations in spleen across species.
The proportions of total mercury in spleen that were found to be methylmercury were less variable than absolute mercury species concentrations, both among individuals and across groups. All fish species tested had methylmercury as the predominant form of mercury in the spleen (Figure 4.6). No significant differences were found across species for the percentage of total mercury as methylmercury in the spleen.
Figure 4.6. Spleen percent of total mercury as methylmercury (Mean±SD, n=3 for all groups)

Determination of percentage recoveries were very close to CRM published values and lend confidence to the accuracy of mercury speciation analysis. Total liver mercury was highest in the gar and lowest in the bowfin. Liver inorganic mercury proportions were highest in the gar, but were closely followed by the catfish and bowfin, and were lowest in the bass (significantly lower than all other groups). Spleen total and methyl mercury concentrations mirrored each other, with concentrations highest in the bass and gar, and lowest in the bowfin and catfish. No significant differences were found for proportions of methylmercury among groups in spleen.

Methylmercury was found to be the majority of total mercury in the spleens of all groups of Caddo fish. Total mercury was found to be higher in the liver of all groups of fish than in spleen.

4.3.2 Total Mercury and Selenium Analysis in Liver

Gar showed higher levels of total liver mercury than the catfish, but all other comparisons of a group failed to be associated with a significant difference (ANOVA, p=0.036, followed by A

97A
Tukey’s Test (Figure 4.7). Bowfin were found to have significantly higher levels of selenium than all other groups of fish (Figure 4.8, ANOVA p=0.001, followed by Tukey’s Test).
The ratio of mercury liver concentrations to selenium concentrations were found to be significantly lower for bowfin than all other fish (ANOVA, p=0.0006, followed by Tukey's Test) (Figure 4.9).

![Figure 4.9. Ratio of selenium to mercury (Mean ± SD, n)](image-url)
No obvious pattern of association was found between total mercury and selenium concentrations in fish livers across species (Pearson correlation, $p = 0.35$) (Figure 4.10), nor within species except in the gar. In the gar, an increasing mercury was found to be associated with an increased selenium (Pearson correlation, $p < 0.0001, r = 0.9878$) (figure 4.11). The lack of an apparent association of mercury and selenium concentrations in fish other than gar may be due to the low variation in mercury levels in other fish. Gar have individuals with liver mercury concentrations similar to those of other Caddo fish groups, but also have individuals with mercury levels that are more than seven times higher.
Figure 4.11. Association of Gar Liver Selenium and Total Mercury.

Ratios of selenium to mercury of 1 or lower, that ratio often associated with demethylation activity in the literature, are only achieved in two gar and one bass in this study. Other fish range in selenium to mercury ratio from 1.45 in a bass to a high of 70.23 in a bowfin and an general selenium concentration is higher in liver tissue from Caddo fish than is mercury (Table 4.2).
Table A4.2A

Selenium to Mercury Ratios by Fish Species

<table>
<thead>
<tr>
<th>Species</th>
<th>Number of fish</th>
<th>Selenium to Mercury Ratio (Unitless)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Largemouth Bass A</td>
<td>10A</td>
<td>4.3A</td>
<td>3.3A</td>
</tr>
<tr>
<td>Channel Catfish A</td>
<td>9A</td>
<td>5.5A</td>
<td>4.0A</td>
</tr>
<tr>
<td>Bowfin A</td>
<td>7A</td>
<td>27.7A</td>
<td>10.0A</td>
</tr>
<tr>
<td>Spotted Gar A</td>
<td>9A</td>
<td>2.5A</td>
<td>0.7A</td>
</tr>
</tbody>
</table>

4.4 Discussion

Mercury speciation (inorganic and organic mercury proportions) in the livers of fish has been shown to vary both by species and within species in both marine and freshwater. Studies from the North Sea show percentages of methylmercury as low as 3% to as high as 100% in fish liver. Dogfish from the North Sea were shown to vary in methylmercury content from 30-80% (n=10) and hornback rays (n=10) were likewise shown to vary from 36-100% methylmercury content. Blackmouth dogfish (Galeus melastomus) were found to vary in the Adriatic Sea near Italy were found to vary in percentages of methylmercury from 25-100%, while values from the Adriatic Sea near Albania was found to be 67%. In the same study blackmouth dogfish were found to range in liver methylmercury content from 12.6-52.1% in the Ionian Sea and from 18.8-63.0% in the Aegean Sea (Storelli & Marcotrigiano, 2002). Freshwater fish have also been shown to show variability in percentages of methylmercury in the livers of fish. In Caddo Lake, fish liver methylmercury percentages have been found to range from 23-80% (n=8) (D. Y. Yang, Chen, & A. Belzile, 2010). In Caddo Lake, fish liver methylmercury percentages have been shown to range from 56% in spotted gar, 56% in red-eared sunfish, and 74% in largemouth bass.
Few studies in the literature detail the speciation of mercury in fish spleen. Despite the variability between individuals in absolute concentrations of mercury species in tissues, the variability of the proportionality of total mercury as methylmercury (and thus relative mercury speciation) is much lower and is consistent with a pattern of high proportions of methylmercury in the livers of bass over catfish, gar, and bowfin. Caddo gar are shown to be high in total mercury, as was shown in earlier work (M. Chumchal, Rainwater, Cobb, & Smith, 2008; M. M. Chumchal, et al., 2011), but other fish, with lower levels of total mercury show a similar proportionality of organic to inorganic mercury in the liver. This suggests that mercury content, itself, is not the prime driver for the difference in mercury species proportionality. Spleen data seem to show that the mechanism responsible for increased proportions of inorganic mercury in the livers of catfish, gar, and bowfin are not at work in this tissue, and no evidence for species specific differences in mercury form is evident from analysis of this tissue.

Melanomacrophages, fixed tissue macrophages of fish, are expected to follow a specific tissue tropisms based on phylogenetic relationships. These centers of macrophage aggregates occur predominantly in spleen and kidney of teleostean fish, and in the livers of preteleostean fish (Agius, 1980). They are found in abundance in the spleen of teleostean fish, such as bass and catfish, and in the livers of preteleostean fish, such as gar and bowfin. The findings from this study are not consistent with the pattern expected in mercury speciation under the hypothesis that the primary driver of demethylation of methylmercury is melanomacrophage centers. One would expect to find a predominance of inorganic mercury in the livers of the
preteleostean gar and bowfin, while anadromous teleostean catfish and bass, an organomercury antagonist. The current findings reiterate those of Chumchal et al. (2011) in that gar are found to have very high proportions of inorganic mercury in liver, while bass have high proportions of organomercury. The high proportion of inorganic mercury in livers of catfish is unexpected.

One primary determinant of mercury concentration in fish is stable nitrogen isotopic ratios, with higher trophic levels correlating with higher $^{15}N$ (Cabana & Rasmussen, 1994), which itself correlates with mercury content in fish tissues (Boudou & Ribeyre, 1997; K. A. Kidd, et al., 1995). Some studies have also found that the nature of the base of the food chain from which organisms are feeding affects mercury concentration. This can be estimated through the measuring of carbon isotopic ratios ($^{13}C$ and $^{12}C$). High $^{12}C$ correlates with feeding from a food chain based in phytoplankton, while high $^{13}C$ indicates a carbon source in periphyton (France, 1995; Hecky & Hesslein, 1995). Phytoplankton-based food webs have associated with higher mercury concentrations when compared with same trophic level species that feed on periphyton-based communities (Gorski, et al., 2003; K. A. Kidd, et al., 2003; A Power, et al., 2002). A work at Caddo Lake has failed to show stable carbon isotopic ratios as significant determinants of mercury concentrations in fish tissues (Chumchal MM & Hambright KD, 2009). In addition to the concentration of total mercury in fish tissue with trophic level, the proportion of total mercury in animal tissue that is represented by methylmercury is thought to increase with increasing trophic level (Bowles, Apte, Maher, Kawei, & Smith, 2001; M. M. A. Chumchal, et al., 2011).
Catfish from Caddo Lake have been estimated to occupy a lower trophic level (3.3 ± 0.4) than largemouth bass (4.1 ± 0.1) or spotted gar (4.5 ± 0.1) based on stable nitrogen isotopic ratios (Chumchal MM & Hambright KD, 2009). While the predominance of inorganic mercury in the liver of the catfish may be explained by feeding behavior and trophic level, the findings from a spleen are more difficult to explain.

This study finds no difference between proportions of methylmercury in the spleens of the species tested. Under the hypothesis that macrophage centers are responsible for demethylation of methylmercury, one would expect to find high proportions of inorganic mercury in the spleens of the teleostean bass and catfish and low proportions in the preteleostean gar and bass. In the current study all fish tested had a predominance of methylmercury in spleen tissue. The overall concentration of mercury was found to be lower in the spleens than liver of all species tested, and it is possible that this low total mercury content is below some threshold for active demethylation in the tissue. Thresholds for active demethylation have been hypothesized to occur in the livers of seabirds and marine mammals and the mechanism, and hence the threshold for demethylation of methylmercury may be a similar in liver and spleen tissue (Eagles-Smith et al., 2009; J. Yang et al., 2007).

It is widely accepted that selenium has a protective effect against mercury toxicity, but the underlying mechanisms for this protective effect are not known. Some studies have found selenium exposure to increase mercury elimination but others to have no effect. Recently, high selenium diets have been found to decrease assimilation efficiency in fish orally exposed to inorganic mercury, but not organic mercury (Dang & Wang, 2011). An additional administration of methylmercury and selenium has been found to decrease biliary mercury
Increased levels of mercury have also been associated with increased selenium in mammalian and bird tissues, and selenium:mercury ratios of 1:1 have been specifically linked to increased demethylation (Urano et al., 1997). Ratios of mercury and selenium with selenium in molar excess have been associated with those individuals below the threshold for demethylation, while 1:1 or even mercury excess have been associated with demethylation (Eagles-Smith et al., 2009; J. Yang et al., 2007; Yoneda & Suzuki, 1997).

However, it has been suggested that this 1:1 ratio is more appropriately associated with selenium: inorganic mercury than total mercury. Detoxification is thought to culminate in the formation of complexes of inorganic mercury bound with selenoproteins or selenium salts in crystals (Khan & Wang, 2009; J. Yang et al., 2007). Selenoproteins include the important antioxidant protein glutathione peroxidase which is upregulated in oxidative stress (Reeves & Hoffmann, 2009). A

High concentrations of selenium have been shown to be toxic in fish, and recent research suggests that excess selenium induces oxidative stress in trout hepatocytes (Misra & Niyogi, 2009). A tissue-based criterion for selenium in aquatic life of 4.0 μg/g has been suggested based on conservative extrapolation of health effects in fish from whole-body laboratory and field data (Hamilton, 2002). The proposed issue-based criterion as lower than most of the liver concentrations in Caddo fish, however, this health-based estimate refers to whole-body burden and liver and kidneys are routinely found to have high concentrations relative to other tissues. Liver concentrations in marine fish have been found to range from 2.6 to 150 μg/g (30 μg/g fresh weight) to 4150 μg/g (40 μg/g dry weight) in heavily contaminated waters (30 μg/g fresh weight).
In carp the proportion of liver to whole body selenium was found to be 3.6 times higher, so an estimate of liver concentrations associated with a whole body burden of 4 μg/g would be about 14.4 μg/g (Eisler, 1985). Selenium levels in bass and catfish are below the concentrations estimated to be associated with health effects, but gar and bowfin may be at risk from selenium-related toxicity.

Selenium concentrations were found to be highest in the livers of gar and bowfin, and these were estimated to be at a level that could be associated with selenium toxicity. Selenium to mercury ratios of 1 or less were found only in one bass and the two highest levels of mercury. Molar excess of selenium does not appear to be associated with the predominance of inorganic mercury in the livers of Caddo Lake fish, as has been suggested in other fish and marine mammals. No evidence for a selenium-driven mechanism for demethylation of methylmercury was found in this study.

Concentrations of total mercury above the proposed 8.51 μg/g for the threshold of demethylation in birds (Yang et al., 2007) were only achieved in two bass and two gar, threshold concentration associated with demethylation in Dall’s porpoises (20–30 μg/g) (Yang et al., 2007) were only achieved in the two highest gar, and no fish in the present study achieved the liver total mercury concentration of 100 μg/g proposed as the demethylation threshold in striped dolphins (Palmisano et al., 1995).
fish from Caddo Lake are not as high as those found in other species of birds and mammals. Nonetheless, high proportions of inorganic mercury in the livers of all fish except largemouth bass were found. It is unlikely that the mechanism responsible for the predominance of inorganic mercury in the livers of gar, catfish, and bowfin is driven by the same kinds of thresholds suggested in earlier studies in other species.

In general, the pattern of mercury speciation in the livers and spleens of fish from Caddo Lake does not show the high proportions of methylmercury in the livers and low proportions of methylmercury in the spleens of channel catfish and largemouth bass expected if melanomacrophages are primary demethylators in these tissues. Currently, no evidence for a pattern of demethylation consistent with a primary role for melanomacrophages in the tissues of fish was found. This study shows, in gar, the fish species with the broadest range of mercury concentration, a correlation between mercury concentration and selenium concentration. This is also the fish with the highest proportion of inorganic mercury. An increasing selenium concentration with increasing mercury may be directly related to the mechanism of methylmercury demethylation, or it may be secondary to the binding and inactivation of inorganic mercury. No evidence for a correlation of mercury speciation with selenium was found in the current study.

The current study fails to show a pattern of mercury speciation in the livers and spleens suggestive of a primary role for melanomacrophages in the demethylation of mercury. This study is the first to show a departure in fish from the selenium to mercury ratio that has been associated with mercury demethylation in birds and mammals. This difference in selenium to mercury ratio was found in the current study.
mercury ratio in fish with high proportions of hepatic inorganic mercury may suggest differences in mercury demethylation mechanisms or mechanisms of mercury retention in fish from those active in birds and mammals.
CHAPTER 5

GENERAL DISCUSSION

The four fish species from Caddo Lake represent teleostean and preteleostean groups and were chosen to test the hypothesis that inorganic mercury content in the tissues of fish is directly related to the presence and number of macrophage centers in those tissues. Teleostean bass and catfish were found to have lower numbers of macrophage centers in their livers than the preteleostean bowfin and gar. Spleens of teleostean fish were found to have more macrophage centers than those of preteleostean fish. These findings were expected, were the basis of their choice for hypothesis testing, and are in agreement with the literature (C Agius, 1980).

The morphology of macrophage centers in spleen and liver were found to vary based on fish species. These differences are reflected in estimates of center number and relative surface area of tissue sections. Bowfin and gar tended to have many small centers while bass and catfish tended to have smaller numbers of larger macrophage centers.

Mercury speciation data showed that livers of catfish, gar, and bowfin were not significantly different from one another in the percentage of total mercury as inorganic mercury, but bass were much lower in inorganic mercury fraction than the other species. Two gar with the highest levels of liver total mercury were found to have higher proportions of inorganic fraction than other gar. Gar and bowfin were expected to have a predominance of inorganic mercury under the macrophage metabolism hypothesis, but the high inorganic mercury fraction in catfish was not predicted and is at variance with this hypothesis. It could be...
argued that this predominance of inorganic mercury is the result of dietary input of inorganic mercury rather than methyl mercury (prehepatic hypothesis). In Caddo Lake, channel catfish have been shown to occupy a lower trophic position than largemouth bass or spotted gar by stable nitrogen isotope but all feed from a littoral foodweb (Chumchal MM & Hambright KD, 2009). As was discussed in Chapters 1 and 4, the percentage of methyl mercury in issue is thought to increase with increasing trophic level. The possibility remains that catfish oral exposures may be partially responsible for their high proportion of inorganic to organic mercury.

All spleen issues showed a predominance of organic mercury fraction despite differences in macrophage content. Under the macrophage metabolism hypothesis, inorganic mercury fractions were expected to be higher in the spleens of bass and catfish than in gar and bowfin. These findings fail to support the macrophage metabolism hypothesis. However, since spleen total mercury concentrations (744+727) are significantly lower than those experienced in the liver (8232+37800) on an ng/g basis (Welch-corrected unpaired t-test, p=0.0257, n=12, speciation estimates), it is possible that there is a threshold for demethylation as suggested in some of the literature, that concentrations never reach a level consistent with significant demethylation activity. The current data do not support hypotheses I or II:

Hypothesis I: Methyl mercury demethylation is much more active in the liver of preteleostean fish than teleostean fish.

The current data do not support hypotheses I or II.
Hypothesis: Methylmercury demethylation is much more active in the spleen of teleostean fish than in preteleostean fish. In Caddo Lake, channel catfish have been shown to occupy a lower trophic position than largemouth bass or spotted gar by stable nitrogen isotopic ratio (Hambricht KD, 2009). The possibility remains that at fish oral exposures may be partially responsible for their high proportion of inorganic to organic mercury. All spleen tissues showed a predominance of organic mercury fraction despite differences in macrophage content. Under the macrophage metabolism hypothesis inorganic mercury fractions were expected to be higher in the spleens of bass and catfish than in gar and bowfin. These findings fail to support the macrophage metabolism hypothesis. However, since spleen total mercury concentrations (744±727) are significantly lower than those experienced in the liver (8232±3780) on a ng/g basis (Welch-corrected unpaired t-test, p=0.0257, n=12, A speciation estimates), it is possible that there is a threshold for demethylation as suggested in some of the literature, that concentrations never reach a level consistent with significant demethylation activity. The relative area of macrophage centers was around a increase with increasing mercury in all species examined. The slope of increase in macrophage center area per unit mercury was greater for gar and bowfin than for catfish and bass. The higher response in the preteleostean fish over teleostean fish may reflect differences in physiology. For preteleostean fish, that have primary fixed macrophages in their livers, response to mercury may produce hypertrophy in centers already present. In the teleostean bass and bowfin, the lower response may be A
reflective of the recruitment of secondary or reactive macrophage centers as mercury levels increase. A

There was a suggestion that total mercury was found to be higher in gar liver than that of other fish species, but this difference only achieved statistical significance when compared to the concentrations in catfish liver. Higher levels of gar liver mercury were due primarily to two fish that were found to have concentrations of mercury much higher than the other gar tested.

The current data do not support hypothesis III: the organic:inorganic mercury ratio is higher in tissue and fish with high abundance of tissue macrophages.
Localization and comparison of relative mercury and selenium content was performed on macrophage centers and surrounding liver parenchyma. All species, with the exception of gar, showed significantly higher concentrations of mercury in macrophage centers than in surrounding tissue. The ratio of macrophage to hepatocyte mercury seems to decrease with increasing total liver mercury, suggesting that the hepatocyte mercury may be more important in the metabolism of mercury than macrophages. These findings support hypothesis IV, but tend to support a hepatic mechanism for methylmercury demethylation, rather than a macrophage mechanism:

Hypothesis IV: Tissue mercury levels are higher in areas of MMCs and deposits than in surrounding parenchyma.

A Histologic examination was carried out to determine possible signs of pathology in fish in relation to the predominant species of mercury that fish were known to have associated with increased liver pathology. Liver parenchymal tissue was found to vary greatly based on species. Catfish were found to have significant inclusions of pancreatic tissue in their livers (hepatopancreas), while pancreatic tissue was rare in the livers of other fish species examined. Gar and Caddo fish were found to have germinal centers in perivascular cuffs while these were not apparent in other fish species.

A Macrophage centers were found to vary not only in size and number, but were also different in pigment content. Gar and bowfin macrophages can truly be called melanomacrophages and are characterized by having a very high melanin content, with gar melanin content higher than bowfin. Catfish and bass had very low levels of melanin in their livers.  

A
All macrophage centers were found to contain iron, and this was the predominant pigment along with lipofuscin in bass and catfish macrophages. No evidence of histopathology was observed in Caddo fish over reference fish with H&E staining. As was discussed in Chapter Two, this is not surprising since average liver concentrations in the Caddo fish were than those found in the literature that were associated with histopathologic lesions. Fish populations have also been found to develop tolerance to mercury exposure in contaminated environments (Klerks & Weis, 1987; A.S. Weis & Weis, 1984, 1989; P. Weis, 1984). Incidental findings included a benign tumor in one bowfin liver, consistent with biliary adenoma, and parasitic worm infection in Caddo and reference bass. Vacuolation of reference fish livers was noted, but later found to be a likely result of increased glycogen storage by PAS stain. Iron deposition (hemosiderin) was found in Caddo, but not in reference fish hepatocytes. The severity of iron deposition by group appeared to be associated with those groups having high proportions of inorganic mercury. The data findings on hepatic hemosiderosis tend to support Hypothesis V:

Hypothesis V: An active demethylation of methylmercury in the liver will be found.

Overall, the data analyzed support a lack of primary role for macrophages in the demethylation of methylmercury in the liver. Little evidence was found for thresholds for demethylation similar to those found in aquatic mammals or birds in the ratio of selenium to mercury. Suggestive evidence supports a possible role of hepatocytes in the demethylation of methylmercury in the form of increasing proportions of total mercury in...
hepatocytes with increasing total liver mercury and hemosiderosis in hepatocytes that may be linked to oxidative stress in those cells.

Continued research into the underlying cause of differences in the proportionality of mercury in livers of different fish species continues to be warranted. The possibility that demethylation of mercury in the liver reduces mercury burden in edible portions and other tissues of fish is important for human health risk assessment and through presumptive protection of gonads and CNS to ecological risk assessment. These studies found evidence of a reduction in liver to muscle tissue total mercury proportions with increasing liver proportions of inorganic mercury (Figure 5.1). The ratio of muscle to liver total mercury was found to decrease significantly as the percentage of liver inorganic mercury increased in gar, catfish, and bass (Pearson correlation $r^2 = 0.876, p = 0.0002$). In bowfin this trend was not found (Pearson correlation for all fish $r^2 = 0.076, p = 0.387$) and may point to species-specific differences in mercury absorption. The finding of strong correlation between liver mercury speciation ratios and muscle to liver total mercury ratios in some species may indicate sequestration of orally absorbed mercury in liver tissue over time.
The age of the fish used for this study should be determined. Otoliths and gular plates were collected from the fish collected for histologic examination and these are stored at -80°C for future work. While the decreasing proportion of mercury in muscle tissue with increasing inorganic mercury deposition as inorganic mercury complexed to selenium in liver tissue, the possibility remains that this may actually be the cause of high inorganic mercury concentrations in some fish. The correlation between selenium in gar livers and mercury may support this mechanism. Despite the lack of evidence for macrophage involvement in demethylation of methylmercury in the tissues so far examined, the hypothesis remains attractive for scientific consistency with reactive oxygen species mechanisms for demethylation and consistency with a...
Comparison of speciation values from CVAFS with the DMA method developed by Ben Barst, and recently submitted for publication, should be made on the samples collected for this study. A number of individuals could be greatly extended. The current speciation is based on only three individuals per species due to the cost of CVAFS methodology, but verification of those samples and extension to all liver samples taken from Caddo Lake would increase the sample number to 10 catfish, 10 bass, 9 gar, and 7 bowfin.

Continued research should focus on speciation of mercury and selenium in situ. This has been done using x-ray absorbance near edge structure (XANES) in the tissues of whales and fish (George et al., 2011; Kuwabara, Arai, Topping, Pickering, & George, 2007). The technique requires the use of a synchrotron light source to produce soft, tunable x-rays (Cooke Andrews, 2006; C. Kim, 2005), but these light sources are free to use with petition since they were paid for by US tax dollars. The localization and speciation of mercury in liver frozen sections would allow much greater identification of structures associated with different mercury species and may suggest a mechanism of mercury demethylation. This technique would allow the identification of inorganic mercury, mercury metal, mercurial cysteine, ethylmercury, and other forms, as well as allowing scanning and image production in whole tissue slices. The production of microscope slides and even drying or freeze drying of tissues for species analysis has some chance of altering the form of mercury in tissues. Certainly the form of mercury extracted from tissues in mercury speciation methods is an approximation of what is found naturally in the
living tissue due to the chemical process of extraction, and the possibility that this may affect mercury speciation. The XANES method is also non-destructive and allows other methods to be used after this method has been applied. Light microscopic images of tissues could be made after XANES imaging, for example.
REFERENCES


122A


spectrometry (LA ICP-MS) analysis of selenium in otoliths. Environmental science & technology, 41(10), 3679-3683.


