HEPATOTOXICITY OF MERCURY TO FISH

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Tissue samples from spotted gar (*Lepisosteus oculatus*) and largemouth bass (*Micropterus salmoides*) were collected from Caddo Lake. Gar and bass livers were subjected to histological investigation and color analysis. Liver color (as abs at 400 nm) was significantly correlated with total mercury in the liver ($r^2 = 0.57$, $p = 0.02$) and muscle ($r^2 = 0.58$, $p = 0.01$) of gar. Evidence of liver damage as lipofuscin and discoloration was found in both species but only correlated with liver mercury concentration in spotted gar. Inorganic mercury was the predominant form in gar livers. In order to determine the role of mercury speciation in fish liver damage, a laboratory feeding study was employed. Zebrafish (*Danio rerio*) were fed either a control (0.12 ± 0.002 µg Hg g⁻¹ dry wt), inorganic mercury (5.03 ± 0.309 µg Hg g⁻¹ dry wt), or methylmercury (4.11 ± 0.146 µg Hg g⁻¹ dry wt) diet. After 78 days of feeding, total mercury was highest in the carcass of zebrafish fed methylmercury (12.49 ± 0.369 µg Hg g⁻¹ dry wt), intermediate in those fed inorganic mercury (1.09 ± 0.117 µg Hg g⁻¹ dry wt), and lowest in fish fed the control diet (0.48 ± 0.038 µg Hg g⁻¹ dry wt). Total mercury was highest in the viscera of methylmercury fed zebrafish (11.6 ± 1.86 µg Hg g⁻¹ dry wt), intermediate in those fed inorganic diets (4.3 ± 1.08 µg Hg g⁻¹ dry wt), and lowest in the control fish (below limit of detection). Total mercury was negatively associated with fish length and weight in methylmercury fed fish. Condition factor was not associated with total mercury and might not be the best measure of fitness for these fish. No liver pathologies were observed in zebrafish from any treatment.
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CHAPTER 1
LITERATURE REVIEW

General Background

Mercury is a transition metal found naturally in the Earth’s crust, and is a toxic pollutant. Mercury is found in the environment in several forms, including elemental [Hg(0)], inorganic [Hg(II)], and organic (methylmercury). Mercury is primarily released due to human activity, however significant natural sources exist. Natural mercury deposits largely occur as chlorides, sulfides, or oxides, which when heated allow the release of Hg(0) into the atmosphere as vapor (Lin and Pehkonen 1999). Natural sources include volcanoes and forest fires, while anthropogenic sources are related to burning coal and the incineration of waste (Lin and Pehkonen 1999).

Almost 95% of the mercury in the atmosphere is Hg(0), which can be oxidized to Hg(II) by common atmospheric constituents such as ozone, HClO, HSO₃⁻, or OH⁻ (Morel et al. 1998; Lin and Pehkonen 1999). Hg(0) remains in the atmosphere for 1-2 years, while Hg(II) has a much shorter residence time (Lin and Pehkonen 1999). The long residence time, and slow transition of atmospheric Hg(0) to Hg(II) makes its release a global problem as even remote wilderness areas are subject to mercury contamination (Drevnick et al. 2007). Mercury is removed from the atmosphere through both dry and wet deposition. Once in surface waters, Hg(II) is readily reduced to Hg(0) and returned to the atmosphere. The primary mechanism by which this occurs is via photoreduction, thus maintaining a constant flux of mercury between surface waters and the atmosphere (Lin and Pehkonen 1999). Inorganic mercury can be sequestered in the sediments of lakes where sulfate-reducing bacteria create organic mercury species such as methylmercury (MeHg) (Morel et al. 1998) (Figure 1). The rate of methylation
is critical in aquatic environments because it determines the amount of mercury, which will be most bioavailable for aquatic organisms. A variety of parameters such as temperature, pH, dissolved oxygen (DO), and the amount of dissolved organic carbon (DOC) can affect the level of methylation in water bodies (Gilmour and Henry 1991; Ullrich et al. 2001).

![Figure 1](image1.png)

Figure 1. Biogeochemical cycling of Hg in the environment (adapted from Fitzgerald et al. 2007).

In particular, low pH, high DOC, low DO waters show increased levels of methylation. Increasing the input of sulfate to an aquatic system can also increase the rate of methylation by providing additional substrate for sulfate-reducing bacteria (Gilmour et al. 1992). Since these parameters vary among aquatic systems, mercury bioaccumulation can be habitat specific (Chumchal et al. 2008). Although both Hg(II) and MeHg accumulate in aquatic biota, MeHg is of particular importance in aquatic systems because of its greater propensity to bioaccumulate and biomagnify (Devlin 2006). For this reason, approximately 90% of the mercury found in fish
is MeHg (Bloom 1992). Larger, older fish feeding at higher trophic positions often have the highest mercury loads (Chumchal et al. 2008).

MeHg avidly binds to sulfur containing compounds in the cell, such as protein thiols. Since sulfur-containing compounds exist in a variety of locations within the body, there are many prospective sites of interaction (Mela et al. 2007). MeHg has been shown to negatively affect survival, growth, behavior, and reproduction in fish (Hammerschmidt et al. 2002; Scheuhammer et al. 2007). An increase in apoptotic ovarian follicular cells in fathead minnows (Pimephales promelas) fed dietary MeHg provides a possible mechanism for decreased reproduction (Drevnick et al. 2006). Freidmann et al. (1996) noted that walleye (Stizostedion vitreum) had a decrease in both immune function and growth after being reared on MeHg-spiked diets. Evidence of hindered predator avoidance behavior in golden shiner (Notemigonus crysoleucas) fed MeHg was observed as well (Webber and Haines 2003).

Berntssen et al. (2003), noted that MeHg caused significant increases in lipid peroxidation, a type of oxidative stress, in Atlantic salmon (Salmo salar). Lipid peroxidation results from reactive oxygen species (ROS), which oxidize the hydrophobic membranes in a cell. Unsaturated fatty acids are often susceptible to attack by free radicals within a lipid membrane. The primary oxidation is self-propagating from the point of initial oxidation. This results in destruction of the lipid membrane by means of a series of radical-radical reactions. It has been suggested that increased ROS generation by MeHg occurs by the uncoupling of oxidative phosphorylation and the electron transport chain during aerobic metabolism in the mitochondria (Daré et al. 2001).

Fish liver and kidneys are key areas for pathological inspection, as these organs have shown evidence of toxicity after short-term dietary exposure. These organs can, therefore, be
used as indicators of toxicity through histopathological inspection (Mela et al. 2007). Both field and laboratory based studies have recently shown a positive correlation between mercury exposure and liver pathologies in fish organs (Mela et al. 2007; Raldúa et al. 2007; Drevnick et al. 2008). Mela et al. (2007) described increased leukocyte infiltration, increased melanomacrophage aggregations, and necrosis in the livers of *Hoplias malabaricus* fed contaminated prey fish (0.075 µg MeHg g⁻¹ wet wt) for 70 days. The kidneys of these fish contained phagocytic areas, necrotic regions, melanomacrophage aggregates, and atypical cells. Raldúa et al. (2007) showed an increased prevalence of lipofuscin, pycnotic nuclei, and macrophage aggregates in the livers of fish downstream of a chlor-alkali plant.

A recent study involving northern pike (*Esox lucius*) showed that variation in liver color, due to differences in lipofuscin, was positively correlated with total mercury concentration in the edible fillets (Figure 2) (Drevnick et al. 2008). Lipofuscin is the granular collection of fluorescent pigments composed of oxidized protein and lipids which accumulates naturally with age and as a result of oxidative stress (Terman and Brunk 2004). In this study, age as a covariate was accounted for and shown not to have a significant influence on the presence of hepatic lipofuscin in pike. Furthermore, an inverse relationship between body condition factor and total Hg in the livers of northern pike was described. Pike with the highest concentrations of Hg in the liver tended to have more lipofuscin granules, decreased lipid reserves, and appeared emaciated. The authors also noted interesting differences in Hg speciation between pike muscle and liver. When livers had total Hg concentrations below 0.5 µg g⁻¹ wet wt, the majority of Hg was MeHg. Conversely when total Hg concentrations exceeded 0.5 µg g⁻¹ wet wt, MeHg made up only 28 to 51 % of the Hg in pike liver (Drevnick et al. 2008). Demethylation of MeHg has been reported in bacteria, birds, and mammals, but has not been described in fish (Oremland et
al. 1991; Dock et al. 1994; Palmisano et al. 1995; Pak and Bartha 1998; Scheuhammer et al. 2007; Shapiro and Chan 2008; Eagles-Smith et al. 2009). Rather than attributing a low percentage of MeHg in livers to a hepatic demethylation mechanism, Drevnick et al. (2008) suggested that pike were obtaining inorganic Hg by feeding on macroinvertebrates, with high inorganic Hg content. Hepatic demethylation of MeHg was further ruled out, since MeHg concentrations in liver were comparable to MeHg in muscle, suggesting that a conversion of MeHg to inorganic Hg was not occurring.

Figure 2. There is a positive correlation between liver color (as absorbance at 400 nm) and total mercury concentration in skin-on edible fillets of northern pike from lakes at Isle Royale, Michigan. $y = 2.0067x + 0.0952$, $r^2 = 0.5107$ (Drevnick et al. 2008).

Caddo Lake (TX/LA) is located within 250 km of some of the highest Hg emitting power plants in North America and has some of the highest Hg concentrations in Texas freshwater fish (TCEQ 2004; Chumchal et al. 2008a). There are noted differences in Hg accumulation between habitat types within the lake, with fish sampled from wetland areas tending to have higher Hg
concentrations (Chumchal et al. 2008b). Caddo Lake is therefore, an interesting area to study the relationship between Hg and liver pathology in fish.

Previous research involving Hg has often been centered on risks to human health by the consumption of mercury-laden food, however relatively little exists on the effects to fish and wildlife (Wiener and Spry 1996; Drevnick and Sandheinrich 2003; Scheuhammer et al. 2007). Of the studies which focus on the effects to fish, many have used aqueous exposures with concentrations, which were not always environmentally relevant (Wiener and Spry 1996; Hammerschmidt et al. 2002; Scheuhammer et al. 2007). Since fish accumulate Hg primarily through their diet, aqueous exposures are not representative of environmental conditions. There is, therefore, a need for more relevant dietary studies (Scheuhammer et al. 2007). Liver color is used as a measure of fish health, and has been shown to be altered by environmental contaminants, such as Hg (Carls et al. 1998; Drevnick et al. 2008) In order to provide a link causative, rather than a correlative, association between Hg, liver color, and pathologies in fish, a controlled laboratory study is needed.
CHAPTER 2

RELATIONSHIP BETWEEN LIVER COLOR AND MERCURY IN FISH FROM CADDO LAKE (TX/LA)*

Introduction

Mercury (Hg) is a widespread contaminant, which is found in three forms in the environment: elemental, inorganic, and organic (Lin and Pehkonen 1999). Bacteria, in aquatic environments, convert inorganic Hg into methylmercury (MeHg), its most bioavailable and toxic form (Morel et al. 1998; Ullrich et al. 2001). MeHg bioaccumulates and biomagnifies and is the majority of Hg in fish (Bloom 1992). Hg has a high binding affinity for sulfur, which is distributed throughout cells. For this reason Hg has many molecular targets and is capable of disrupting physiological processes (Clarkson and Magos 2006).

In fish, Hg has been shown to negatively impact survival, growth, and reproduction (Hammerschmidt et al. 2002; Drevnick and Sandheinrich 2003; Drevnick et al. 2006; Scheuhammer et al. 2007; Crump and Trudeau 2009). Hg has also been shown to alter predator avoidance behavior and decrease immune function in fish (Friedmann et al. 1996; Webber and Haines 2003). Several studies have associated Hg with increased occurrence of hepatic and renal pathologies (Mela et al. 2007; Raldúa et al. 2007; Drevnick et al. 2008). Raldúa et al. (2007) noted increased lipofuscin and melanomacrophage aggregations, which varied with Hg concentration, in barbels (Barbus graellsii) and bleak (Alburnus alburnus) downstream of a chlor-alkali factory. Lipofuscin is the granular collection of fluorescent pigments composed of oxidized protein and lipids, which can accumulate due to oxidative stress and has been used as a marker for contaminant exposure in fish (Weinstein et al. 1997; Terman and Brunk 2004).

* Data from this study appear in Roberts, A. P., Barst, B.D., Gevertz, A.K., Chumchal, M.M., Rainwater, T.R., and Drevnick, P.E. (In Review). "Evidence of Mercury Induced Liver Pathologies in Fish from Caddo Lake (TX/LA, USA)." Environmental Toxicology and Chemistry.
Drevnick et al. (2008) described an increase in the presence of hepatic lipofuscin in northern pike (*Esox lucius*) from Isle Royale National Park (MI), which accounted for the majority of the variation in their liver color (as absorbance at 400 nm).

Caddo Lake straddles the border of Texas and Louisiana, and is within 250 km of several of the highest Hg emitting power plants in North America. Caddo Lake has some of the highest Hg concentrations in Texas freshwater fish (TCEQ 2004). Hg concentrations in fish and invertebrates differ between habitat types within the lake, with fish from the open water Louisiana side of the lake generally having lower concentrations than fish sampled from the wetland habitat in Texas (Chumchal et al. 2008a; Chumchal et al. 2008b; Chumchal and Hambright 2009). The high Hg concentrations in fish from this region make the lake a useful area to study effects of Hg toxicity. The goal of this research was to quantify liver color in Caddo Lake fish and then determine if a correlation between lipofuscin, Hg concentration, and liver discoloration exists in these fish as it did in northern pike from Isle Royale National Park.

**Methods**

**Study Site and Sampling**

Caddo Lake, including contiguous wetlands, occupies an area of 10,850 hectares within Texas and Louisiana. The western portion of the lake is shallow (<1 m mean depth) and dominated by wetland plant species. The eastern portion of the lake is deeper (mean depth= 1.4 m) and is predominately open water (Chumchal et al. 2008b).

Dr. Matthew Chumchal, from Texas Christian University, provided all fish samples used in this study. The species provided included 14 spotted gar (*Lepisosteus oculatus*), and 22 largemouth bass (*Micropterus salmoides*). Fish were collected with a boat-mounted electro
fishing unit from both the wetland and open-water habitats of the lake. Fish were euthanized immediately after capture and morphometric data (length and weight) was recorded and muscle and liver were collected. Liver samples for histopathology analysis were immediately preserved in neutral buffered formalin. Remaining muscle and liver samples, for Hg and color analysis, were separated into individual labeled plastic bags and maintained at -20°C. Samples for Hg analysis were dried in a 60°C oven and homogenized to a flour-like consistency using a ball-mill grinder in preparation for Hg analysis.

All 14 gar and 22 bass were tested for liver color, but only a subset of these fish livers was tested for all three of the endpoints in this study (Hg concentration, presence of lipofuscin, and liver color). This included 10 spotted gar and 16 largemouth bass.

**Hg Analysis**

Dr. Matthew Chumchal conducted total Hg analysis on livers and edible fillets using a Direct Mercury Analyzer 80 (Milestone Inc., Monroe CT), which makes use of thermal decomposition, gold amalgamation, and atomic absorption spectroscopy (Chumchal et al. 2008b; Chumchal and Hambright 2009). A subset of livers and fillets was sent for MeHg analysis using Hg-Thiourea complex Liquid Chromatography–Cold Vapor Atomic Fluorescence Spectrometry (Quicksilver LLC., Lafayette CO.).

**Histopathology**

Fish livers were assessed for the presence of lipofuscin in the Roberts laboratory at the University of North Texas using methods described in Drevnick et al. (2008) (courtesy Ms. Amanda Gevertz). To eliminate bias, livers were assigned random numbers and blind evaluated.
Formalin-fixed livers were embedded in paraffin, cut in 6 µm sections, and placed on glass slides. One section from each liver was deparaffinized, rehydrated, and stained with eosin and hematoxylin. One unstained slide from each liver was viewed with fluorescence microscopy (using an excitation filter 355-425nm with suppression at 460nm) at 20X magnification to identify lipofuscin. A Zeiss Axio compound microscope with an Axiocam High Resolution camera (Carl Zeiss Inc., Berlin, Germany), was used to photograph each liver at 200X magnification. A digital grid created in Adobe Photoshop (Adobe Systems Inc., San Jose, California) consisting of 130, 25 X 25 µm squares was superimposed on the image. A random number generator was used to select 50 of the 130 total squares. The occurrence or lack of lipofuscin was recorded in each of the 50 squares. The total number of squares containing lipofuscin, out of the 50 selected, was used to generate proportion lipofuscin values. A total of 3 digital images was created and assessed, for each of the livers. The 3 proportions were then averaged to generate 1 mean lipofuscin value for each sample.

Liver Color Analysis

Liver color was assessed according to Drevnick et al. (2008). 100 mg of liver tissue was taken from each fish (14 spotted gar and 22 largemouth bass), placed in a 1.5 ml microcentrifuge tube and homogenized in 1ml of deionized water for 1 minute using a mini bead beater (Biospec Products Inc., Bartlesville, OK). 200 µL of chloroform were added to each tube. Tubes were vortexed and centrifuged for 15 minutes at 12,000 rpm. A 200 µL aliquot of each sample was placed in a 96-well microplate and absorbance was read at 400 nm on a BioTek Synergy 2 spectrophotometer (Winooski, VT, USA). Each liver was analyzed in duplicate and absorbance readings were averaged to obtain a mean absorbance for each sample.
Statistical Analyses

Least-squares regression analyses were performed using SPSS version 17.0 software. Body condition factor was calculated using the formula: \((100,000 \times g/cm^3)\) Hg data were \((\log_{10})\) transformed to meet assumptions of normality and homogeneity of variance. Analysis of variance (ANOVA) followed by a Tukey’s post-hoc test was used to test for the effect of habitat and fish species on the occurrence of lipofuscin and fish mercury concentrations. Significance was based upon an \(\alpha = 0.05\) for all tests.

Results

Hg Content

Total mercury concentrations in largemouth bass and spotted gar muscle tissue were similar and MeHg comprised approximately 85% of the total Hg in the muscle of both species (Table 1). Interestingly, there were differences in Hg content and composition in the livers of the two species of fish. While MeHg concentrations in the fish livers were similar, total Hg concentrations and Hg speciation were very different (Table 1).

<table>
<thead>
<tr>
<th>Common name</th>
<th>Total Hg in muscle</th>
<th>MeHg in muscle</th>
<th>Total Hg in Liver</th>
<th>MeHg in Liver</th>
</tr>
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<td>Largemouth bass</td>
<td>1,718 ± 386 (8)</td>
<td>1,442 ± 469 (6)</td>
<td>997 ± 154 (5)</td>
<td>736 ± 22.5 (3)</td>
</tr>
<tr>
<td>Spotted gar</td>
<td>2,611 ± 478 (5)</td>
<td>2,224 ± 374 (4)</td>
<td>30,171 ± 12,377 (5)</td>
<td>610 ± 309 (4)</td>
</tr>
</tbody>
</table>

Table 1. Mercury content and speciation in a subset of muscle and liver tissues of spotted gar and largemouth bass from Caddo Lake, TX/LA, USA, adapted from Chumchal et al. (in review). Values are expressed as means (ng/g dry wt) ± 95% confidence interval, and n is included in parentheses.
MeHg was only 2% of the total Hg found in spotted gar livers while it was 74% of the total Hg found in largemouth bass livers. The two species occupy similar habitats, and stable isotope analyses indicate that they occupy similar vertical trophic positions (Chumchal and Hambright 2009). Liver concentrations of total Hg were higher in wetland sampled fish (both spotted gar and largemouth bass) than open-water sampled fish ($p < 0.01$) (Figure 3). Total Hg concentrations in muscle tissue were also higher in wetland sampled spotted gar ($p < 0.01$) but not in largemouth bass ($p > 0.05$) (Figure 4).

![Figure 3](image-url)

Figure 3. Mean total mercury concentrations in spotted gar ($n = 10$) and largemouth bass ($n = 16$) livers were significantly different between wetland and open water habitats of Caddo Lake ($p < 0.01$). Asterisks denote a significant difference between means. Error bars represent ± 1 standard error.
Figure 4. Mean muscle total mercury concentrations were significantly different between open water and wetland habitats in spotted gar (n = 10, p < 0.01), but not largemouth bass (n = 16, p > 0.05) from Caddo Lake. Asterisks denote a significant difference between means. Error bars represent ± 1 standard error.

Liver Pathology

Habitat-specific differences in lipofuscin were observed for spotted gar (Figure 5). Spotted gar sampled from wetland habitats had approximately five times higher occurrences of lipofuscin than those sampled from open water areas (p = 0.04). No significant habitat-specific differences were observed in largemouth bass (p = 0.1). In largemouth bass, the occurrence of lipofuscin was much less compared to spotted gar. Lipofuscin occurred at rate of 1.7% in open water habitat, 0.5% in wetland areas, and in a maximum of 5% of the histological grids in a single individual. Lipofuscin occurrence in largemouth bass was not significantly correlated with either total Hg in the muscle ($r^2 = 0.026, p = 0.55$) or total Hg in the liver ($r^2 = 0.016 p = 0.64$).

The mean occurrence of lipofuscin was higher in gar from wetland habitat (mean = 9.02 percent) than gar from open water (mean = 0.32 percent). Percent hepatic lipofuscin in spotted
gar was positively correlated with total Hg in the liver ($r^2 = 0.565, p = 0.012$) (Figure 6) and muscle ($r^2 = 0.697, p = 0.003$). This relationship did not exist in largemouth bass ($r^2 = 0.005, p = 0.795$). When data were pooled for gar (n = 14) and largemouth bass (n = 22) there was a significant positive correlation ($r^2 = 0.71, p = 0.000$) (Figure 7) between liver color and total liver Hg. When liver color was assessed on a species specific basis it became clear that this relationship was likely driven by gar ($r^2 = 0.564, p = 0.02$) (Figure 8) rather than largemouth bass ($r^2 = 0.001, p = 0.0.877$) (Figure 9). Liver coloration in spotted gar was also positively correlated with total Hg in the muscle ($r^2 = 0.58, p = 0.01$) (Figure 10). The relationship between liver color and lipofuscin was marginally significant in gar ($r^2 = 0.355, p = 0.068$) (Figure 11).

Figure 5. Occurrence of hepatic lipofuscin was significantly different between open water and wetland habitats in spotted gar (n = 10, $p = 0.04$) but not largemouth bass (n = 16, $p = 0.1$) of Caddo Lake. Asterisks denote a significant difference between means. Error bars represent ± 1 standard error.
Figure 6. There is a significant positive correlation between percent lipofuscin and total mercury concentration in the livers of spotted gar (n = 10) from Caddo Lake ($r^2 = 0.565, p = 0.012$).

Figure 7. There is a significant positive correlation between liver color (as absorbance at 400 nm) and log total mercury concentration in the livers of largemouth bass (n = 22) and spotted gar (n = 14) from Caddo Lake ($r^2 = 0.711, p = 0.000$).
Figure 8. There is a significant positive correlation between liver color (as absorbance at 400 nm) and log total mercury concentration in the livers of spotted gar (n = 14) from Caddo Lake ($r^2 = 0.565$, $p = 0.02$).

Figure 9. There is no significant correlation between liver color (as absorbance at 400 nm) and total mercury concentration in the livers of largemouth bass (n = 22) from Caddo Lake ($r^2 = 0.001$, $p = 0.877$).
Figure 10. There is a significant positive correlation between liver color (as absorbance at 400 nm) and total mercury concentration in muscle of spotted gar (n = 10) from Caddo Lake ($r^2 = 0.58, p = 0.01$).

Figure 11. There is a marginally significant positive correlation between liver color (as absorbance at 400 nm) and percent lipofuscin in the livers of spotted gar (n = 10) from Caddo Lake ($r^2 = 0.355, p = 0.068$).
Discussion

*Hg Speciation*

MeHg and total Hg were analyzed in a subset of spotted gar and largemouth bass. The majority of Hg in the muscle of both species was organic Hg which is consistent with other studies (Bloom 1992; Drevnick et al. 2008). Interestingly while MeHg comprised the majority of Hg in largemouth bass livers, inorganic mercury was the predominant form in gar livers (Table 1). Gar and bass were sampled from the same sites and have been shown to occupy similar vertical trophic positions through stable isotope analysis (Chumchal and Hambright 2009). An initial explanation for higher inorganic Hg in gar livers might point to dietary differences or differences in Hg metabolism between species. Drevnick et al. (2008) noted increased concentrations of inorganic Hg relative to MeHg, in the livers of northern pike from Isle Royale National Park. The authors hypothesized that the Hg content in the livers of these fish was a result of feeding on invertebrates high in inorganic Hg. Inspection of gut contents and stable isotope analysis revealed that gar and bass from Caddo Lake share similar diets and therefore it is not likely that the two species differ in inorganic mercury content of their livers because of dietary sources (Chumchal and Hambright 2009).

A species-specific hepatic demethylation mechanism could be the reason for differences in inorganic Hg in the livers of gar and bass from Caddo Lake. This mechanism would result in the generation of inorganic Hg from MeHg, thus reducing the amount of MeHg in the liver. A demethylation mechanism has not been well described in fish (Wiener and Spry 1996), however studies have suggested its existence (Cizdziel et al. 2003; Gonzalez et al. 2005). Cizdziel et al. (2003) suggested that high concentrations of total Hg in the livers of striped bass (*Morone saxatilis*) from Lake Mead could be due to conversion of MeHg to inorganic Hg. Gonzalez et
al. (2005) reported a decrease in the percentage of hepatic MeHg, in zebrafish, over a 63 day exposure to food containing 13.5 µg Hg g$^{-1}$ dry wt, and attributed this to demethylation.

MeHg demethylation has been speculated on in birds as well. A recent study showed that American avocets (*Recurvirostra americana*) and stilts (*Himantopus mexicanus*) had a reduced percentage of MeHg in their livers above a threshold concentration of 9.91 ± 1.29 µg g$^{-1}$ dry wt, while Caspian terns (*Hydroprogne caspia*) had a threshold concentration of 7.48 ± 1.48 µg g$^{-1}$ dry wt, suggesting that a demethylation mechanism in waterbirds is both threshold dependent and species specific (Eagles-Smith et al. 2009).

At the cellular level, MeHg and inorganic Hg are thought to be dealt with in different ways. MeHg enters the liver where it readily binds with the thiol-rich antioxidant, glutathione, and is then excreted in bile (Dutczak and Ballatori 1994; Mugesh and Singh 2000). Inorganic Hg is thought to couple with selenium forming polyatomic ions which associate with binding proteins in the liver (Palmisano et al. 1995; Eagles-Smith et al. 2009; Khan and Wang 2009). These proteins keep inorganic Hg sequestered in the liver. If a mechanism like this exists in gar then accumulated MeHg would be converted into inorganic Hg which would then be sequestered in the liver (Schomburg et al. 2004; Khan and Wang 2009; Reeves and Hoffmann 2009).

A hypothesized mechanism for demethylation raises interesting evolutionary questions about the potential costs and benefits of converting MeHg into inorganic Hg. Spotted gar is a more archaic species than largemouth bass, having diverged approximately 225 MYA (Helfman et al. 1997). If a demethylation mechanism exists in gar and not largemouth bass it could be because gar experienced some selection pressure from MeHg, which was not present during the time of bass divergence. Since MeHg is more neurotoxic than inorganic Hg, a demethylation mechanism could have been selected for to protect the fish brain. If a demethylation mechanism
explains the ratio of inorganic Hg to MeHg in gar, which in turn leads to liver damage, then largemouth bass may have lost the ability to demethylate to prevent liver damage.

Liver Pathology

Liver damage was apparent in the livers of Caddo Lake spotted gar and largemouth bass, the two top predatory fish in the ecosystem. Gar and bass were also the fish species with the highest concentrations of total Hg (Chumchal In Review). There were strong correlations between liver damage (lipofuscin and color) and Hg concentrations in the muscle and liver of spotted gar, which indicates a possible causative link. Since this was a field study, other factors cannot be ruled out as the cause of liver damage in spotted gar. Lipofuscin has been referred to as the “age pigment” because it tends to accumulate not only from oxidative stress but also as organisms grow older (Terman and Brunk 2004). Age was not accounted for in this study; however Drevnick et al. (2008) showed that age was not an important determinant for the presence of hepatic lipofuscin in northern pike from Isle Royale.

Previous research has demonstrated higher concentrations of Hg in spotted gar and largemouth bass in the wetland habitat of Caddo Lake (Chumchal et al. 2008b; Chumchal and Hambright 2009). Wetland areas are typically associated with increased Hg methylation due to the better conditions (low pH, high dissolved organic carbon, low dissolved oxygen) for sulfate-reducing bacteria (Chumchal et al. 2008b). Lipofuscin occurrence was also higher in gar from the wetland area. The relationship between lipofuscin and habitat type is further evidence linking Hg to liver pathology. Elevated levels of lipofuscin in fish from the highest trophic levels, is consistent with a biomagnifying contaminant such as Hg. It is unlikely that another contaminant, capable of biomagnification, would vary in such a way with habitat type. For
example, organochlorines biomagnify (Oliver and Niimi 1988; Kidd et al. 1995) and have been linked to liver damage in fish by several studies (Eller 1971; Miranda et al. 2008; Fisher et al. 2008). However, organochlorines are likely deposited evenly across the lake and do not rely on wetland- methylation as Hg does.

Liver color is used as a measure of fish health, and has been shown to be altered by environmental contaminants (Carls et al. 1998; Drevnick et al. 2008). Liver color and both total Hg in liver and muscle were positively correlated in spotted gar from Caddo Lake. Drevnick et al. (2008) demonstrated a significant positive correlation between liver color and occurrence of lipofuscin in northern pike. In this study, liver color and occurrence of lipofuscin were weakly related, which could be due to the small sample size of this study.

Hepatic lipofuscin was identified in largemouth bass from Caddo Lake, but not as frequently as in spotted gar. Liver damage was observed in only ~30 % of largemouth bass compared to ~70 % of spotted gar. There was no significant relationship between largemouth bass lipofuscin and habitat type in Caddo Lake, even though previous reports described habitat-specific differences in Hg concentrations (Chumchal et al. 2008b). Also, there was no significant relationship between liver color and Hg concentration in the livers of bass. Largemouth bass from Caddo Lake have some of the highest concentrations of Hg in freshwater fish from Texas. It is, therefore unlikely that lack of liver damage is related to low Hg concentrations. Hg speciation could explain the differences in pathology between gar and bass. While the two species had comparable concentrations of MeHg in the muscle tissue, gar had much less MeHg and much higher inorganic in the liver. This suggests that high concentrations of inorganic Hg lead to the liver damage seen in spotted gar but not in largemouth bass. This is
consistent with Drevnick et al. (2008) in which northern pike from Isle Royale had elevated levels of hepatic lipofuscin in conjunction with high levels of inorganic Hg.

Body Condition

Drevnick et al. (2008) showed that northern pike with the highest hepatic Hg concentrations had the fewest hepatic lipid reserves, lowest body condition factors, and appeared emaciated. In Caddo Lake largemouth bass, there was a positive correlation between body condition factor and muscle Hg concentration likely due to larger fish, older fish accumulating more MeHg (Chumchal and Hambright 2009). In spotted gar, body condition factor was not significantly correlated with Hg concentration in liver or muscle. There was a significant negative correlation between length and total hepatic Hg in gar, but no other associations existed between liver Hg and mass or muscle mercury and any of the growth metrics employed (mass, length, body condition factor). It is possible that body condition factor, length, and mass are not the best metrics of fitness in these fish. Other more subtle measures of fitness, like reproduction, were not assessed in this study, yet the Hg concentrations in bass and gar from Caddo Lake are within ranges that have been shown to negatively impact reproduction elsewhere (Crump and Trudeau 2009). More studies are necessary to further explain Hg’s role in liver pathology and influence on fish fitness.
CHAPTER 3

HEPATOTOXICITY OF DIETARY INORGANIC MERCURY AND METHYLMERCURY TO ZEBRAFISH (*Danio rerio*)

Introduction

Previous research involving mercury (Hg) has most often dealt with the risks to human health by the consumption of Hg-laden food, however little exists on the effects to fish and wildlife (Drevnick and Sandheinrich 2003). Of the relatively few studies investigating the toxicity of Hg to fish, many have employed aqueous exposures which were not always environmentally relevant (Wiener and Spry 1996; Hammerschmidt et al. 2002; Scheuhammer et al. 2007). Since fish accumulate Hg primarily through their diet, aqueous exposures are not always representative of environmental conditions (Scheuhammer et al. 2007). Laboratory and field studies, have recently shown positive associations between Hg and pathologies in renal and hepatic fish tissues (Mela et al. 2007; Raldúa et al. 2007; Drevnick et al. 2008). Mela et al. (2007) noted increased leukocyte infiltration and occurrence of melanomacrophage aggregations (MAs) in the liver and necrosis in liver and kidney of fish exposed to dietary MeHg. Raldúa et al. (2007), noted increased pycnotic nuclei, MAs, and morphological changes which varied with total Hg, in livers of barbels (*Barbus graelli*) and bleaks (*Alburnus alburnus*) downstream of a chlor-alkali factory. Drevnick et al. (2008) described changes in liver color and increased lipofuscin in the livers of northern pike from Isle Royale National Park. Positive associations between hepatic lipofuscin, liver discoloration, and Hg concentrations have also been reported in spotted gar from Caddo Lake, TX/LA. Interestingly, both northern pike from Isle Royale and spotted gar from Caddo Lake had high concentrations of inorganic Hg in livers, which suggests Hg metabolism and speciation might play an important role in liver damage to fish (Drevnick et al. 2008; Roberts In Review). The objective of this study was to determine the effects of dietary
inorganic Hg and MeHg on liver pathology in zebrafish (*Danio rerio*), in order to elucidate Hg’s role in liver damage to fish, with regard to speciation.

Materials and Methods

**Chemicals**

Unless otherwise noted, all chemicals used in the laboratory analyses were obtained from Sigma Chemical (St. Louis, MO, USA).

**Experimental Design**

Zebrafish were used as a model organism, due to their rapid maturation period of 90 days and the abundance of genetic and physiological information available on the species. Zebrafish were raised according to an approved University of North Texas Institutional Animal Care Use Committee protocol. All glass aquaria, utensils, and hatching tanks were acid washed with 15% HNO₃ before use with fish. Acid washing of utensils was performed between uses as well. Zebrafish eggs were purchased from Aquatica Tropicals Inc (Plant City, FL) and hatched in 2 L plastic aquaria. All eggs hatched within 7 days of age of one another. After hatching, larvae were divided among 20 L holding tanks until 30 days post hatch. At this time 30 fish were transferred randomly to 1 of 9, 40-L static renewal aquaria. Three replicate aquaria were designated for each treatment, with 3 treatments for a total of 9 aquaria and a total of 270 fish. Larvae were raised on flake food (Ocean Star International Inc., Snowville, UT) for the first 47 days. Detritus from tanks was siphoned daily, algae were removed from glass as needed, and a 50% water change occurred weekly. Each tank was equipped with separate sponge filter and air stone. Cleaning utensils were kept separate between treatments to prevent chemical or biological
contamination between tanks. Zebrafish cultures were maintained at 24 °C in reconstituted hard water. Zebrafish were exposed to dietary Hg beginning at 48 days post hatch until termination of the experiment at 125 days post hatch, for a total Hg exposure of 78 days. Temperature, DO, pH, conductivity, and hardness were measured weekly (Table 2).

Table 2. Average water chemistry values for 78 day zebrafish feeding study (mean ± 1 SE).

<table>
<thead>
<tr>
<th>Conductivity (µS/cm)</th>
<th>Hardness (mg CaCO3/L)</th>
<th>DO (mg/L)</th>
<th>Temperature (°C)</th>
<th>pH</th>
<th>Alkalinity (mg CaCO3/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>309.4 ± 2.58</td>
<td>82.0 ± 1.45</td>
<td>7.4 ± 0.33</td>
<td>23.7 ± 0.90</td>
<td>7.7 ± 0.12</td>
<td>59.5 ± 0.89</td>
</tr>
</tbody>
</table>

MeHg diets were prepared according to Drevnick et al. (2003), by dissolving MeHgCl in 100% ethanol. This mixture was then added to flake food on a glass petri dish. Inorganic Hg diets were created by dissolving HgCl₂ in deionized water. Flake food was then soaked in this solution. Control diets consisted of flake food and ethanol only. In all cases, the liquid (ethanol or water) was allowed to evaporate overnight under a fume hood. Both inorganic and MeHg diets were created well above the target concentration of 4 to 5 µg Hg g⁻¹. The adulterated food was analyzed for total Hg and then diluted with control food to reach target concentrations. Three subsamples of each diet were analyzed again for total Hg, as a form of quality control, before feeding. Flake food for each treatment was stored in a foil-wrapped glass vial at room temperature.

Fish were fed diets consisting of (mean ± 1 SE of total Hg) 0.12 ± 0.002 (control), 5.03 ± 0.309 (inorganic Hg), or 4.11 ± 0.146 (MeHg) µg Hg g⁻¹ dry weight for 78 days. For the first 21
days of the study, fish were fed twice daily. Feeding was increased to three times daily for the remainder of the experiment, to facilitate growth. The MeHg diet represents a relevant environmental concentration, which might be found in forage fish from low alkalinity lakes (Hammerschmidt et al. 2002). This concentration has been used in other studies, which have shown negative reproductive effects in fathead minnows (Hammerschmidt et al. 2002; Drevnick et al. 2006). Fish were not fed for two days before they were sacrificed, to allow time for all food to exit the gut tract. Fish were sacrificed using MS-222, and weight (g) and length (cm) information were recorded for each fish. Five fish from each tank (15 per treatment) were randomly selected for pathological inspection and preserved in neutral buffered formalin for 96 hours. All other fish were wrapped in foil and placed in plastic bags at -80°C.

*Hg Analysis*

Prior to Hg analysis, thawed fish were eviscerated using a sterile disposable hypodermic needle. Needles were acid washed with 15% HNO₃ between fish. Viscera and carcass were placed into separate 1.5 ml centrifuge tubes and lyophilized overnight using a Freezone 6 vacuum lyophilizer (Labconco, Kansas City, MO). Lyophilized samples were weighed to one thousandth of a milligram using a CAHN C-31 microbalance (CAHN Instruments, Inc., Cerritos, CA).

Hg analysis was performed at Texas Christian University using a direct Hg analyzer (DMA-80, Milestone Inc. Monroe, CT) that uses thermal decomposition, gold amalgamation, and atomic absorption spectrometry (Chumchal et al. 2008b; Chumchal and Hambright 2009). Quality assurance included analysis of reference materials for every ten samples analyzed (MESS-3, DOLT-3). Mean recovery for reference materials was 101%.
Liver Pathology

Fixed zebrafish were dehydrated and paraffin infiltrated under vacuum using a microwave rapid histoprocessor (RHS1, Milestone Inc. Monroe, CT). Whole fish were embedded in paraffin, cut into 6 µm sections, and mounted on glass slides. Alternate sections were deparaffinized, rehydrated, and stained with eosin and hematoxylin. A Zeiss Axio compound microscope with an Axiocam High Resolution camera (Carl Zeiss Inc., Berlin, Germany), was used to photograph each liver at 200X magnification.

Statistical Analyses

Least-squares regression analyses were performed to compare Hg concentration with fish length, weight, and body condition factor. Kruskal-Wallis tests were used to compare mean Hg concentrations between treatments. These were followed by a Tukey’s post-hoc test. SPSS version 17.0 software was used to perform statistical tests. Hg data were (log10) transformed to meet assumptions of normality and homogeneity of variance. An α = 0.05 was used for all tests.

Results

Hg Analysis

Mean total Hg concentrations, in viscera, were significantly different between treatments (Kruskal-Wallis, p < 0.0001) and belonged to three statistically distinct groups (Tukeys, Methyl > Inorganic > Control). Hg content was highest in viscera of zebrafish fed diets containing MeHg (mean = 11.6 ± 1.86 µg Hg g⁻¹ dry wt), intermediate in zebrafish fed diets containing inorganic Hg (mean = 4.3 ± 1.08 µg Hg g⁻¹ dry wt), and lowest in those fed the control diet (Below Limit of Detection) (Figure 11). The detection limit for the analyzer was calculated as
0.349 ng Hg. To do this, seven DORM-3 samples, each weighing 0.0026 g, were analyzed for total Hg using the DMA-80. The weight was selected, because in 0.0026 g of DORM-3 there is a certified value of 1 ng Hg, which was the expected detection limit of the analyzer. The seven obtained values were then averaged and a standard deviation was obtained. The standard deviation was then multiplied by the t-value (0.314) for the 99% confidence interval, to obtain the detection limit.

Mean total Hg concentrations in carcass, were significantly different between treatments (Kruskal-Wallis, \( p < 0.0001 \)) and were separated into statistically distinct groups (Tukeys, Methyl > Inorganic > Control) (Figure 13). Hg content was highest in the carcass of zebrafish fed diets containing MeHg (mean = 12.49 ± 0.369 µg Hg g\(^{-1}\) dry wt), intermediate in zebrafish fed diets containing inorganic Hg (mean = 1.09 ± 0.117 µg Hg g\(^{-1}\) dry wt), and lowest in those fed the control diet (mean = 0.48 ± 0.038 µg Hg g\(^{-1}\) dry wt) (Table 3).

**Growth**

Body condition factor was not significantly associated with total Hg in the carcass \( (r^2 = 0.007, p = 0.836) \) (Figure 14) of zebrafish raised on the control diet. Fish length and total Hg content in the carcass were positively associated in fish fed control diets \( (r^2 = 0.58, p = 0.017) \) (Figure 15), but there was no determinable relationship between fish length and total Hg in the viscera. There was no significant association between fish weight and total Hg in viscera or carcass \( (r^2 = 0.34, p = 0.10) \) (Figure 16) of zebrafish fed control diets.

There was no significant association between body condition factor and total Hg in carcass \( (r^2 = 0.007, p = 0.83) \) (Figure 17) or viscera \( (r^2 = 0.137, p = 0.326) \) (Figure 18) of zebrafish fed an inorganic Hg diet. There was no significant association between length and total
Hg in viscera ($r^2 = 0.062, p = 0.52$) (Figure 19) or carcass ($r^2 = 0.074, p = 0.48$) (Figure 20) of fish fed inorganic Hg diets. Weight and total Hg were not significantly associated in carcass ($r^2 = 0.07, p = 0.49$) (Figure 21) or viscera ($r^2 = 0.007, p = 0.83$) (Figure 22) of zebrafish fed inorganic Hg diets.

Body condition factor and total Hg were not significantly associated in carcass ($r^2 = 0.018, p = 0.73$) (Figure 23) or viscera ($r^2 = 0.23, p = 0.19$) (Figure 24) of zebrafish fed a MeHg diet. For fish fed the MeHg diet, there was no significant association between length and total Hg concentration in carcass ($r^2 = 0.03, p = 0.66$) (Figure 25), but a negative association existed in viscera ($r^2 = 0.75, p = 0.003$) (Figure 26). Weight and total Hg were negatively associated in viscera ($r^2 = 0.71, p = 0.005$) (Figure 27), but not in carcass ($r^2 = 0.057, p = 0.54$) (Figure 28) of fish fed MeHg diets.

When data were pooled across treatments, body condition factor was marginally significantly associated with total Hg in the carcass of zebrafish ($r^2 = 0.12, p = 0.071$), while fish weight and total Hg were marginally positively associated ($r^2 = 0.12, p = 0.071$). Length and total Hg in carcass were positively associated ($r^2 = 0.15, p = 0.045$) across treatments.
Figure 12. Mean total Hg concentrations in zebrafish viscera (n = 9 per treatment) were significantly different between treatments ($p < 0.0001$). Asterisks denote a significant difference between means. Error bars represent ± 1 standard error.

Figure 13. Mean total Hg concentrations in zebrafish (n = 9 per treatment) carcass were significantly different between treatments ($p < 0.0001$). Asterisks denote a significant difference between means. Error bars represent ± 1 standard error.
Figure 14. Total Hg in carcass and body condition factor were not associated in zebrafish fed a control diet \((0.12 \pm 0.002 \, \mu g \, Hg \, g^{-1} \, dry \, weight)\) for 78 days \((r^2 = 0.007, p = 0.836)\).

Figure 15. Total Hg in carcass and length were significantly positively associated in zebrafish fed a control diet \((0.12 \pm 0.002 \, \mu g \, Hg \, g^{-1} \, dry \, weight)\) for 78 days \((r^2 = 0.58, p = 0.017)\).
Figure 16. There was no significant association between weight and total Hg in the carcass of zebrafish fed a control diet (0.12 ± 0.002 µg Hg g\(^{-1}\) dry weight) for 78 days \((r^2 = 0.34, p = 0.10)\).

Figure 17. Total Hg concentration in the carcass and body condition factor were not associated in zebrafish fed an inorganic Hg diet (5.03 ± 0.309 µg Hg g\(^{-1}\) dry weight) for 78 days \((r^2 = 0.007, p = 0.83)\).
Figure 18. Total Hg in viscera and body condition factor were not significantly associated in zebrafish fed an inorganic diet (5.03 ± 0.309 μg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.137, p = 0.326$).

Figure 19. Total Hg concentration in viscera and length were not significantly associated in zebrafish fed an inorganic diet (5.03 ± 0.309 μg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.06, p = 0.52$).
Figure 20. Total Hg concentration in carcass and length were not significantly associated in zebrafish fed an inorganic Hg diet (5.03 ± 0.309 µg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.0741, p = 0.48$).

Figure 21. Total Hg concentration in carcass and weight were not associated in zebrafish fed an inorganic Hg diet (5.03 ± 0.309 µg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.0695, p = 0.49$).
Figure 22. Total Hg concentration in viscera and weight were not associated in zebrafish fed an inorganic Hg diet (5.03 ± 0.309 µg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.007, p = 0.83$).

Figure 23. Total Hg concentration in carcass and body condition factor were not associated in zebrafish fed a MeHg diet (4.11 ± 0.146) for 78 days ($r^2 = 0.018, p = 0.73$).
Figure 24. Total Hg concentration in viscera and body condition factor were not associated in zebrafish fed a MeHg diet (4.11 ± 0.146 µg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.23$, $p = 0.19$).

Figure 25. Total Hg concentration in carcass and length were not associated in zebrafish fed a MeHg diet (4.11 ± 0.146 µg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.03$, $p = 0.66$).
Figure 26. Total Hg concentration in viscera was negatively associated with length of zebrafish fed a MeHg diet (4.11 ± 0.146 µg Hg g\(^{-1}\) dry weight) for 78 days \((r^2 = 0.75, \ p = 0.003)\).

Figure 27. Total Hg concentration in viscera was negatively associated with weight in zebrafish fed a MeHg diet (4.11 ± 0.146 µg Hg g\(^{-1}\) dry weight) for 78 days \((r^2 = 0.71, \ p = 0.005)\).
Figure 28. Total Hg concentration in carcass was not associated with weight in zebrafish fed a MeHg diet (4.11 ± 0.146 µg Hg g⁻¹ dry weight) for 78 days ($r^2 = 0.057$, $p = 0.54$).

Table 3. Comparison of mean mercury contents of fish from three studies. Sample size is marked in parentheses.

<table>
<thead>
<tr>
<th>Common name</th>
<th>Total Hg in muscle/carcass</th>
<th>Total Hg in Liver/viscera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Northern Pike</td>
<td>0.214*</td>
<td>0.237*</td>
</tr>
<tr>
<td>Largemouth bass</td>
<td>1.718 (8)</td>
<td>0.997 (5)</td>
</tr>
<tr>
<td>Spotted gar</td>
<td>2.61 (5)</td>
<td>30.17 (5)</td>
</tr>
<tr>
<td>Zebrafish (control)</td>
<td>0.48 ± 0.038 (9)</td>
<td>BDL (9)</td>
</tr>
<tr>
<td>Zebrafish (inorganic)</td>
<td>1.09 ± 0.117 (9)</td>
<td>4.3 ± 1.08 (9)</td>
</tr>
<tr>
<td>Zebrafish (MeHg)</td>
<td>12.49 ± 0.369 (9)</td>
<td>11.6 ± 1.86 (9)</td>
</tr>
</tbody>
</table>

*Wet Weight
Liver Pathology

No lipofuscin was identified after pathological inspection of zebrafish livers. Zebrafish livers appeared normal with little evidence of pathology (Figures 29, 30, and 31). Some of the livers showed increased amounts of vacuolization, however there was no relationship with the type of diet fish received (Figure 32).

Figure 29. Photograph (200X) of a liver taken from a zebrafish fed a control diet for 78 days. There is no apparent liver damage.
Figure 30. Photograph (200X) of a liver taken from a zebrafish fed an inorganic mercury diet for 78 days. There is no apparent liver damage.

Figure 31. Photograph (200X) of a liver taken from a zebrafish fed a methylmercury diet for 78 days. There is no apparent liver damage.
Figure 32. Photographs (200X) of a highly vacoulized liver (left) and a less vacoulized liver (right).

Discussion

Hg Concentrations

Hg concentrations were highest in the MeHg fed zebrafish, intermediate in the inorganic, and lowest in the control treatment. These findings are consistent with MeHg’s greater capacity to bioaccumulate (Morel et al. 1998; Devlin 2006; Crump and Trudeau 2009). MeHg makes up the majority of total Hg in higher trophic levels (Bloom 1992), even though inorganic Hg is more abundant in aquatic ecosystems. MeHg is assimilated more efficiently and is eliminated more slowly than inorganic Hg (Morel et al. 1998; Devlin 2006).

Drevnick et al. (2008) reported mean concentrations of 0.214 $\mu$g total Hg g$^{-1}$ wet wt in the edible fillets and 0.237 $\mu$g total Hg g$^{-1}$ wet wt in the livers of northern pike from Isle Royale National Park. Spotted gar from Caddo Lake had mean concentrations of 2.61$\mu$g total Hg g$^{-1}$ dry wt in muscle and 30.17 $\mu$g total Hg g$^{-1}$ dry wt in liver. Largemouth bass from Caddo Lake had mean concentrations of 1.72 $\mu$g total Hg g$^{-1}$ dry wt in muscle and 0.997 $\mu$g total Hg g$^{-1}$ dry wt in
liver. In this study, zebrafish fed MeHg, had a mean Hg concentration higher than northern pike from Isle Royale and largemouth bass from Caddo Lake. Only spotted gar liver had a higher mean total Hg concentration than MeHg fed zebrafish. Since Hg concentrations in zebrafish were above those in wild fish with liver pathologies it seems likely that Hg concentration alone is not to blame for liver pathologies.

**Body Condition**

Drevnick et al. (2008) described a negative correlation between condition factor and total Hg in the livers of northern pike. In spotted gar from Caddo Lake, total Hg content of liver was negatively correlated with length; however condition factor was not associated with total Hg as it was in Isle Royale pike. Condition factor of largemouth bass was positively correlated with total Hg in the muscle, but not with total Hg in liver. In comparison, condition factor was not significantly associated with total Hg in either viscera or muscle of zebrafish. Total Hg in the viscera of zebrafish fed a MeHg diet was negatively correlated with fish length as was seen in spotted gar, while total Hg in carcass of zebrafish fed a control diet was positively correlated with fish length as seen in largemouth bass. This positive association was likely due to larger fish accumulating more Hg though the consumption of larger amounts of food.

**Liver Pathology**

The absence of hepatic lipofuscin in zebrafish fed Hg diets could be explained several ways. There is much correlative evidence to suggest Hg’s role in liver damage (Mela et al. 2007; Raldúa et al. 2007; Drevnick et al. 2008; Roberts In Review) however little causative evidence that dietary Hg leads to the accumulation of hepatic lipofuscin. Since a causative link has not
been described previously to and no evidence of causation was found in this study, it is necessary to acknowledge that dietary Hg may not cause the development of hepatic lipofuscin in fish and another environmental contaminant could be at work.

Although both spotted gar and largemouth bass were shown to occupy the same vertical trophic position (Chumchal and Hambright 2009) and have similar prey items they had very different inorganic Hg concentrations in their livers. This suggests that a species-specific difference in Hg metabolism might be the cause of the discrepancy. Gar could possess a demethylation mechanism that bass do not have. Zebrafish, like bass, may not demethylate MeHg and thus may not produce the inorganic ion in the liver. Zebrafish fed the inorganic diet showed no evidence of liver damage, which might mean that a higher concentration of inorganic Hg is needed to produce an effect. Again, a demethylation mechanism is not well described in fish. Eagles-Smith et al. (2009) have recently described a threshold dependent demethylation mechanism in water birds. Here the authors show that above a threshold concentration of total Hg in the liver, demethylation of MeHg occurred resulting in progressively lower ratios of MeHg to total Hg in the liver. This threshold concentration was different across the waterbird taxa examined in this study. Based on these results, if a demethylation mechanism exists in fish, it could be that fish taxa begin demethylation at different total Hg concentrations in the liver. This could account for high concentrations of inorganic Hg in gar livers and not in bass livers. This could also explain why strong relationships between Hg and pathologies existed in pike and gar but not in bass and zebrafish.

Another possibly reason for the lack of lipofuscin in lab fed zebrafish and presence in field sampled fish, could be dietary in nature. Laboratory fish, which are fed ad libitum, are less limited by diet and are thus less likely to have dietary deficiency. Commercial fish feed is
designed to supply fish with a nutritionally complete diet. Because of this, zebrafish in this study could have been provided with enough antioxidants to counteract the prooxidant effects of dietary Hg. This might account for the lack of lipofuscin in zebrafish.

Finally, it is possible that if exposed to dietary Hg for longer than 78 days, zebrafish might show signs of hepatic injury. Certain individuals from all three field-caught fish had hepatic lipofuscin. These individuals were likely exposed to Hg for a longer period than the zebrafish fed for 78 days. A longer exposure to dietary Hg could result in a gradual overwhelming of antioxidant defenses resulting in lipofuscin accumulation as was seen in Atlantic salmon fed Hg diets for a 4-month period which showed increased concentrations of antioxidant enzymes below a threshold concentration of 10 mg MeHg kg⁻¹ dry wt, but above the threshold antioxidant enzymes decreased resulting in greater pathological damage (Berntssen et al. 2003).
CHAPTER 4  
CONCLUSIONS

There is a wealth of evidence which suggests that Hg is linked to liver damage in fish (Mela et al. 2007; Raldúa et al. 2007; Drevnick et al. 2008). Drevnick et al. (2008) demonstrated strong relationships between Hg concentration and measures of liver damage (liver color and lipofuscin) in northern pike. Spotted gar from Caddo Lake displayed a similar relationship between total Hg concentration and liver damage. Although largemouth bass from Caddo Lake had Hg concentrations above pike from Isle Royale, there was no relationship between total Hg and liver damage in this fish. Both spotted gar and northern pike had elevated levels of inorganic Hg in their livers, which suggests that Hg speciation plays a role in liver damage. Northern pike most likely accumulated the inorganic Hg by feeding on invertebrates high in inorganic Hg (Drevnick et al. 2008). Spotted gar and largemouth bass share similar trophic positions and feed on similar prey items within the Caddo Lake ecosystem (Chumchal and Hambright 2009). It is unlikely that their difference in inorganic Hg concentrations is due to their diet, but rather due the way Hg is metabolized in these fish. It is possible that a hepatic demethylation mechanism is the reason for the discrepancy in inorganic Hg in these fish, and future studies should be focused on determining whether this is occurring.

Previous studies have noted that selenium (Se) seems to have a protective effect against Hg (Potter and Matrone 1974; Eisler 1985). Numerous studies have noted that Hg and Se tend to coaccumulate (Heisinger et al. 1979; Eisler 1985; Wang et al. 2001; Khan and Wang 2009). Selenium is a micronutrient which is incorporated into important antioxidant proteins such as glutathione peroxidase (Khan and Wang 2009). Palmisano et al (1995) described a two stage MeHg demethylation mechanism in dolphins which involved Se. The true nature of Se’s
protective effect against Hg is not well understood and future studies should be aimed at elucidating this relationship (Eisler 1985). If Se is involved in Hg demethylation and provides a protective effect, then future field studies would be strengthened by measuring Hg (total and MeHg) and Se in fish tissues.

Eagles-Smith et al. (2009) recently described a demethylation mechanism in waterbirds which was both threshold dependent and species-specific. If a demethylation mechanism is species specific in fish, then it could mean that some fish are more susceptible to liver damage than others. Future studies could determine if a species specific mechanism exists in fish, and determine which species might be more susceptible to liver damage.
REFERENCES


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