# MATERNAL TRANSFER OF DIETARY METHYLMERCURY AND IMPLICATIONS FOR EMBRYOTOXICITY IN FATHEAD MINNOWS (*Pimephales promelas*)

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Bridges, Kristin N. *Maternal Transfer of Dietary Methylmercury and Implications for Embryotoxicity in Fathead Minnows (Pimephales promelas)*, Doctor of Philosophy (Biology), December 2016, 107 pp., 4 tables, 13 figures, chapter references.

Mercury (Hg) is a ubiquitous environmental contaminant, which is capable of global atmospheric transport. As a result, even the most pristine aquatic ecosystems are affected by atmospheric Hg deposition, following which microbial transformation yield organic Hg forms, the most concerning of which is methylmercury (MeHg). Methylmercury is capable of bioaccumulation and biomagnification in food webs, resulting in potentially toxic body burdens due to regular dietary exposure in long-lived organisms at higher trophic levels. It is also a molecular mimic of some endogenous amino acids, providing a route of transfer from mother to offspring via large amino acid transporters. Exposure during neurodevelopment can lead to serious, irreversible neurological dysfunction, associated with a variety of cognitive and motor abnormalities across species. The present studies evaluate the effects of maternally-transferred dietary MeHg, at environmentally relevant concentrations on early life stage fathead minnows (*Pimephales promelas*). Embryos were collected from adult fatheads exposed to one of three diets with varying concentrations of MeHg for 30 days. Adult reproductive metrics were also monitored over the course of the study, with results indicating no effects on spawning frequency, clutch size, or total egg output. In embryos, Hg concentration was a function of female diet and the duration (number of days) of female exposure. Offspring spawned in tanks administered the low Hg diet displayed altered embryonic movement patterns (hyperactivity), decreased time to hatch, decreased mean larval size, and alterations to several metabolite abundances when compared with controls. Significantly altered metabolites

include those associated with cellular energetics, fatty acid metabolism, and polyamine synthesis, indicating current environmental exposure scenarios are sufficient to disrupt important cellular pathways. Dysregulation of the dopaminergic system of embryos is also characterized, and may be a possible mechanism by which hyperactive behaviors are observed in these embryos. Offspring from tanks administered the high Hg diet exhibited delayed hatching, increased mortality, and physiological abnormalities. Brain tissue of exposed adults from the low diet were dissected into regions, and also evaluated for alterations in dopamine cycling. Collectively, these results indicate current exposure scenarios in North American lakes and rivers are sufficient to cause reductions in fitness and survival of early life stage fish. The potential for community structure impacts exists, as sensitive individuals and species become disproportionately affected by chronic, low-level MeHg exposure.

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

# CHAPTER 1

# INTRODUCTION

## Mercury in the Environment

Mercury is a toxic metal that is released into the environment through a variety of natural processes as well as anthropogenic activities. It is currently estimated that environmental mercury levels are three to six times higher than pre-industrial levels, [1], and current surface water concentrations range from 0.1- 0.8 ng/L [2, 3]. Natural processes that release mercury into the environment include; weathering of soils, ore deposits, and hydrocarbons that contain naturally occurring elemental mercury, geothermal activity, and re-emissions [4, 5]. Re-emissions are characterized as the re-release of previously deposited mercury from all natural and anthropogenic sources combined [6]. This makes it difficult to characterize the exact amount of mercury that has been deposited due to human activity, though estimates attribute 70% of the annual release (~ 1,980 metric tons in 2010) to human activity [1, 7]. The sources of these anthropogenic emissions include the mining and smelting of a number of metals (in which mercury is found as an impurity), industrial chemical production, cement production, the combustion of fossil fuels, and incineration of solid wastes and biomass [6].

Though mercury emissions are regulated in the US and many other developed nations, rising mercury levels are still being observed. Increasing concentrations are a global issue, occurring even in remote areas of North America [8]. This is attributed to long range transport of metallic mercury vapor from coal burning facilities in Asia, where over half of anthropogenic

mercury emissions originate [8]. Metallic mercury is highly volatile, and its vapor can remain suspended in the atmosphere for up to 2 years. The volatility of this element, coupled with a long atmospheric half-life, has led to a global deposition problem stemming from long range atmospheric transport. A combination of chemical reactions with atmospheric constituents, such as ozone, HClO, $HSO^{3-}$, or $OH^{\cdot}$, and photoactivation can cause the oxidation of the uncharged form, $Hg^0$ to $Hg^{2+}$. The oxidized form of mercury has a much shorter half-life than $Hg^0$ [9], and may bind to aerosol particulates, precipitate out of the atmosphere via wet or dry deposition, and sorb to aquatic sediments and other particles.

Following deposition into aquatic systems, mercury partitions into sediments where it can be chemically or biologically transformed into organometallic forms (methylmercury). These include dimethylmercury and monomethylmercury produced primarily by sulfate or iron reducing bacteria found in aquatic sediments under reducing conditions [10]. The rate of mercury methylation is determined by a variety of factors in the aquatic environment. High levels of dissolved organic carbon, low pH, high sulfate levels and the availability of metabolic electron donors and acceptors in an aquatic environment will increase the rate of mercury methylation, while the converse conditions will slow the conversion process [10, 11].

## Mercury in Biota

Methylmercury is a potent neurotoxicant that exerts both lethal and sub-lethal effects in aquatic and terrestrial biota, and may have human health implications for sensitive groups such as pregnant women and children. Because of the relative lipophilicity, approximately 95% of the methylmercury ingested through diet is transported across the gut and enters the blood

stream. Due to the affinity of methylmercury for sulfur containing amino acids, dietary methylmercury will accumulate in muscle tissue where it is stored [12], and may reach concentrations $10^6$-$10^7$ times higher than surface waters. Wiener *et al*. [13] speciated mercury in yellow perch (*Perca flavescens*) from the Great Lakes region and found 95% of the total body burden was bound to proteins in axial muscle, 99% of which was in the MeHg form. Methylmercury accumulated by prey items can be transferred to higher trophic levels, and biomagnify up the food chain.

Dietary sources account for 90% of methylmercury uptake in freshwater fish, while waterborne exposure accounts for very little [14]. As a result of trophic transfer, the highest concentrations of methylmercury are generally found in top predators, larger, and older aquatic organisms [12]. Because these fish are often preferred as food sources, most human exposure to methylmercury occurs through the consumption of contaminated fish and seafood. In fact, all 50 states have fish consumption advisories in place, 81% of which are due to mercury contamination [15]. Furthermore, methylmercury contamination in some waters of the Great Lakes region has degraded the quality of yellow perch, a commercially and recreationally fished species, for human consumption [13]. Recent studies have shown effects on humans are elicited at lower MeHg concentrations than previously thought, and that the risk associated with MeHg exposure is often greatly underestimated in human studies [16, 17]. Consequently, communities of people who subsist on fish based diets are particularly at risk of exposure to harmful levels of methylmercury [18, 19].

Sandheinrich and Wiener [20] report that the tissue concentrations that lead to adverse effects in fish are much lower than previously proposed. Current risk assessments attribute

sublethal effects in individual fish health to wet weight Hg concentrations of 0.2-0.3 ppm, concentrations which are not uncommon to aquatic environments in North America [14, 21, 22]. A recent survey of mercury concentrations of yellow perch in the great lakes region found wet weight axial muscle concentrations ranging from 0.01-2.6 ppm, while an assessment of walleye *(Sander vitreus),* northern pike *(Esox Lucius)*, smallmouth bass *(Micropterus dolemieu)* and largemouth bass (*Micropterus salmoides*) concluded a majority of over 2,000 locations sampled in the Great Lakes region have potentially harmful body burdens of methylmercury, due to contamination [13, 23]. Many of the concentrations observed in these fish are sufficient to induce changes in reproduction, as they exceed wet weight Hg concentrations of 0.2 ppm [21, 22, 24]. Piscivorous fish in acidic lakes and newly flooded reservoirs commonly contain axial muscle concentrations ranging from 0.5-3.0 ppm wet weight, far above the LOEC of 0.2 ppm [25]. Flooding of new reservoirs can increase fish tissue concentrations by up to 10 times the pre-flood values, requiring 20-30 years to return to reference values [20]. Studies investigating methylmercury toxicity to fish have displayed numerous neurological, physiological, developmental and reproductive changes associated with chronic MeHg exposure [12, 19, 26].

## Mechanisms of Toxicity

### *Oxidative Stress*

Methylmercury presence leads to increased oxidative stress, as cellular antioxidants such as glutathione are depleted. This leads to a reduction in the cell's ability to rid itself of reactive oxygen species (ROS) which may lead to lipid peroxidation, dysfunction of enzymes and

direct damage to DNA [27]. Mercury has been shown to accumulate in the mitochondria, where it disrupts the membrane potential by increasing permeability to calcium and other ions, and inhibits oxidative phosphorylation [28-30]. The resulting mitochondrial dysfunction leads to ATP production collapse and additional ROS generation, which may result in tissue pathologies, necrosis, and apoptosis [29]. Oxidative damage due to chronic, low level MeHg exposure has shown to be particularly abundant in the liver, kidneys, muscle, and gonads [22, 26, 31].

### *Reproductive Toxicity*

Ovarian apoptosis was increased three fold in fathead minnows (*Pimephales promelas)* exposed to 0.87 ppm dietary methylmercury, and six fold at a concentration of 3.93 ppm [26]. Increased apoptosis was also observed in gonads of male fathead minnow at 0.87 ppm MeHg [32]. The concentrations leading to increased apoptosis in gonad tissue are not uncharacteristic of concentrations found in those of wild invertivorous and piscivorous fish in North America, and have been associated with significant effects on fish reproduction [3, 24].

Fathead minnows chronically exposed to a diet containing 3.93 ppm MeHg for 600 days showed a general stress response, which included downregulation of many genes involved in protein processing, testosterone and estrogen production. Upregulation of vitellogenin mRNA was observed in male fatheads while downregulation occurred in females [32]. In addition to gene expression changes that can affect reproduction, it is suggested that necrosis and apoptosis of liver tissues may also play a large role in MeHg induced reproductive changes. The liver is critical to the metabolism of key reproductive hormones, such as testosterone and

estrogen, as well as the production of vitellogenin, and is highly susceptible to MeHg instigated tissue pathologies [22, 31, 33].

Fathead minnows fed a diet dosed with MeHg concentrations as low as 0.86 ppm displayed dose dependent delays in spawning, decreased spawning activity and declines in egg production [3]. A study performed by Latif *et al*. [2] showed that MeHg in the environment, even at nanogram levels may also elicit a decline in hatching success. These effects were observed in walleye (*Sander vitreus*) from three lakes in Ontario, and were representative of mercury exposures relevant to current aquatic ecosystems. Evidence from these studies suggest adverse consequences for wild fish populations are likely [3]. Average egg concentrations from adult females caught in the lakes ranged from <10 ppb- 988 ppb, and egg concentrations were positively correlated with maternal concentrations, averaging 181.5- 2,701 ppb [2]. Maternal exposure to MeHg is the primary exposure pathway of embryos in the wild, as structural similarities with methionine enable MeHg to use methionine transporters to cross the embryonic barrier, placental, and blood-brain barriers [31, 34].

### *Neurotoxicity*

As a result of molecular mimicry, the brain is particularly susceptible to damage caused by the presence of methylmercury [19]. In a feeding study performed by Berntssen [27], Atlantic Salmon (*Salmo salar*) fed a diet spiked with 4.35 ppm MeHg for four months, showed upregulated production of superoxide dismutase and glutathione peroxidase in response to increased ROS production when compared with control fish. Dissection of the fish showed severe vacuolization and necrosis in the brains of exposed fish. At a diet of 10 ppm, the fish

showed significantly reduced neural enzyme activity, in addition to a seven-fold increase in lipid peroxidation in the brain. The results indicate brain pathologies in adult fish are elicited by dietary methylmercury, but do not represent consequences of exposure to embryos, as the developing CNS is far more sensitive to some toxicants [16, 35].

Exposure to MeHg in early stages of fish development can have profound, irreversible effects that are elicited at much lower concentrations than in adults [36]. Locomotor deficits were observed in adult zebrafish *(Danio rerio)* embryonically exposed to MeHg at concentrations as low as 0.015 ppm [37]. Reduced heart rate, survival of embryolarval stages, and dose–dependent decreases in hatching success were observed in eggs collected from Walleye in Clay Lake, Ontario [2]. Smith *et al*. [38] observed significantly decreased cell body density in the dorsal and lateral telencephalon of adult zebrafish exposed to MeHg at experimental concentrations in excess of 0.01 μM during embryo neurogenesis. The teleost telencephalon processes geometrical attributes of surroundings to reorient body position, as well as acoustic, lateral line and gustatory information [39]. It is a critical region for spatial associations and learning responses necessary to identify foraging and nesting locations, initiate startle responses, and navigate the environment [38, 40]. Therefore, functional reductions due to the presence of MeHg in the telencephelon may have serious implications for proper imprinting of fish behaviors associated with biological fitness.

### *Behavioral Toxicity*

Alterations in swimming, foraging, competition and predator evasion can be a sensitive indicator of ecosystem stress due to aquatic contaminants, such as MeHg [41]. Behavioral

changes due to MeHg exposure can greatly reduce the ability of a fish to compete for resources or avoid predators in wild populations where food is not as readily available as in laboratory studies [14]. A feeding study performed on golden shiners *(Notemigonus crysoleucas)* using a range of environmentally relevant MeHg concentrations concluded that several predator avoidance behaviors were altered after a 90 day exposure [42]. Among the behaviors affected were vertical dispersal and increased time to settle, both indicative of hyperactivity. Separate feeding studies involving rainbow trout (*Oncorhynchus mykiss*) and largemouth bass both found an increase in nervous behavior and aimless movement or agitation [43, 44]. These nervous behaviors may affect the ability of the fish to compete for food and mates, as well as impact the likelihood of survival, as appropriately executed behavioral responses to environmental stimuli enable fish to detect and avoid predators [45].

Behavior of adult fish arises from simpler behaviors imprinted during embryonic development [45]. Alterations to behavioral imprinting due to developmental exposure to MeHg may have profound, lifelong consequences for fish. There is a scarcity of studies examining transgenerational effects of MeHg on the behaviors of early life stage fish, however, available data suggests hyperactivity due to MeHg exposure is elicited in early life stage fish at much lower exposure concentrations [45-47]. Mora-Zamorano *et al.* [47] found increased locomotor output in larval zebrafish exposed to maternally derived MeHg at environmentally relevant dietary concentrations [47]. Zebrafish embryos exposed to 10 ppb MeHg via waterborne exposures exhibited premature hatching, delayed mortality syndrome 6 days post hatch, and reduced prey-capture abilities [37]. Embryonic fathead minnows display the same startle response seen in adult fish avoiding predation. In embryos, this response serves as a

mechanism to distribute hatching enzymes, and is necessary for breakdown of the egg chorion [48]. An increase in embryonic motor activity provides a likely explanation for earlier hatching times, which has been associated with reductions in survival [37, 49]. When coupled with evidence from the study by Mora-Zamorano *et al.* [47], which suggests these hyperactive behaviors can be expected to persist, the expectation of reductions in offspring fitness due to maternal transfer of dietary MeHg appears likely.

## Study Objectives

The primary form of mercury exposure for fish in natural waters is persistent low levels of dietary MeHg, which bioaccumulates in older and larger fish, and biomagnifies up trophic levels [50]. MeHg is an endogenous mimic of sulfur containing amino acids, and is therefore actively transported across the embryonic membrane, placental, and blood-brain barriers via amino acid transporters [31, 51].

Transgenerational effects of dietary MeHg, remain largely understudied in spite of the potential impacts on community structure. As a result of the lack of research in this area, sublethal effects of MeHg on long-term development and survival of juvenile fish are largely unknown. Furthermore, the available studies investigating effects of MeHg on early life stage fish have often used exposure routes that are not environmentally relevant, exposure concentrations in excess of those found naturally, or have used acute exposure scenarios [3, 12, 46].

The amount of mercury transferred to offspring has been shown to be determined by concentrations present in prey items consumed during oogenesis, rather than maternal body

burdens [52]. The present study endeavors to mimic the most environmentally relevant exposure scenarios across each life stage. Adult exposures occur through chronic dietary exposure to MeHg concentrations commonly found in aquatic ecosystems. All developmental/larval exposures resulted from maternal transfer of dietary MeHg to eggs during oogenesis, with the aim of examining the MeHg induced behavioral alterations associated with reductions in fitness, during this extremely sensitive life stage [34, 36]. In addition to the aforementioned endpoints, a highly conserved, neurological mechanism by which the observed behavioral changes may occur in vertebrates is proposed.

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# CHAPTER 2

# EMBRYOTOXICITY OF MATERNALLY TRANSFERRED METHYLMERCURY TO FATHEAD MINNOWS *(Pimephales promelas)*[1]

## Introduction

Mercury is a widespread environmental contaminant released through a variety of natural processes and anthropogenic activities. Following deposition into aquatic systems, mercury can be transformed by microbes into methylmercury, a form which bioaccumulates and biomagnifies through aquatic food webs. Methylmercury (MeHg) results in effects on fish health at concentrations exceeding 0.2-0.3 ppm wet weight in the whole body [1, 2]. These concentrations are not uncommon to aquatic food webs in North America, suggesting that MeHg concentrations in wild prey may decrease overall fish health, as well as alter reproduction in some fish [2-4].

A feeding study examining effects of MeHg spiked diets (0.455 ppm Hg wet weight, 0.959 ppm Hg wet weight) on predator avoidance behavior of adult golden shiners (*Notemigonus crysoleucas*), concluded that several predator avoidance behaviors were altered after a 90 day exposure when compared with controls [5]. Among the behaviors affected were vertical dispersal and increased time to settle, both indicative of hyperactivity. These nervous behaviors may affect the abilities of adult fish to avoid predation and compete for food or

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[1] This entire chapter is adapted from Bridges KN, Soulen BK, Overturf CL, Drevnick PE, Roberts AP. 2016. Embryotoxicity of maternally-transferred methylmercury to fathead minnows (Pimephales promelas). *Environmental Toxicology & Chemistry,* with permission from John Wiley and Sons.

mates. A review by Depew et al. [1], which incorporated data from feeding studies utilizing commercially prepared diets spiked with MeHg, prey items injected with MeHg, as well as prey items naturally contaminated with environmental MeHg, concluded that fish consuming diets greater than ~0.2 ppm dry weight dietary Hg are at risk for reproductive effects. These include decreases in gonadosomatic index (GSI), sex steroid production, spawning success, spawning behavior, fertilization success, fecundity, and increases in ovary apoptosis. Drevnick et al. [6] report that plasma testosterone in males and 17β-estradiol in female fathead minnows were significantly decreased in fish fed a MeHg spiked diet containing 0.87 ppm (dry weight) Hg compared to low mercury controls. Female fathead minnows from the same study also showed inhibited gonadal development, reduced spawning success, and increased time to spawn.

In addition to altering reproductive success in adult fish, MeHg may alter the hatching success rates and survival of embryo-larval stages. Birge et al. [7] determined that mercury concentrations 0.07-0.10 ppm (wet weight) in eggs of rainbow trout led to increased embryo mortality. Transgenerational effects of maternally derived MeHg are understudied, and it is believed that early life stages of fish are more sensitive to the effects of MeHg toxicity than adults [1, 8, 9].

Nearly all of the mercury in adult fish and their eggs is MeHg [10]. Because mercury has a strong bonding affinity for reduced sulfur atoms, methylmercury forms conjugates with sulfur containing biomolecules, resulting in compounds that resemble endogenous amino acids. This molecular mimicry provides a mechanism of transfer for dietary methylmercury from adult female fish to eggs through amino acid transporters [11]. Previous research has shown the diet

of the maternal adult during oogenesis, rather than adult body burden, is the principal source of mercury in eggs [12].

The goal of the present study was to evaluate the effects of maternally derived dietary methylmercury on embryonic development, hatch, and survival in fathead minnows. We fed adult fathead minnows diets containing environmentally relevant concentrations of MeHg that result in axial muscle concentrations commonly found in piscivorous fish in North American lakes, and characterized several reproductive and embryonic metrics [6, 12, 13].

## Materials and Methods

### *Animal Care*

All procedures using fish were approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC) under protocol #1303-3. Reproductively active, adult fathead minnows were obtained from Aquatic Biosystems (Fort Collins, CO), and divided among fifteen, 21 L glass aquaria. Each tank contained three females (control: 2.01± 0.51 g, low: 1.89± 0.45 g, and high: 1.81 ± 0.49 g wet weight) and one male fish (control: 4.17± 1.8 g, low: 4.21 ± 1.2 g, and high: 3.97 ± 1.6 g wet weight), as well as two breeding tiles constructed from halved PVC pipe sections. All tanks were equipped with activated carbon filtration systems, heaters, and were on a photoperiod of 16h light: 8h dark. Tanks were filled with reconstituted moderately hard water (24±1 ºC, pH 7.2-7.8, DO 5, mg/L, hardness 80-100 mg/L $CaCO_{3)}$ and maintained by performing daily 25% water changes, and 50% water changes every three days. Water quality and temperature were monitored using salinity and pH probes in addition to test strips for nitrates/nitrites. All tanks received the same food (Skretting starter crumble, Tooele,

UT) for two weeks in order to establish baseline reproduction. Waste and food debris were siphoned daily from each aquarium. At the conclusion of the study adult fatheads were euthanized using 250 ppm MS-222 (buffered with equal masses of NaHCO3), according to IACUC protocol.

*Experimental Design*

Following the two-week acclimation period, aquaria were randomly assigned either a control diet containing 0.02 ± 0.002 ppm Hg dry weight (n= 5), or one of two experimental diets containing 0.87 ± 0.08 (low, n= 5), or 5.5 ± 0.6 (high, n= 5) ppm Hg dry weight. These concentrations were selected as they have been found to correspond with the range of concentrations seen in benthic invertebrates and zooplankton in some North American lakes, when normalized to caloric density [6, 13]. With 15 aquaria in total, there were 5 replicates per treatment. All aquaria were administered similar quantities of food two times per day for 30 days. Diets were prepared by mixing fish food with methylmercury chloride (Sigma-Aldrich) dissolved in reagent ethanol (Fisher) in an acid washed glass dish. Ethanol was removed by evaporation in a fume hood. Food was prepared in accordance with the methods used by Hammerschmidt et al. [13]. Samples of each diet were analyzed for Hg as described in the mercury determination section. Waterborne MeHg from unconsumed food pellets was not considered a significant route of Hg exposure for adult fathead minnows, or their offspring. Waste and unconsumed food were siphoned from tanks daily, regular water changes were performed, and all clutches were removed from tanks and placed in clean reconstituted moderately hard water (RMHW) shortly after spawn. Hammerschmidt et al. [13] also previously demonstrated very little dissociation of MeHg from food prepared using the

methods and concentrations employed in the present study occurs in static well water. It is therefore reasonable to conclude that adult exposure to MeHg was a result of dietary sources, and exposure to eggs occurred via maternal transfer.

Sexually mature fathead minnow ovaries contain oocytes of all developmental stages, as they are asynchronous spawners. Total maturation time for each oocyte is approximately 3 days [14]. In order to ensure all eggs used in the present study were exposed to maternally transferred MeHg throughout all stages of oogenesis, eggs produced during the first five days of the experimental diet administration were not included in the data. Thereafter, breeding tiles from each tank were inspected for attached embryos every morning. Frequency of spawning, clutch size, and total egg production for each diet were tabulated by first finding a mean for each replicate (n=5), then finding the mean of the replicates to obtain a treatment mean. Subsamples of twenty fertilized embryos (determined by microscopic examination) from each clutch were transferred to a crystallizing dish containing RMHW and methylene blue (to discourage fungal growth), and gently aerated with an air stone. The remainder of each clutch was then dried and analyzed for Hg content.

Effects of maternally-transferred MeHg on embryo development were assessed by comparing the mean number of movements per minute observed in subsamples of embryos daily for 7 days post fertilization (DPF). In order to attain mean number of movements per minute, each clutch was transferred to a petri dish containing RMHW under an Olympus dissecting scope equipped with a Canon Vixia HF G30 video camera. After a 2 minute acclimation period on the stage of the microscope, each clutch was recorded for 1.5 minutes. Movement counts were observed for all 20 embryos in each clutch subsample over a consistent

time frame within the recording. This was done to ensure standardization of environmental factors affecting startle responses. Mean movements per minute for each diet were calculated using the means of each individual clutch (control diet: n=5, low diet: n=5 and high diet: n=6).

At 5 pm each day until 7 DPF, the number of hatched embryos in each clutch were counted and used to calculate a cumulative percent hatch rate for each clutch. Mean hatch rates for diets were calculated using the mean observed hatch rate for each tank in the treatment.

*Mercury Determination*

Skretting starter crumble, experimental diets, embryos and adult tissues (muscle and gonads) were analyzed for total mercury with a DMA-80 Direct Mercury Analyzer (Milestone Inc., Monroe, Connecticut) according to US EPA Method 7473. Briefly, samples loaded in quartz boats were heated in a decomposition furnace, dried, then thermally and chemically decomposed. Products are carried to the catalytic section of the furnace, where oxidation is completed. A stream of oxygen carries remaining products to an amalgamator, were mercury is trapped. As the amalgamator is then rapidly heated, mercury vapor is carried through absorbance cells of a single wavelength (253.7 nm) atomic absorption spectrophotometer [15].

Calibration curves were generated using three reference materials from the National Research Council of Canada: MESS-3 (marine sediment: certified value = 0.091 ± 0.009 ppm dry weight), TORT-2 (lobster hepatopancreas: certified value = 0.270 ± 0.060 ppm), and DOLT-4 (dogfish liver tissue: certified value = 2.580 ± 0.220 ppm). Quality assurance included blanks, duplicate samples, and reference samples. Blank (empty boats) were analyzed every 20 samples, and concentrations for all blanks (n=38) were below the method detection limit

(MDL). The MDL, determined as the standard deviation of seven replicates multiplied by 3, was estimated to be 0.002 ppm in a 0.02 g sample. Duplicate samples were analyzed every 20 samples with a mean relative percent difference of 10.2 % (range = 12.5 %, n = 12). Reference samples (MESS-3, TORT-2 and DOLT-4) were analyzed every 10 samples with a mean percent recovery for each of: MESS-3 101.7 ± 5.2% (n= 6), TORT-2 109.6 ± 3.2% (n = 7), and DOLT-4 105.1 ± 1.3% (n=7).

### *Statistical Analyses*

Data were analyzed using JMP 11.1 (Cary, NC). Normality of the data was confirmed using the Shapiro-Wilk test. Adult muscle and gonad total Hg concentrations were evaluated for differences using a two factor ANOVA followed by a Tukey's *post hoc* test, with gender and food mercury concentration as factors. Single factor ANOVA models were used to compare data for spawning frequency, mean clutch size, and mean egg output, using food mercury concentration as a factor. Embryonic movement was evaluated using a repeated measures ANOVA followed by a Tukey's *post hoc* test. Median time to hatch ($ET_{50}$) for each diet was calculated using an inverse prediction of the 95% confidence interval data in JMP. An $\alpha = 0.05$ was used to determine significance for all statistical analyses.

## Results and Discussion

### *Accumulation of Mercury in Adults and Eggs*

Gonad and muscle Hg concentrations in both genders were significantly different among all three diets (ANOVA, DF= 2, F= 68.6, $p < 0.01$ and ANOVA, DF= 2, F= 322.2, $p < 0.01$, respectively). Mean gonad Hg concentrations (Table 2.1) were significantly different between

genders in fish administered the 5.5 ppm diet (ANOVA, DF= 5, F = 77.2, $p < 0.01$). Males had significantly higher gonad concentrations than females, which may be attributed to losses of contaminants due to maternal transfer of dietary methylmercury to eggs [17-18]. Mean muscle Hg concentrations (Table 2.1) did not significantly vary between genders (ANOVA, DF= 5, F = 197.6, $p > 0.5$) with mean dry weight concentrations of 0.32 ppm (control), 3.02 ppm (low), and 16.03 ppm (high). These dry weight concentrations are roughly equivalent to wet weight concentrations of 0.06 ppm (control), 0.60 ppm (low), and 3.21 ppm (high), and are similar to concentrations in piscivorous freshwater fish in North America. A comprehensive analysis of Hg concentrations in freshwater fish collected from over 5,000 sites in Canada reported mean wet weight Hg concentrations in 104 fish species, including Walleye, Northern Pike, and Lake trout, three important sport fish in Canada. The median Hg concentrations for these three species were 0.41, 0.38, and 0.28 ppm, with maximum concentrations of 10.4, 10.9, and 10 ppm, respectively [16]. Wet weight muscle concentrations measured in fish fed the low MeHg diet in this feeding study are comparable to mean concentrations observed in piscivorous freshwater fish in North America. Wet weight muscle concentrations in fish administered the high MeHg diet in the present study are still well below maximum reported tissue concentrations in wild caught fish.

Table 2.1. Mean total mercury concentrations (± 1 SD) in muscle and gonad from adult fathead minnows fed one of three experimental diets.

| Diet | Gender | n | Mean Muscle Hg (ppm DW) | Mean Gonad Hg (ppm DW) |
|---|---|---|---|---|
| 0.02 ppm | Female | 15 | 0.33 ± 0.13 | 0.068 ± 0.01 |
| | Male | 5 | 0.23 ± 0.06 | 0.032 ± 0.04 |
| 0.87 ppm | Female | 15 | 3.11 ± 0.34 | 1.17 ± 0.09 |
| | Male | 5 | 2.72 ± 0.47 | 1.36 ± 0.30 |
| 5.5 ppm | Female | 15 | 16.50 ± 1.38 | 11.00 ± 2.78* |
| | Male | 5 | 14.89 ± 3.43 | 16.90 5.06* |

*Denotes significant differences in Hg concentrations between genders of the same treatment.

Mean dry weight Hg concentrations for clutches in each treatment were obtained by finding the mean Hg concentration of all clutches spawned on days 5-30 of the dietary administration from each replicate (tank), which were then used to calculate a treatment mean. The mean dry weight Hg concentration for the low diet was 0.13 ± 0.04 ppm (n=5), the mean for the high diet was 1.65 ± 0.35 ppm (n=5), and all control clutches (n=5) were below the detection limit. As previously mentioned, eggs from the control and low treatments remained relatively constant for the duration of the feeding study, while eggs from the high diet continued to increase over time. It is worth noting that the continued rise of Hg concentrations in eggs from the high treatment introduces a larger degree of variance within each replicate, not reflected in the SD associated with the treatment mean. The proportion of mercury

mobilized to eggs increased with increasing dietary concentrations (Figure 2.1). The concentrations of Hg in embryos rapidly increased in a dose dependent manner following the first administration of MeHg dosed food, indicating egg concentrations are largely a function of maternal dietary concentrations.

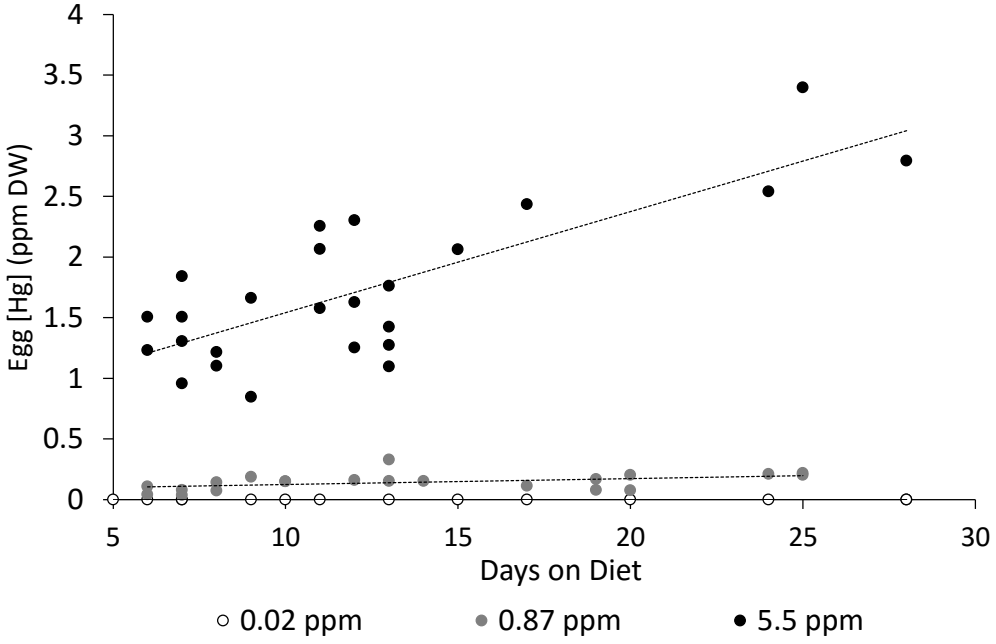


Figure 2.1. Concentration of total mercury in eggs from adult fathead minnows fed one of three methylmercury spiked diets for 30 days. Each data point is representative of mean Hg concentration measured in an individual clutch.

Clutches from the low treatment tanks quickly reached an equilibrium concentration following the first several days of the feeding study, however, the concentrations measured in eggs from the high diet continued to increase for the duration of the study (Figure 2.1). As fish in all treatments were exposed to unchanging dietary concentrations at consistent intervals for

the duration of the study, time of egg exposure (duration of oogenesis) remains relatively constant [14], and uptake of Hg by oocytes is thought to be transporter dependent [17-19], a plateau in egg Hg concentrations would be anticipated in all treatments if maternal transfer were dependent only on dietary Hg sources. The association between increasing exposure length and increasing egg concentrations suggests maternal body burden may also contribute to the total concentration of Hg transferred to eggs, albeit to a much lesser extent than maternal diet during oogenesis. The increasing Hg concentrations observed over the course of the study in eggs from the high treatment make comparison with female muscle concentrations more difficult to accurately characterize. Nonetheless, the available data indicates egg mercury concentrations are approximately 4.3% (low), and 9.9% (high) of the mean muscle Hg concentration found in the females in each treatment (sampled at the conclusion of the study), and were significantly different by dietary mercury concentration (ANOVA, DF= 2, F= 220.9, $p < 0.01$).

A study by Stefansson et al. [17] employed the use of stable MeHg isotopes to investigate the sources of mercury transferred to eggs. Adult sheepshead minnows (*Cyprinodon variegatus*) were exposed to one of three MeHg spiked diets containing 1, 5, or 10 ppm Hg (dry weight). The diets administered during the pre-oogenesis stage contained different MeHg isotopes than the diet administered during oogenesis, allowing for characterization of the proportion of Hg in eggs derived from maternal body burden versus maternal diet during oogenesis. Results indicated that a constant percentage of maternal body burden was transferred to eggs across all treatments, however the majority (range 84-94%) of total Hg found in eggs was from recent maternal dietary exposure. Consequently, in fish with

high body burdens, such as those seen in the 5.5 ppm treatment in the present study, historical exposure may be an important source of maternally transferred MeHg in eggs. These findings offer a possible explanation for the increasing concentrations seen in eggs, as maternal body burden would have continued to increase over the 30-day administration of MeHg spiked diets.

*Reproduction*

No significant effects of dietary MeHg were observed on any reproductive endpoints measured in adults during the 30-day feeding study (Figure 2.2), including mean clutch size (ANOVA, DF= 2, F= 3.2, p = 0.08), mean egg output (ANOVA, DF= 2, F= 0.54, p = 0.59), and spawning frequency (ANOVA, DF= 2, F= 1.0, p = 0.41). Although muscle Hg concentrations resulting from experimental diets in the present study exceeded the 0.2 ppm wet weight threshold thought to be protective against changes in reproduction, this lack of effect was anticipated [1, 4]. Penglase et al. [18] demonstrated that zebrafish fed MeHg spiked diets (12 ppm Hg dry weight) displayed increased mating (+66%) and overall reproductive success (+100%) when compared with controls during the first 100 days of the feeding study, after which both metrics declined steadily until the conclusion of the study. By day 206, mating success in fish exposed to dietary Hg was 49% lower than controls, while reproductive success was 37% lower when compared to control fish. Hammerschmidt et al. [13] also found that spawning success of adult fathead minnows is not affected by short-term exposure to dietary MeHg concentrations similar to those used in the present study, but rather by long-term exposure as juveniles. Fish administered one of three MeHg spiked diets (0.88 ppm Hg, 4.11 ppm Hg, or 8.46 ppm Hg dry weight) as juveniles, followed by control diets as adults, displayed

reduced spawning success for the duration of the study. Conversely, fish fed a control diet until sexual maturity, followed by MeHg spiked diets thereafter, did not display reduced spawning success in spite of increasing body burdens during the 136-day exposure period. All fish used in the present study were sexually mature prior to dietary exposure, and exposure duration was significantly shorter (30 days). Therefore, it is not surprising that no effects on reproductive metrics were observed as concentrations used in the present study were comparable to the low, and medium diets used by Hammerschmidt et al. [13].

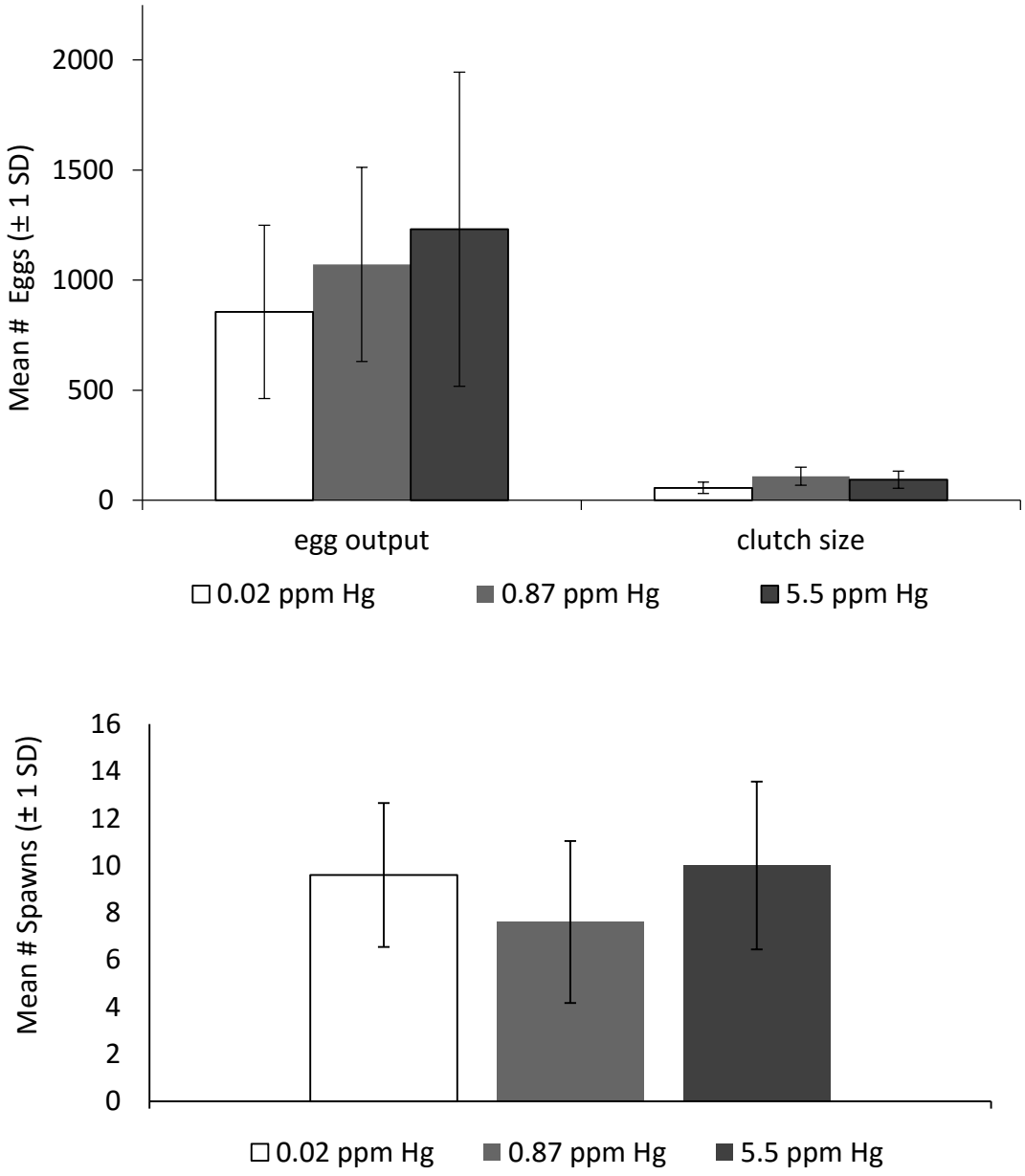


Figure 2.2. Reproductive metrics from adult fatheads fed one of three methylmercury spiked experimental diets. No significant effects on mean clutch size ($p = 0.10$), mean egg output ($p = 0.59$), or mean number of spawns ($p = 0.41$) were observed between diets. Mean egg output and mean clutch size were both calculated using means from each replicate tank ($n = 5$) within each dietary treatment.

*Embryonic Movement and Survival*

Patterns in movement frequency in embryos followed a similar trend in all mercury diet treatments (Figure 2.3). This consisted of little to no movement 0-1 DPF, followed by a spike to the maximum number of movements per minute 2 DPF, and then decreasing movement each DPF until hatch. However, the 0.87 ppm Hg treatment embryos had greater number of movements 2 DPF (Figure 2.3) than the other two treatments (ANOVA DF= 21, F= 34.85, $p < 0.01$). At 3 DPF, the 0.87 treatment eggs still displayed a higher number of movements than the other two treatments (similar number of movements to the maximum for the other 2 treatments which occurred 2 DPF).

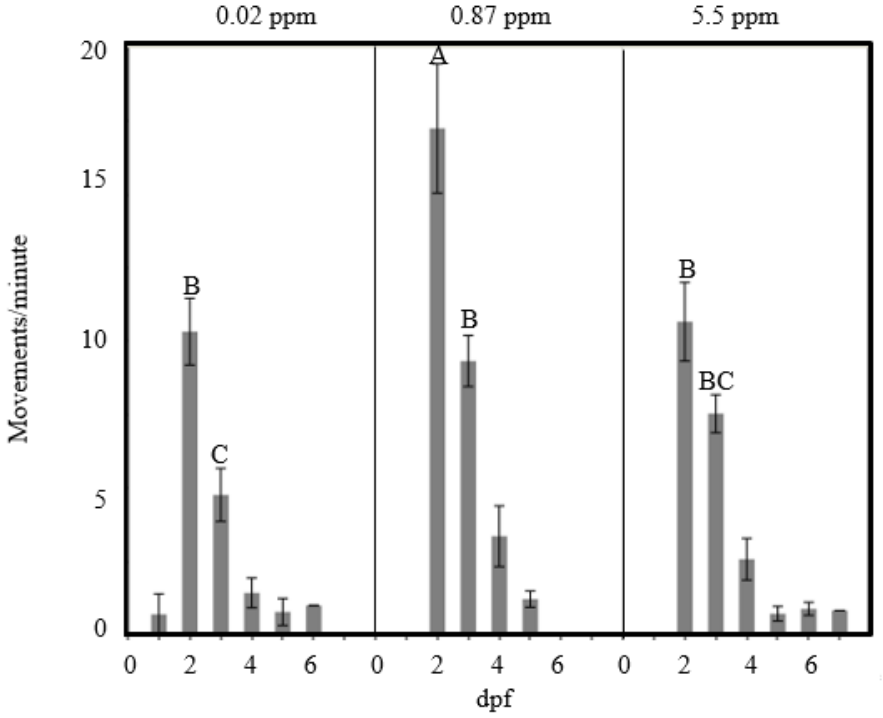


Figure 2.3. Mean embryonic movement per minute, by treatment (± 1 SE). Letters denote statistically different groups. Mean movements per minute for each treatment were calculated using means of individual clutches (control: n= 5, low: n= 5, high: n= 6).

A study conducted by Webber [5] found a 90 day exposure to environmentally relevant concentrations of dietary MeHg (range 0.46 ppm-0.96 ppm) disrupted several predator avoidance behavior patterns, and led to hyperactivity in golden shiners. Fish startle responses involved in predator evasion are initiated by Mauthner neurons, followed by recruitment of many other neurons to complete the response. Mauthner cells develop very early during fish embryogenesis, and are responsible for initiating embryonic movements analogous to adult startle responses [19]. Considering the results of the present study, in conjunction with the findings of Webber [5], it is possible that the significantly increased embryonic movement may be a result of MeHg induced neurotoxicity interfering with Mauthner neuron function.

In conjunction with this increased movement pattern, a decrease in time to hatch was observed in the 0.87 ppm Hg diet. The calculated $ET_{50}$ (± 95% CI) for hatch in the 0.87 ppm treatment (4.04 ± 0.12 DPF) was approximately a full day sooner than the control (4.95 ± 0.11 DPF) and high (5.40 ± 0.12 DPF) treatments (Figure 2.4). Startle responses in fish embryos serve as a mechanism to distribute hatching enzymes responsible for breakdown of the egg chorion, and the significantly earlier hatch time is possibly a result of increased enzyme distribution due to increased embryonic movement beginning 2 DPF [19]. Premature hatch was also observed in zebrafish *(Danio rerio*) embryonically exposed to waterborne concentrations as low as 0.010 ppm MeHg [20]. Our data suggest that maternally transferred mercury, a more ecologically relevant mode of exposure compared to waterborne dosing, also affects embryonic development in fish.

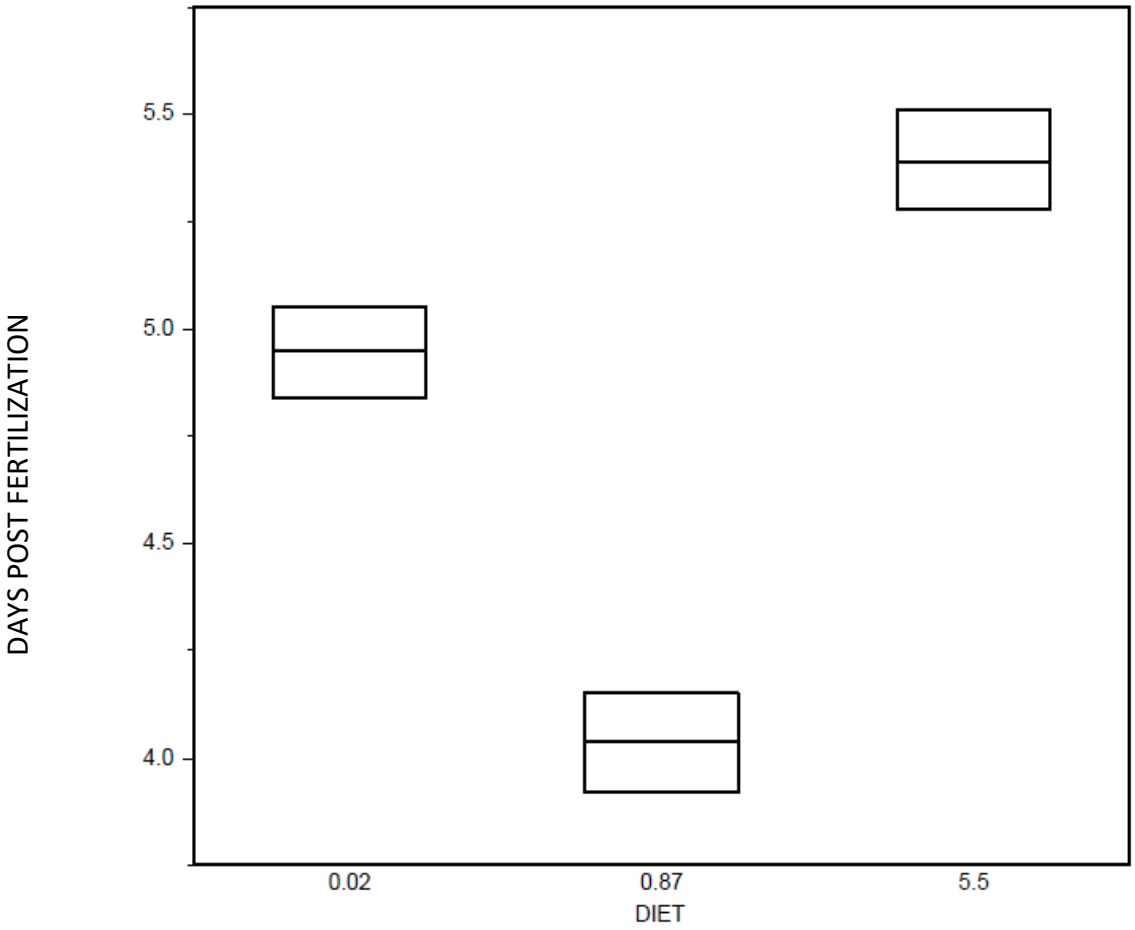


Figure 2.4. Median time to 50% hatch (ET50) for egg clutches from adult fathead minnows fed one of three experimental mercury diets. Boxes represent 95% confidence intervals.

Embryos from the 5.5 ppm diet experienced a delayed hatch as demonstrated by the $ET_{50}$ values (Figure 2.4). Embryo-larval mortality during the 7 DPF observation period was significantly higher in offspring from the 5.5 ppm treatment (41.6% ± 16.6, Tukey's HSD $p < 0.04$) when compared with the offspring from the 0.87 (21.5% ± 2.3) and control diets (24.6% ± 9.0) (ANOVA, DF= 2, F= 5.7, p = 0.02). Anecdotally, a large number of surviving offspring from the 5.5 ppm treatment also displayed spinal deformities (Figure 2.5) and circular swimming patterns, alterations that could have affected movement patterns within the chorion. These

results suggest individuals from the 5.5 ppm diet may have been compromised during development. Several other studies have shown physiological alterations in developing fish associated with increased mortality can occur at very low MeHg exposures. For example, reduced heart rates, reduction in survival of embryo-larval stages, and dose–dependent decreases in hatching success were observed in eggs from walleye in Clay Lake, Ontario following exposure to increasing environmental concentrations of waterborne MeHg (0.1-7.8 ppm). However, in contrast to the results of the present study, hatching success remained unaffected by exposure to maternally transferred MeHg [9]. Zebrafish exposed to waterborne concentrations as low as 0.010 ppm MeHg also displayed dose dependent reductions in survival, culminating in 100% mortality in a 0.5 ppm exposure group 24 hours post fertilization [20]. The mean Hg concentration of fathead minnow eggs from adults fed the 5.5 ppm diet was well above (1.72 ± 0.63 ppm MeHg) concentrations shown to cause physiological changes and increased mortality in zebrafish.

Figure 2.5. Offspring from adults administered the 5.5 ppm Hg diet displayed physiological abnormalities, such as the spinal deformities observed here.

## *Conclusions*

Assessments concerning the effects of MeHg on fish health have largely focused on toxicity to adult animals [1, 4, 9]. However, it is generally accepted that early life stage exposure to most toxicants can lead to irreversible, adverse impacts at much lower concentrations than those seen in adults [1, 2]. Here we show that maternal transfer of dietary MeHg led to concentrations in eggs that significantly altered movement behaviors associated with survival in embryonic fish during a 30-day exposure. Consequently, significant effects on hatch and survival were observed. These results indicate dietary MeHg concentrations at environmentally relevant concentrations may contribute to reduced recruitment of young. Further research is needed to determine the extent to which these changes in behavior and survival may affect individual fitness, and population structure.

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# CHAPTER 3

# EFFECTS OF DIETARY METHYLMERCURY ON THE DOPAMINERGIC SYSTEM OF ADULT FATHEAD MINNOWS AND THEIR OFFSPRING[2]

## Introduction

Mercury is a ubiquitous environmental contaminant released through a variety of anthropogenic activities and natural processes. Following volatilization into the atmosphere, mercury can undergo long-range transport. Once oxidized by atmospheric components, wet or dry deposition can introduce mercury into aquatic ecosystems, where it partitions into sediments. In sediment, inorganic forms of mercury may be transformed by bacteria into organic forms [1]. Organic mono-methylmercury (MeHg), is a highly bioavailable form capable of bioaccumulation and biomagnification. As a result, long-lived organisms at higher trophic levels, such as piscivorous fish, have the potential to accumulate high body burdens of this potent neurotoxin. Fish consumption is the primary route of human exposure to MeHg, and is of particular concern in populations that rely on fish as a key source of dietary protein [2, 3].

Methylmercury has a strong bonding affinity for reduced sulfur atoms, and forms conjugates with sulfur containing biomolecules. When bound to these biomolecules, the resulting compounds may resemble endogenous amino acids, which are actively transported by amino acid transporters [4]. This provides a mechanism of transfer for dietary methylmercury across the blood brain barrier, from adult fish to eggs during oogenesis, and from maternal circulation to fetus across the placenta in humans [3, 5, 6]. A study relating maternal MeHg intake to fetal cord blood in humans demonstrated that fetal levels of MeHg were actually

higher than those in maternal circulation, possibly due to unidirectional active transport via amino acid transporters across the placenta [7].

Though neurotoxicity due to MeHg exposure occurs in adults, it is of particular concern to developing organisms undergoing neurogenesis [5, 8]. The developing nervous system is particularly susceptible to the toxic effects of MeHg, resulting in a range of neurological defects at much lower exposure concentrations [9]. Data on delayed developmental milestones collected from children exposed to MeHg in utero following a mass poisoning in Iraq concluded the human nervous system had a 5-fold greater vulnerability to MeHg during early development [10]. Though mass poisonings such as the one in Iraq are uncommon, studies are emerging that suggest even low levels of prenatal exposure to MeHg, such as the levels associated with regular fish consumption, may result in neurocognitive effects in early childhood, and may also be associated with Attention Deficit Hyperactivity Disorder (ADHD) [2, 11]. In light of the serious implications for human health and development, there is a clear need for research into mechanisms by which MeHg causes developmental neurotoxicity.

Transgenerational effects of maternally derived MeHg in fish are also understudied, and the exact mechanisms by which neurological insult occurs are not fully understood [12-15]. Similar to the association of ADHD with developmental MeHg exposure, we previously observed significant hyperactivity in fathead minnow embryos maternally exposed to MeHg at environmentally relevant concentrations. The significant increase in embryonic movement was correlated with decreases in hatching times, attributed to increased distribution of hatching enzymes [16]. Consistent with these results, Mora-Zamorano et al [17] found increased locomotor output in larval zebrafish exposed to maternally derived MeHg. A mechanism for

the observed hyperactivity in embryonic and larval fish exposed to maternally derived MeHg has not yet been proposed to our knowledge, though early life stage exposure in rats has been shown to alter the function of the dopaminergic system, leading to changes in dopamine (DA) receptor-mediated motor activity [18, 19].

Spontaneously hypertensive rats (SHR) are widely used as a model for ADHD, and numerous studies have linked altered activity of the dopaminergic system to hyperactivity [20]. Increased transcription of the D1 DA receptor in the brain has been characterized in SHR rats, and treatment with D1 receptor antagonists has been show to significantly reverse hyperactive behaviors [20, 21]. Optimal doses of ADHD medications (methylphenidate and atomoxetine) also improved ADHD symptoms in rats and monkeys, the effects of which were reversed following administration of D1 receptor antagonists [22, 23]. These results indicate dopaminergic activity plays a central role in the manifestation, and treatment of hyperactivity in higher vertebrates. Given the highly conserved nature of the dopaminergic system across taxa, it is likely hyperactivity in fish may also be explained by alterations to this neurotransmission system.

Dopamine (DA) is associated with cognition, attention, reward-motivated behaviors, and motor function, and MeHg has been shown to increase spontaneous release of DA in larval mummichogs, and adult walking catfish exposed to waterborne MeHg [24, 25]. Neurotransmitter cycling is a complex process, and it is widely accepted that exposure to neurotoxicants, such as MeHg, can alter these cycles. Dopamine concentrations at the synapse are mediated by vesicular release mechanisms, numerous transporters and receptors, and enzymatic degradation [6, 18, 26]. Modulation of any one of these control mechanisms can

have implications for neurotransmission. One of these mechanisms that has previously been shown to be negatively affected by the presence of MeHg, is enzymatic degradation of DA by monoamine oxidase (MAO). Adult walking catfish exposed to 40 ppb waterborne MeHg for 40 days, and adult salmon exposed to 10 ppm dietary MeHg for four months showed significant reductions in brain MAO activity [24, 26, 27].

Here we evaluate the effects of dietary methylmercury on DA concentrations and MAO in fathead minnow offspring exposed to MeHg via maternal transfer. Both DA and MAO are highly conserved components of neurotransmission across species, and the goal of the present study is to elucidate possible mechanisms which may explain hyperactive behaviors observed in several species of organisms exposed to MeHg during neurodevelopment [28, 29]. We also characterized DA concentrations in various brain regions of adult fathead minnows, as studies examining MeHg exposure and changes in brain DA concentrations in these fish have typically provided whole-brain measurements which may obscure potentially significant regional changes [30]. Additionally, long-term exposure to MeHg has been shown to decrease reproduction in teleosts, and both DA and MAO are key modulators in neuroendocrine control of reproduction [30, 31].

## Materials and Methods

### *Animal Care*

All procedures using fish were approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC) under protocol #1303-3. Reproductively active, adult fathead minnows were obtained from Aquatic Biosystems (Fort Collins, CO), and divided among 20, 21 L glass aquaria. Each tank contained three females (control: 0.94 ± 0.18 g, Hg treated:

0.97 ± 0.30 g wet weight) and one male fish (control: 2.19 ± 0.43 g, Hg treated: 2.56 ± 0.15 g wet weight) as well as two breeding tiles constructed from halved PVC pipe sections. All tanks were equipped with bio sponge foam filters, heaters, and were on a photoperiod of 16h light: 8h dark. Tanks were filled with reconstituted moderately hard water (24±1 ºC, pH 7.2-7.8, DO 5, mg/L, hardness 80-100 mg/L $CaCO_{3)}$ and maintained by daily siphoning of waste and debris, and 25% water changes every two days. Water quality and temperature were monitored using salinity and pH probes in addition to test strips for nitrates/nitrites. All tanks received the same food (Skretting starter crumble, Tooele, UT) for two weeks in order to establish baseline reproduction. At the conclusion of the study adult fatheads were euthanized using buffered MS-222 at a concentration of 250 ppm, according to the IACUC protocol.

*Experimental Design*

Following the two-week acclimation period, aquaria were randomly assigned either a control diet containing 0.02 ± 0.0005 ppm Hg dry weight, or a MeHg spiked experimental diet containing 0.72 ± 0.013 ppm Hg dry weight. The concentration of the MeHg spiked diet was selected, as it represents the range of concentrations seen in benthic invertebrates and zooplankton in some North American lakes when normalized to caloric density. In adult fish, exposure to MeHg is primarily through diet, and for larval fish almost all exposure to MeHg is through maternal transfer. Therefore, these were the exposure routes chosen in order to ensure environmentally relevant exposure scenarios [5, 32, 33]. All aquaria were administered similar quantities of food two times per day for 30 days. Diets were prepared by mixing fish food with methylmercury chloride (Sigma-Aldrich) dissolved in reagent ethanol (Fisher) in an

acid washed glass dish. Ethanol was removed by evaporation in a fume hood. Food was prepared in accordance with the methods used by Hammerschmidt et al. [32]. Samples of each diet were analyzed for Hg as described in the mercury determination section. Waterborne MeHg from unconsumed food pellets was not considered a significant route of Hg exposure for adult fathead minnows, or their offspring [32, 34].

Fathead minnows spawn asynchronously and ovaries of sexually mature fathead minnow ovaries contain oocytes of all developmental stages, with a total maturation time approximately 3 days per oocyte [35]. In order to ensure all eggs used in the present study were exposed to maternally transferred MeHg throughout all stages of oogenesis, eggs produced during the first seven days of the experimental diet administration were not included in the data. Thereafter, breeding tiles from each tank were inspected for attached embryos every morning. Frequency of spawn, clutch size, and total egg production for each diet were calculated by finding a mean for each replicate (n = 10), then finding the mean of the replicates to obtain a treatment mean. Clutches were transferred from breeding tiles to a crystallizing dish containing RMHW and methylene blue (to discourage fungal growth) 0 days post fertilization (DPF), and gently aerated with an air stone. In order to avoid fluctuations in environmental factors, all clutches were cultured at 23° C in an environmental chamber on a 16:8 h photoperiod. Every 5th day, whole clutches from each treatment were dried and analyzed for Hg content via DMA analysis in order to monitor Hg concentrations in eggs for the duration of the study.

In order to confirm increased embryonic motor activity seen 2 DPF in a previous study, we compared the mean number of movements per minute observed in subsamples of fertilized

embryos, daily, up to 3 DPF using the methods described in Chapter 2 [34]. Mean movements per minute for each diet were calculated using the means of each individual clutch (control diet: n=9, Hg diet: n=9).

### *Mercury Determination*

Skretting starter crumble, experimental diets, embryos and adult tissues (muscle, gonad, and brain) were analyzed for total mercury using the methods and quality assurance protocols described in Chapter 2 [34]. The MDL, determined as the standard deviation of seven replicates multiplied by the corresponding t-value for the 95% confidence interval, was estimated to be 0.001 ppm in a 0.02 g sample. Duplicate samples were analyzed approximately every 10 samples, with a mean relative percent difference of 3.5 % (range = 0.26 – 4.73%, n = 10). Reference samples (MESS-3, TORT, DORM and DOLT-4) were analyzed every 10 samples with a mean percent recovery for each of: MESS-3 102.6 ± 5.77 % (n = 8), TORT 105.28 ± 3.12 % (n = 6), DORM 104.38 ± 2.49 % (n = 4) and DOLT-4 102.64 ± 5.86 % (n = 8).

### *Dopamine Extraction*

Entire clutches of eggs were collected for analysis of DA concentrations either 1, 2, or 3 DPF. Fertilized eggs from each clutch were transferred from crystalizing dishes into individual, pre-weighed microcentrifuge tubes, and immediately frozen at -80 degrees. Once completely frozen, clutches were weighed, and homogenized using a motorized pellet pestle. Homogenized eggs were then placed in a refrigerated (4°C) centrifuge for 5 minutes at 13,300 RPM. Supernatant was immediately transferred to a new microcentrifuge tube, and spiked with an appropriate volume of a 1 ppm stock solution of 1,1,2,2-D4 dopamine obtained from

Cambridge Isotope Laboratories Inc. (Tewksbury, MA) as an internal standard (IS), to reach a final concentration of 100 ppb. A liquid-liquid extraction was then performed with cold acetonitrile at a volume to mass ratio of 5:1 (Sigma Aldrich), followed by repeated cycles of freezing and centrifugation (4°C, 13,300 RPM) until no precipitate was observed.

Brains from sacrificed fish were immediately dissected on ice into three sections. The olfactory bulb/telencephalon (FB), cerebellum/pons/medulla (HB) were removed from the brains, and remaining tissue was categorized as mid-brain (MB). Dissected brain regions were transferred to microcentrifuge tubes, weighed, and snap frozen in in liquid nitrogen. Samples were then stored in a -80 freezer to avoid degradation of analytes. A subsample of brain tissue from each diet was used for DMA analysis, and all remaining samples were either analyzed for DA concentrations, or used to determine MAO activity.

Brain tissue samples used for analysis of DA were weighed, spiked with appropriate volumes of 25 ppm IS to reach a final sample concentration of 0.5 ppm, and homogenized using a motorized pellet pestle. Cold acetonitrile (Sigma-Aldrich) was added to the microcentrifuge tubes at a 5:1 volume to mass ratio, followed by incubation in a -80 freezer for ~45 minutes. Samples were centrifuged for 5 minutes (13,300 RPM at 4°C), and the supernatant was removed and transferred to a new microcentrifuge tube. Cycles of freezing and centrifugation were repeated until no precipitate was observed. All samples were stored in a -80 freezer until LC-MS/MS analysis.

*LC-MS/MS Analysis of Dopamine*

Concentrations of DA in extracts were obtained using a Waters Model 2695 HPLC with a Micromass Quattro Ultima triple quadrupole mass spectrometer at the University of North

Texas, fitted with a Phenomenex Hydro-RP column (150 mm x 3.0 mm) (Torrance, CA). The binary mobile phase (0.2 mL/min) consisted of an ultrapure water (A) and methanol (B) gradient, both with 0.1% formic acid. The gradient was held at 100% A until DA eluted at 5 min. and was then ramped over 10 min. to 100% B where it was held for 5 min and then ramped over 5 min. to starting conditions of 100% A where it was held for 5 min for equilibration. One µL of extract was injected and the following transitions were monitored: D4-DA: 158>147 and DA 154>137. For analysis of embryos, a 9-point standard curve ranging from 6 ppb-1600 ppb DA ($R^2$=0.999) was run prior to sample analysis, and a separate 6-point curve ranging from 50 ppb-1600 ppb was run for brain tissue. The D4-DA IS was held at 500 ppb for both curves. No DA was detected in any unspiked method blanks (n = 15), and method blanks spiked with 500 ppb DA (n = 8) had a mean percent recovery of 97.38 ± 23.704 (range 138.15 – 70.12 %).

*Monoamine Oxidase Activity Determination*

Inhibition of MAO activity in 3 DPF fathead minnow eggs, and adult brain tissue was determined with a MAO Assay Kit (MAK136, Sigma-Aldrich) using a range of MeHg concentrations as an inhibitor. MeHg concentrations used in each test were chosen to span approximate wet weight concentrations measured in eggs, or brain tissue samples from the present study. Clutches of control eggs were pooled to create 5 replicates, which were then homogenized in a microcentrifuge tube using a motorized pestle. The liquid portion of each homogenized sample was transferred to a new tube, which was then subjected to 2 cycles of centrifugation at 14,000 x g for 10 minutes to remove unwanted debris. Egg homogenate was diluted with assay buffer from the MAO kit, and pipetted into individual wells on a 96 well plate

which contained either ultrapure water, or one of 5 concentrations of MeHg (range 0-0.38 ppm) in ultrapure water, and incubated for 10 minutes.

Brains from control fish were homogenized using a disposable pestle, and diluted with assay buffer before centrifugation at 14,000 x g for 10 minutes. Supernatant was transferred into a new microcentrifuge, and centrifugation was repeated. Aliquots of supernatant were added to wells of a 96 well plate containing either ultrapure water, 0.19, 0.38, or 0.76 ppm MeHg (n = 6), and incubated in the dark for 10 minutes.

Following incubation with MeHg, master reaction from the MAO kit was added to all wells, which were incubated in the dark at room temperature for an additional 15 minutes. Fluorescence of all samples and blanks were measured using a plate reader (λ ex: 535, λ em: 590), alongside a 6-point standard curve containing 0- 20 μM $H_2O_2$ ($R^2$ = 0.999). Fluorescence for each replicate was normalized to total protein, obtained using a BCA protein assay kit (Sigma-Aldrich).

### *Statistical Analyses*

All data were analyzed using JMP 11.1 (Cary, NC). Normality of all data was determined using the Shapiro-Wilk test, and all data were determined to be normally distributed unless otherwise stated. Total mercury concentrations for adult muscle and gonad were evaluated for differences using gender and dietary Hg concentrations as factors in a 2-factor analysis of variance (ANOVA) with a Tukey's *post hoc* test. Reproductive data, including frequency of spawn, mean clutch size, and mean egg output was analyzed using a single factor ANOVA, by dietary Hg concentration.

Differences in embryonic activity between treatments by DPF were determined using a repeated measures ANOVA, followed by a Tukey's *post hoc* test. Mean DA concentrations in clutches from each treatment by DPF were analyzed using a 2 factor ANOVA (using diet and DPF as factors), followed by a Tukey's *post hoc* test. Dopamine data from brain regions was determined to be non-normal, and a Kruskall-Wallis test was performed. Data from MAO activity assays were analyzed using a single factor ANOVA. An $\alpha$ of 0.05 was used to determine statistical significance for all tests.

## Results and Discussion

### *Accumulation of Mercury*

The primary route of embryonic exposure to MeHg occurs as a result of maternal transfer to eggs during oogenesis via amino acid transporters [3, 5]. Methylmercury transported into eggs may then elicit neurotoxic effects, including disruption of important neurochemical signaling as the organism develops. Studies suggest that concentrations in the maternal diet, rather than body burden, determine embryonic MeHg exposure [5]. This corresponds with the data obtained in the present study, as Hg concentrations in embryos from treated tanks rapidly increased following the first administration of MeHg dosed food, after which it remained relatively constant. Eggs from tanks fed the Hg spiked diet had a mean Hg (± 1 SD) concentration of 0.23 ± 0.05 ppm dry weight (n = 8), which roughly corresponds to a wet weight Hg burden of 0.074 ppm, assuming 67% of fish eggs mass is attributed to moisture [36]. Control clutch concentrations (n = 8) had a mean dry weight concentration of 0.03 ± 0.02 ppm

Hg (wet weight equivalent = 0.0096 ppm). Mean dry weight Hg concentrations in eggs were significantly different between diets (ANOVA DF = 1, F = 92.27, $p < 0.001$).

Wet weight gonad Hg concentrations (Table 3.1) were significantly different between diets (ANOVA DF = 1, F= 179.37, $p < 0.001$), but not significantly different between genders for either treatment (control: 0.0239 ± 0.007 ppm Hg, n = 15; treated: 0.276 ± 0.071 ppm, n = 13). Wet weight muscle Hg concentrations (Table 3.1) were significantly different between diets (ANOVA DF = 1, F = 237.44, $p < 0.001$). Mean muscle Hg concentrations were significantly higher in females (ANOVA DF = 1, F = 55.23, $p < 0.001$) fed the Hg spiked diet (0.804 ± 0.084 ppm Hg, n = 8) than treated males (0.506 ± 0.039 ppm Hg, n = 5). This may be a result of increased food consumption by females in order to meet the energetic demands of egg production, as MeHg is generally found associated with proteins in the muscle of fish [37, 38]. Mean wet weight muscle concentrations of control fish were not different between genders (0.084 ± 0.017 ppm Hg, n = 13). The wet weight muscle concentrations measured in fish from the present study are similar to concentrations observed in piscivorous freshwater fish in North America, and are well below maximum reported tissue concentrations observed in wild caught freshwater fish [39]. This suggests concentrations and exposure routes used in the present study represent environmentally relevant exposure scenarios. Analysis of brain tissue for total mercury (Table 3.1) yielded average wet weight concentrations of 0.46 ± 0.23 ppm Hg in the treated fish (n = 5). Wet weight concentrations in control fish were 0.07 ± 0.01 ppm Hg (n = 5).

Table 3.1. Mean Hg tissue concentrations (± 1 SD). Letters denote statistically different groups.

| | CONTROL (ppm Hg wet weight) | MERCURY (ppm Hg wet weight) |
|---|---|---|
| FEMALE MUSCLE | 0.084 ± 0.02 (a) (n = 8) | 0.804 ± 0.08 (d) (n = 8) |
| MALE MUSCLE | 0.084 ± 0.02 (a) (n = 5) | 0.506 ± 0.04 (c) (n = 5) |
| FEMALE GONAD | 0.024 ± 0.007 (a) (n = 10) | 0.276 ± 0.071 (bc) (n = 8) |
| MALE GONAD | 0.028 ± 0.01 (a) (n = 5) | 0.204 ± 0.04 (ab) (n = 5) |
| BRAIN TISSUE | 0.07 ± 0.01 (n = 5) | 0.46 ± 0.23 (n = 5) |
| EGGS | 0.0096 ± 0.006 (n = 8) | 0.074 ± 0.02 (n = 8) |

*Embryonic Movement*

Exposure to MeHg during critical periods of neurodevelopment has been shown to affect embryonic motor activity, causing hyperactivity which may lead to early hatch. This is conceivably a result of increased distribution of hatching enzymes [40]. Patterns in movement frequency in embryos from both the control and MeHg spiked treatments followed a similar trend for each observed DPF, with no movement 0-1 DPF (n = 10), followed by maximum activity 2 DPF (n = 10), which dwindled by 3 DPF (n = 5).

Though the overall patterns observed were not different between treatments, the offspring of the tanks administered Hg spiked food showed significantly increased movement 2 DPF (Figure 3.1) when compared with controls (ANOVA, DF = 5, F = 31.84, $p < 0.01$). This confirms previous findings by Bridges et al. [34] in which significantly increased movements were observed 2 DPF in offspring of adults fed a 0.87 ppm diet. Embryonic hyperactivity 2 DPF

was correlated with significantly accelerated hatching rates, presumably due to increased distribution of hatching enzymes led to [34, 40]. Mora-Zamorano et al. [17] also attributed hyperactive behaviors observed in zebrafish larvae to embryonic MeHg exposure, and indicated these behaviors may increase the vulnerability of larvae to predators.

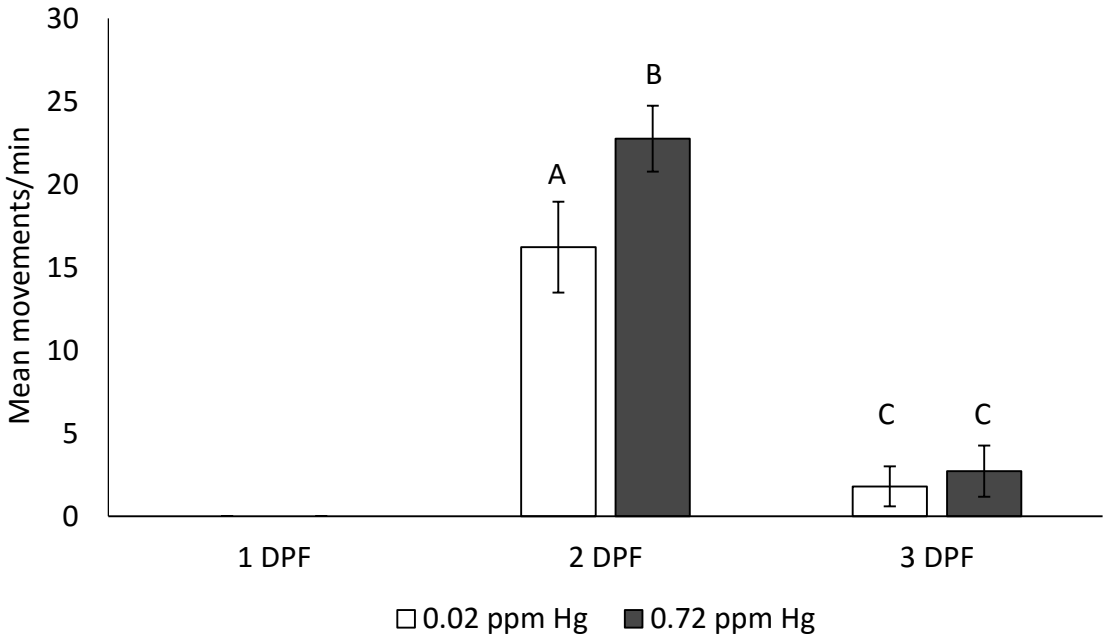


Figure 3.1. Mean number of observed embryo movements per minute (± 1 SE) for each diet by days post fertilization (DPF). Letters denotes statistically different groups ($p < 0.05$), n = 10 clutches for 0-2 DPF, and n = 5 for 3 DPF

*Effects on Dopamine and MAO in Eggs*

Significant differences in DA concentrations were observed between treatments (2-way ANOVA DF= 1, F= 11.48, p = 0.003) (Figure 3.2). Eggs from control tanks had significantly higher DA concentrations 3 DPF (1.927 ± 0.526 ppm DA) when compared with eggs exposed to

maternally transferred MeHg 3 DPF (0.568 ± 0.243 ppm DA), though no significant differences in DA concentrations were detected 2 DPF. These results were not anticipated, as DA is associated with motor activity, and significantly more embryonic movement was observed in treated offspring 2 DPF. As previously described, neurotransmitters, such as DA have complex cycling, and are controlled by tightly regulated systems involving enzymatic degradation, vesicular release mechanisms, a number of receptors, and transporters, all of which can be affected by the presence of MeHg. The concentration of DA at the synapse resulting from the cumulative activity of the aforementioned regulatory mechanisms, rather than overall tissue concentrations, ultimately influences the activity of dopaminergic neurons. It has been shown that vesicular release of DA from synaptosomes in rat hippocampal slices is increased in the presence of MeHg, while results of the present study show significant decreases in MAO activity in eggs incubated with MeHg concentrations as low as 0.048 ppm, when compared with controls (ANOVA, DF= 5, F= 9.43, $p < 0.01$) [19]. The MeHg concentrations used as an MAO inhibitor in the present study spanned those in eggs spawned in treatment tanks, and resulted in decreases in MAO activity that ranged from 9.30% to 17.04% (Figure 3.3). Enzymatic degradation of DA is catalyzed by MAO, consequently significant reductions in MAO activity due to the presence of Hg has negative implications for the rate at which enzymatic degradation of DA at the synapse can occur [29]. When coupled with possible increased release at synaptic terminals, a viable mode of action for the hyperactivity seen in developing embryos and fish larvae is presented.

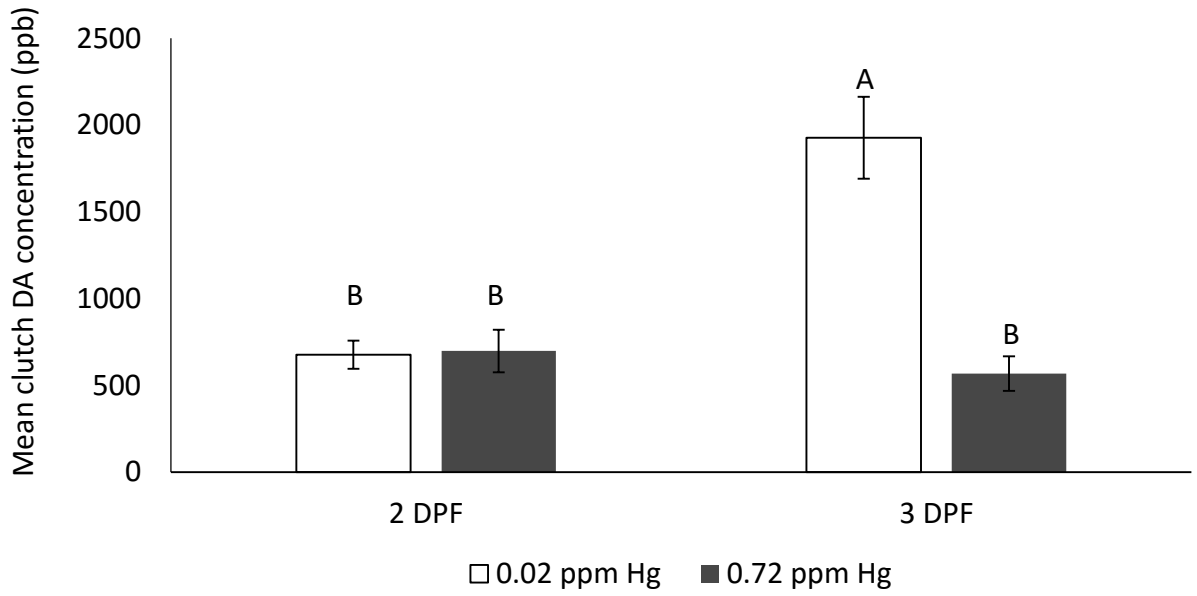


Figure 3.2. Mean dopamine (DA) concentrations in clutches (± 1 SE) for each diet by days post fertilization (DPF). Letters denote significantly different groups ($p < 0.05$), n = 6 for all groups.

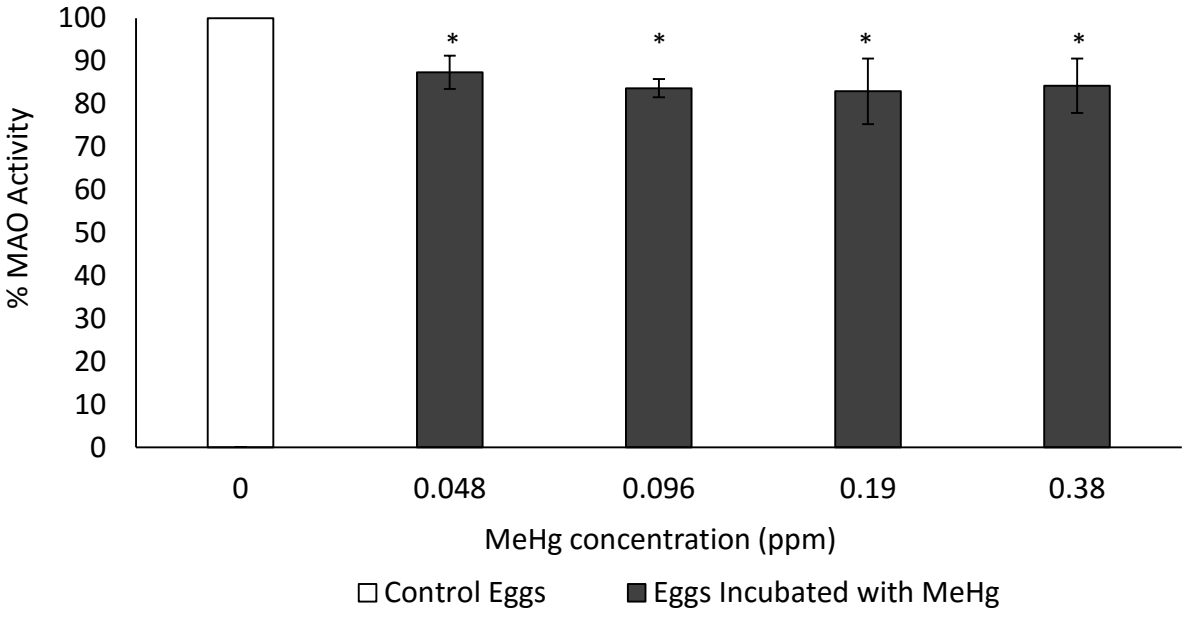


Figure 3.3. Mean % MAO activity (± 1 SD), relative to control eggs, measured in pooled clutches of 3 DPF fathead minnow eggs (n=5) following incubation with a relevant range of MeHg

concentrations. All MeHg exposed egg tissue showed significant reduction in MAO activity relative to controls ($p < 0.01$), but the effect was not dose dependent (* denotes statistical significance).

### *Dopamine Cycling in Adult Brain Tissue*

Brain regions showed significant differences in DA concentrations regardless of diet (Kruskal-Wallis, DF = 5, $X^2 = 17.89$, $p = 0.003$) with the highest concentrations found in the FB (Figure 3.4). Significant differences between treatment DA concentrations were also measured in the forebrain. Mean DA concentrations measured in FB of control fish were determined to be 2.07 ± 0.456 ppm, while those in fish from treated tanks were 1.29 ± 0.411 ppm. No significant differences were detected in any other brain regions, and when specific region values are grouped by diet to represent whole brain DA, no differences in concentrations are indicated. This suggests that whole brain quantification of DA may indeed obscure regional changes which may be associated with alterations in biological function, and behavior.

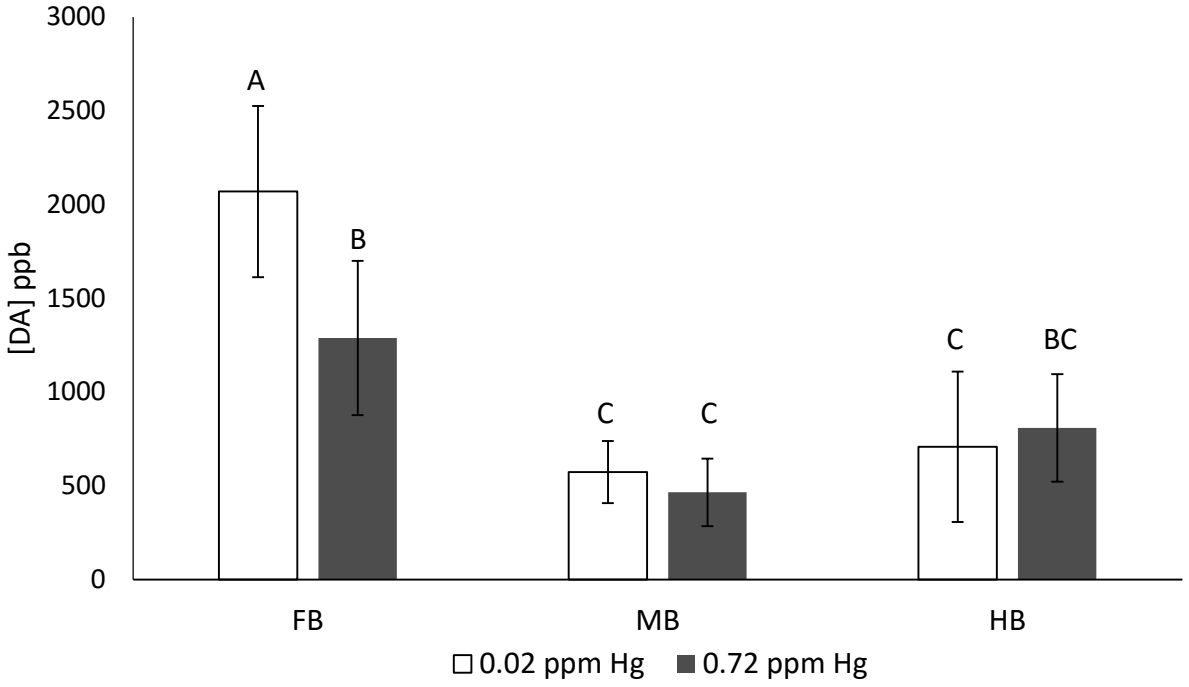


Figure 3.4. Mean dopamine (DA) concentrations in brain regions of adults from each diet (± 1 SD). Dopamine concentrations significantly varied between brain regions regardless of treatment. Mean DA concentrations in FB of control fish were significantly higher than those of Hg treated fish. Letters denote significantly different groups ($p < 0.05$), $n = 5$ for all groups.

Studies examining the function of the teleost telencephalon (categorized FB in the present study) have indicated this brain region processes olfactory information, as well as regulates behavioral outputs in the lower brain centers. Ablation of the telencephalon in teleosts leads to impaired learning and avoidance behavior, and lesions of the dopaminergic projection that connect lower brain centers with the telencephalon lead to reduced locomotor activity [41]. Results of the MAO assay performed on control brain tissue incubated with a range of MeHg concentrations showed that mean % MAO activity was significantly reduced in

all brain tissue exposed to MeHg relative to controls (ANOVA, DF = 3, F = 4.97, p = 0.01), with MeHg exposed brain tissue showing a mean reduction in activity of 17 ± 0.26 % (Figure 3.5). These results are not surprising, as the active site of MAO has been shown to be inhibited by chemicals which bind to sulfhydryl groups [42]. Sequencing of human MAO-A and -B has shown each subunit contains 9 cysteine resides. Of these residues, 2 in MAO-A, and 3 in MAO-B are essential for catalytic activity, as covalent binding of FAD is prevented in mutant forms of MAO which have serine substituted for cysteine [29, 43]. This seems to suggest that inhibition of MAO activity by MeHg may have serious implications for the developing nervous system in higher vertebrates and humans. As mentioned previously, it is possible that decreased enzymatic degradation of DA by MAO may counteract overall decreases in DA concentrations, allowing for short term maintenance of dopaminergic neuron activity in adult brains. It is also probable that vesicular release of DA is increased in brains of fish in response to MeHg exposure, as a similar response to MeHg exposure is indicated in studies using brain tissue from rats [18, 19].

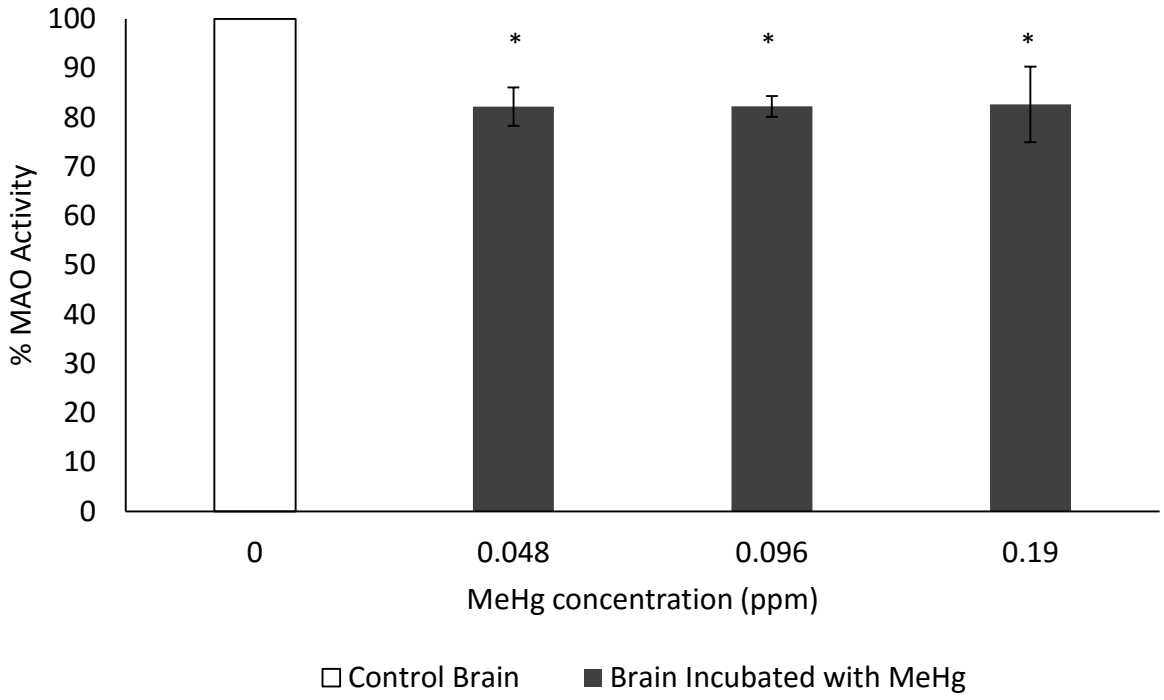


Figure 3.5. Mean % MAO activity (± 1 SD), relative to controls, measured in adult brain regions (n = 6) following incubation with a range of relevant MeHg concentrations. All MeHg exposed tissues, excluding the lowest concentration used in egg analysis, showed significant reduction in MAO activity relative to controls ($p < 0.01$), but the effect was not dose dependent (* denotes statistical significance).

*Reproduction*

No significant effects of dietary MeHg were observed on any reproductive endpoints measured in adults (Figure 3.6) during the 30-day feeding study, including mean egg output (ANOVA, DF = 1, F = 1.40, p = 0.61), mean clutch size (ANOVA, DF = 1, F = 1.89, p = 0.52) or spawning frequency (ANOVA, DF = 1, F = 0.96, p = 0.70). These results were in agreement with those from previous dietary exposures of sexually mature fathead minnows to environmentally

relevant concentrations of MeHg, in which study durations of 30 days did not lead to deleterious effects on reproduction [32, 34].

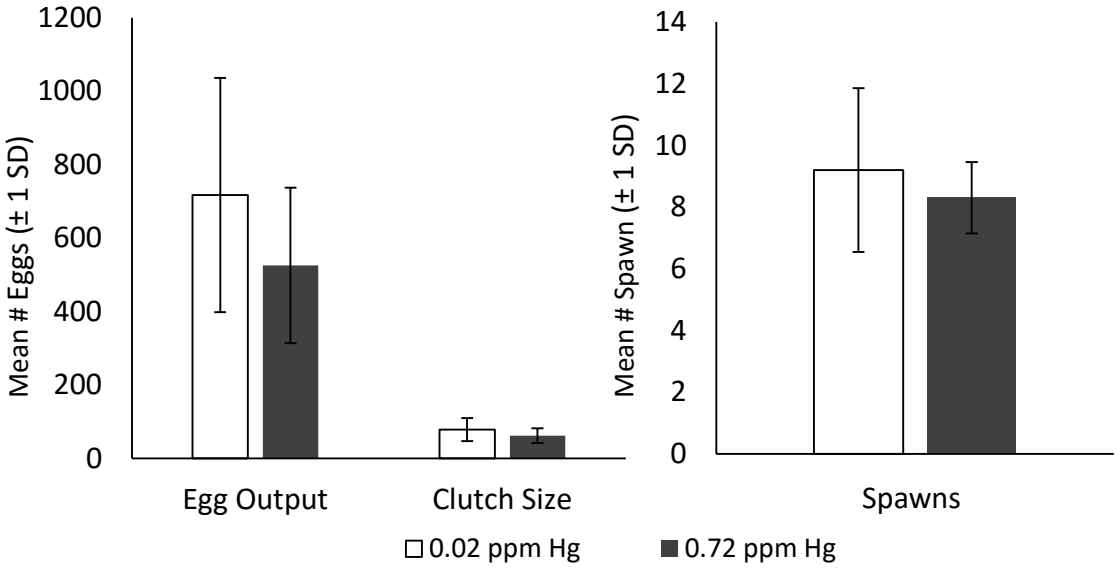


Figure 3.6. Reproductive metrics, including mean clutch size (p = 0.52), mean egg output (p = 0.61), and mean number of spawns (p = 0.52) were not significantly different between treatments (n = 10 for each treatment).

Dopamine has been shown to have a powerful inhibitory effect on reproduction in teleosts, by opposing the stimulatory function of gonadotropin releasing hormone (GnRH). Increases in dopaminergic activity, and/or significant reductions in MAO activity can prevent final oocyte maturation, ovulation/spermiation, as well as sexual maturation in some teleost species [31]. Total brain DA concentrations were not significantly different between diets after 30 days, and the effects of regional differences in overall DA concentrations seen in the FB of

adult fish are likely diluted by the significant reduction in MAO activity. As previously described, catecholamines (including DA) have complex cycling, and it is possible that other mechanisms (transporters, vesicular release) are able to maintain proper DA concentrations at the synapse to prevent biological changes for shorter exposure times, such as those used in the present study. However, the significant inhibition of MAO by the presence of MeHg may help explain the decreases in reproduction that are seen after long term (> 100 days) exposure to low levels of dietary MeHg.

### *Conclusions*

Increased motor activity observed in fathead minnow offspring may be associated with alterations in the dopaminergic system, similar to those seen in rats [18, 19]. Non-mammalian model systems are becoming increasingly useful for studying the role of DA and other neurotransmitters, and there is increasing evidence of overlap in neurochemical profiles between teleosts and mammals [44]. Studies on developmental neurotoxicity due to maternal MeHg exposure in humans tend to underestimate developmental neurotoxicity. This is primarily due to confounding factors inevitable in epidemiological studies, and it is estimated that relative imprecision in such studies may be as high as 50% or greater [45]. This negative bias, coupled with the general lack of available data on developmental neurotoxicity in humans due to MeHg exposure, highlights the need for studies which investigate highly conserved systems that can be extrapolated to higher vertebrates, including humans.

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# CHAPTER 4

# METABOLIC PROFILING OF FATHEAD MINNOW LARVAE EXPOSED TO MATERNALLY TRANSFERRED METHYLMERCURY

## Introduction

Elemental mercury is released into the atmosphere through numerous anthropogenic and natural processes, and can undergo global transport before oxidation into its inorganic form by atmospheric components [1]. Inorganic mercury can then be introduced into aquatic ecosystems via wet or dry deposition. In sediment, inorganic forms of mercury may be transformed by bacteria into organic forms, including mono-methylmercury (MeHg), a highly bioavailable form which bioaccumulates and biomagnifies [2]. The primary route of MeHg exposure for aquatic organisms in adult life stages is through diet resulting in potentially high body burdens of MeHg in long-lived organisms at high trophic levels, such as piscivorous fish [3].

A national dataset of Hg concentrations measured in freshwater fish in Canada reported mean wet weight axial muscle concentrations range from 0.28-0.41 ppm Hg in three important sport fish, with maximum concentrations exceeding 10 ppm for all three species [4]. Similarly, wet weight yellow perch axial muscle concentrations reported from over 2,000 sampling sites in the great lakes regions range from 0.01-2.6 ppm Hg [5]. The concentrations reported in wild fish in North America are of concern, as current estimates suggest sub lethal effects on individual fish health begin to occur at wet weight concentrations as low as 0.2 ppm [3].

Methylmercury has a high affinity for sulfur residues, and forms conjugates with biomolecules containing sulfur, providing a mechanism of transport across the blood brain barrier, as well as from maternal diet to developing offspring [6-8]. Consequently, the primary route of early life stage exposure to MeHg is through maternal transfer [7]. Though MeHg can lead to adverse health effects in adult fish with muscle concentrations exceeding 0.2 ppm, it is of particular concern to developing organisms, as toxicity is observed in early life stages at much lower exposure concentrations than those necessary to affect adults [3, 7, 9, 10]. Behavioral abnormalities (e.g. hyperactivity) associated with significantly decreased time to hatch have been shown to occur in embryos maternally exposed to approximate wet weight Hg concentrations as low as 0.04 ppm during development [10, 11]. Embryos maternally exposed to approximate wet weight concentrations of 0.54 ppm Hg displayed physiological abnormalities, circular swimming patterns, delayed hatch, and a 41% increase in embryo-larval mortality when compared with controls by 7 days post fertilization [10].

In addition to the severe neurological defects ascribed to MeHg exposure, it is also a known endocrine disruptor, and has been shown to alter cell structure and cellular biochemical processes both by generation of reactive oxygen species and by binding to/inactivating critical enzymes and transporters [2]. Among the biochemical processes disrupted by the presence of MeHg is cellular energetics. MeHg has been shown to accumulate in mitochondria, resulting in decreased cell viability and compromised metabolism, which has implications for the growth and development of exposed organisms [2, 12].

Transgenerational effects of maternally derived MeHg in fish are understudied, and the present study seeks to add to the body of knowledge regarding possible modes of toxicity

arising from maternal transfer of MeHg using metabolomics [13]. Metabolomics is useful to simultaneously study a range of organic, low molecular weight metabolites in tissues. Significant changes in metabolite abundance can be extrapolated to changes in cellular processes, and may provide new insight into modes of action for important environmental toxicants, such as MeHg [14, 15]. We fed adult fathead minnows one of two environmentally relevant concentrations of dietary MeHg to ensure realistic exposure scenarios for all life stages of fish. Larvae from each treatment were collected immediately after hatch, and analyzed for dissimilarities in metabolite abundance, and changes in larval growth rates.

## Materials and Methods

### *Animal Care*

All animal care procedures using fish were approved by the University of North Texas Institutional Animal Care and Use Committee (IACUC) under protocol #1303-3 and are described in Chapter 2 [10].

### *Experimental Design*

Aquaria were administered either a control diet containing 0.02 ± 0.0005 ppm Hg dry weight (n = 7), or a MeHg spiked experimental diet containing 0.72 ± 0.013 ppm Hg dry weight (n = 5). Aquaria were randomly assigned to receive either a control or spiked diet, and food was administered twice daily, for 30 days. When normalized for caloric density, the Hg concentration in the spiked diet is representative of concentrations seen in benthic invertebrates and zooplankton in some North American lakes [16]. As previously described,

adult fish are primarily exposed to MeHg through diet, while almost all larval exposure occurs via maternal transfer. These exposure routes were chosen in order to represent environmentally relevant exposure scenarios [7, 16, 17]. Diets were prepared using the methods described in Chapter 2 [10] and were analyzed for Hg as described in the mercury determination section. Waterborne MeHg from unconsumed food pellets was not considered a significant route of Hg exposure for adult or larval fish [10, 16, 18].

In order to ensure all larvae were hatched from eggs exposed to maternally transferred MeHg throughout all stages of oogenesis, eggs were monitored for Hg concentrations for a week following the start of the spiked diet administration using a DMA-80 Direct Mercury Analyzer (Milestone Inc., Monroe, Connecticut) according to USEPA Method 7473 [19]. Mercury concentrations remained stable following the first 4 days of exposure, which accounts for the asynchronous spawning pattern of fathead minnows [20]. Following the monitoring period, individual clutches were gently removed from breeding tiles in exposure tanks every morning, and transferred to a crystallizing dish containing clean reconstituted moderately hard water. Methylene blue was also added to dishes to discourage fungal growth. Dishes were gently aerated with an air stone, and loosely covered with parafilm to help prevent evaporation. Water levels in dishes were checked twice daily, and adjusted as needed to maintain proper salinity. In order to avoid fluctuations in environmental factors, all clutches were cultured at 23° C in an environmental chamber on a 16:8 h photoperiod. Subsamples of 10 larvae from each dish were placed on a microscope slide next to a ruler for scale under an Olympus dissecting scope, and still images were captured for length analysis. Remaining larval

fish were transferred from crystalizing dishes into individual microcentrifuge tubes immediately after hatch, and frozen at -80 degrees until metabolite extraction was performed.

### *Mercury Determination*

Fish food, larvae and adult tissues (muscle, gonad) were analyzed for Hg using the methods and quality assurance protocols described in Chapter 2 [10]. The standard deviation of seven replicates multiplied by the corresponding t-value (for the 95% confidence limit), was used to calculate the method detection limit, which was determined to be 0.001 ppm in a 0.02 g sample. Certified reference materials (MESS-3, DORM and DOLT-4) were analyzed every 10 samples with a mean percent recovery for each of: MESS-3 102.52 ± 5.6 % (n = 9), DORM 104.38 ± 2.49 % (n = 6) and DOLT-4 102.64 ± 5.86 % (n = 8).

### *Length Analysis*

Images of larval fish were analyzed using ImageJ (National Institutes of Health, Bethesda, Maryland). The ruler captured in the image was used to scale the image for larval length analysis. Length was calculated using a line originating at the beginning of the mouth of the larvae, which continued along the spine and ended at the termination of the tail for all ten fish. The ten lengths were then used to find a clutch mean, and clutch means were further averaged to determine the treatment mean length.

### *Metabolite Analysis*

Subsamples of larval fish were transferred to a clean microcentrifuge tube and flash frozen in liquid nitrogen. Larvae were then homogenized in cold 2:5:2

Choloroform:Methanol:Mili-Q™ water solution with a motorized pellet pestle, and then centrifuged at 14000g at 4° C for 8 minutes. Supernatant was removed, and spiked with 10 µL of 150 ppm D-27 Myristate internal standard (IS) before evaporation under a gentle stream of nitrogen. Evaporated samples were derivatized with 50 µL of 15 mg/ml methoxyamine in pyridine solution, vortexed, and heated at 50 ° C for 30 minutes. This was followed by a second derivatization step with the addition of 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS), after which samples were vortexed and heated again at 70° C for 30 minutes. Samples were then analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 6890 GC and 5973 MS)

The Agilent Fiehn Retention Time Locking Library (Agilent part # G1676-90000) was used according to the manufacturer's recommendations for analysis and metabolite identification. Briefly, chromatographic (.D) files were processed using the Automated Mass Spectral Deconvolution & Identification System (AMDIS; National Institute of Standards and Technology, Gaithersburg, Maryland), then submitted to the Agilent Fiehn library to generate semi-quantitative relative response factors ratioed against the response of the IS. A suite of fatty acid methyl ester standards was injected into every 5th sample (n=3) to provide data for the AMDIS Retention Index library used to track slight variations in predicted metabolite retention times.

### *Statistical Analyses*

Reports generated by AMDIS using the Agilent Fiehn Retention Time Locking Library were filtered using an in-house R(v. 2.15.2) program [21] that determined acceptable

metabolite identification based on similarity between sample retention indices and library retention indices, and a net mass spectral quality scores of 70% or greater as calculated by AMDIS. Metabolite results which met all filtration criteria were included in a two tailed Welch's t-test, in order to determine statistically significant differences in RF values between the control and mercury-treated groups.

All other data were analyzed using JMP 11.1 (Cary, NC). Normality of all data was determined using the Shapiro-Wilk test. Mean larval length was determined using a single factor analysis of variance (ANOVA) by dietary Hg concentration. Total mercury concentrations in adult muscle and gonad were evaluated for differences using a 2-factor ANOVA with gender and dietary Hg concentrations as factors. Mercury concentrations in larvae were determined using a single factor ANOVA by adult dietary Hg concentration. An $\alpha$ of 0.05 was used to determine statistical significance for all tests.

## Results and Discussion

### *Mercury Accumulation*

Almost all mercury in fish is methylmercury, and it is primarily found associated with sulfur containing protein residues in skeletal muscle [22]. The muscle concentrations achieved in this study are comparable with those measured in previous dietary studies using the same exposure scenario, and are also representative of mean fish muscle concentrations found commonly in freshwater ecosystems in North America [3, 7, 10, 17, 23]. The concentrations of Hg measured in muscle (wet weight) were significantly different between diets (Table 4.1) (ANOVA DF = 1, F = 237.44, $p < 0.001$). Mean wet weight Hg concentrations were significantly higher (ANOVA DF =

1, F = 55.23, $p < 0.001$) in Hg treated females (0.804 ± 0.084 ppm Hg, n = 8) than treated males (0.506 ± 0.039 ppm Hg, n = 5). The significant difference in muscle concentration between genders likely results from the energetic demands of egg production in females, necessitating increased food consumption [24, 25]. The amount of dietary Hg female fatheads transfer to eggs is small, with current estimates ranging from 1.9-4.3% of maternal body burden. Given this rate of transfer, and the increased sensitivity of the developing central nervous system (CNS), these female muscle concentrations have been shown to be sufficient to induce developmental toxicity in fathead minnow offspring [10, 11]. No significant differences in muscle Hg concentrations (wet weight) were detected between genders in control fish (0.084 ± 0.017 ppm Hg, n = 13).

Mercury concentrations (wet weight) measured in gonads (Table 4.1) were also significantly different between diets (ANOVA DF = 1, F= 179.37, $p < 0.001$). In contrast to muscle tissue, no significant differences in Hg concentrations were detected between genders in either treatment (control: 0.0239 ± 0.007 ppm Hg, n = 15; treated: 0.276 ± 0.071 ppm, n = 13). Larval concentrations of Hg (Table 4.1) were significantly different between diets (ANOVA DF = 1, F = 37.8, $p <0.001$). The mean dry weight concentration of larvae exposed to MeHg via maternal transfer was 0.322 ± 0.11 ppm Hg, which is roughly equivalent to a wet weight concentration of 0.06 ppm. Neurological effects associated with these exposure concentrations during embryogenesis include disruptions in dopamine cycling, premature hatch, and increased motor activity which can be expected to persist into larval stages of fish and beyond [10, 11, 26]. Control larvae had a mean dry weight concentration of 0.065 ± 0.02 ppm Hg, or an approximate wet weight concentration of 0.013 ppm Hg.

Table 4.1. Mean wet weight Hg tissue concentrations (± 1 SD). Letters denote statistically different groups.

| | CONTROL (ppm Hg wet weight) | MERCURY (ppm Hg wet weight) |
|---|---|---|
| FEMALE MUSCLE | 0.084 ± 0.02 (a) (n = 8) | 0.804 ± 0.08 (d) (n = 8) |
| MALE MUSCLE | 0.084 ± 0.02 (a) (n = 5) | 0.506 ± 0.04 (c) (n = 5) |
| FEMALE GONAD | 0.024 ± 0.007 (a) (n = 10) | 0.276 ± 0.071 (bc) (n = 8) |
| MALE GONAD | 0.028 ± 0.01 (a) (n = 5) | 0.204 ± 0.04 (ab) (n = 5) |
| BRAIN TISSUE | 0.07 ± 0.01 (n = 5) | 0.46 ± 0.23 (n = 5) |
| LARVAE | 0.012 ± 0.01 (n = 7 ) | 0.064 ± 0.04 (n = 7) |
| EGGS | 0.010 ± 0.01 (n = 8) | 0.074 ± 0.02 (n = 8) |

*Effects on Early Life Stage Fish*

Embryos spawned in tanks administered dietary MeHg displayed hyperactivity beginning 2 days post fertilization (DPF) as reported in Chapters 2 & 3 [10, 11]. In a previous study, using comparable exposure scenarios, this hyperactivity was associated with significantly earlier hatch [10]. This is likely the result of increased distribution of hatching enzymes, distributed by embryonic movements [10, 27]. These hyperactive behaviors correspond with reduced monoamine oxidase function (MAO), and alterations in dopamine concentrations in embryos, seen also in the telencephalon of adult fathead minnows used in the present study

[11]. Mora-Zamorano et al. [26] confirm these hyperactive behaviors can be expected to continue into larval stages, increasing the vulnerability of larvae to predation.

Larvae from adults administered the 0.72 ppm Hg diet were significantly shorter when compared with control larvae (ANOVA DF = 1, F = 37.1, $p < 0.001$). Mean length (± 1 SD) of larvae (Figure 4.1) from clutches spawned in treatment tanks were 3.66 ± 0.01 mm (n = 6 clutches), while larvae from control clutches (n = 6 clutches) displayed a mean length of 4.24 ± 0.04 mm. Differences in larval length may be attributed to slower growth rates resulting from disruptions to several metabolic pathways discussed below, may result from shorter developmental time due to pre-mature hatch from mercury induced hyperactivity, or a combination of these factors [10]. Larval size is positively related to survival for wild fish, and these reductions in larval length, coupled with hyperactivity, have the potential to greatly diminish individual fitness for fish exposed to maternally derived MeHg [28, 29]. Recruitment of fish populations relies on small differences in individual fitness that increase the probability of survival to sexual maturity [28]. Given the apparent negative effects MeHg has on individual fitness (Figure 4.2) at the low exposure concentrations used in the present study, the potential for effects on community structure at current environmental exposure concentrations exists.

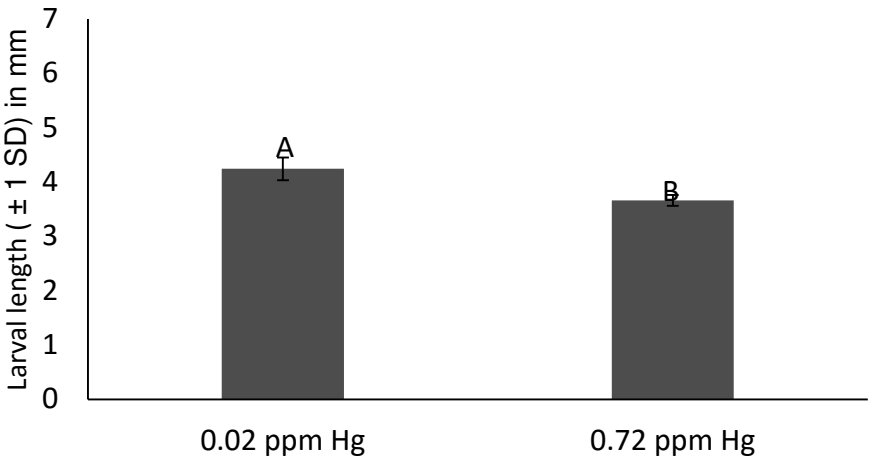


Figure 4.1. Mean treatment length of newly hatched larvae by adult dietary Hg concentration. Treatment means were calculated using average clutch means from each treatment (n = 6). Letters denote statistically distinct groups ( α = 0.05).

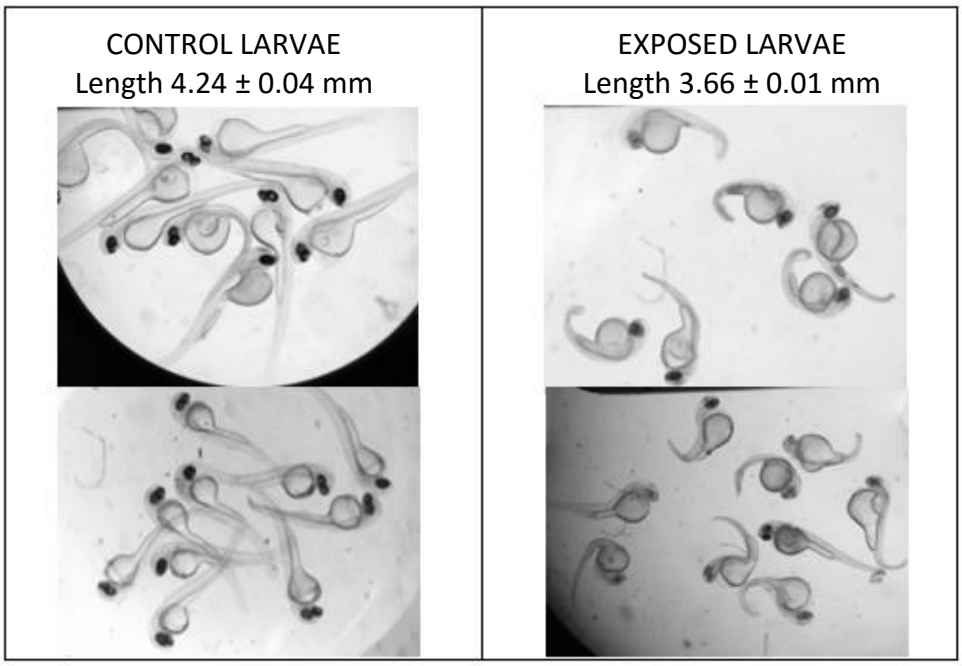


Figure 4.2. Physiological differences, including significantly altered larval length (mean ± 1 SD), between MeHg exposed larvae and controls.

*Metabolite Analysis*

Changes in metabolite response factors provide information about cellular pathways affected by the presence of environmental contaminants, such as MeHg. This information can then be extrapolated to observed physiological alterations. As previously discussed, diminished size in larval fish exposed to maternally transferred MeHg may be attributed to disruptions of cellular pathways involved in cell proliferation and growth, as indicated by significant alterations in specific metabolites.

One such metabolite, putrescine, was significantly increased (T-test, $p < 0.01$) in MeHg exposed larvae, with a mean RF that was roughly twice that of controls (Table 4.2). Putrescine is a polyamine (PA) found in all eukaryotes, and a precursor to other PAs, namely spermine and spermidine. All of the aforementioned PAs have been shown to be essential for cell growth and differentiation [30, 31]. An early response to CNS injury is an induction of PA metabolism, resulting in increased levels of putrescine [31, 32]. The enzymatic conversion of putrescine to spermine, a CNS antioxidant with neuroprotective functions, is catalyzed by S-adenosylmethionine decarboxylase (AdoMetDC) in the presence of the sulfur containing cofactor S-adenosyl-L-methionine (AdoMet) [33]. Depletions of AdoMet, such as those which would likely occur in the presence of sulfur binding MeHg, have been shown to lead to decreased levels of spermine and spermidine, and increased putrescine [33, 34]. Neither spermine or spermidine were present in high enough levels in either treatment to be included in the metabolite profiles (Table 4.2), however the significant increase in putrescine is indicative of both neurological damage in treated offspring, as well as a likely inhibition of production of the higher PAs. Korhonen et al. [33] reported inhibition of AdoMetDC (required for spermine

and spermidine production) in embryonic stem cells caused cell proliferation to completely cease after 48 hours. The altered PA metabolism seen in the treated larvae therefore has clear implications for cell proliferation and growth, and would also appear to potentiate the neurotoxicity of MeHg by depletion of an important CNS antioxidant. The potential for CNS damage is compounded by the inherent toxicity of PAs when present at excessive levels. Polyamine toxicity occurs as they are degraded by oxidation processes. It is known that MeHg generates reactive oxygen species, which would serve to increase the potential for oxidation of putrescine, present at increased concentrations in the MeHg exposed larvae [34].

Table 4.2. Metabolites detected in larvae. An * is used to denote significance between control and Hg exposed larvae (Hg) metabolite response factors (relative to IS).

| Name | N control | Control Mean ± 1 SD | N Hg | Hg Mean ± 1 SD | Hg/Control | P-value |
|---|---|---|---|---|---|---|
| *putrescine | 7 | 74.3 ± 18.9 | 9 | 147.7 ± 50 | 1.99 | 0.01 |
| *L-serine | 5 | 31.8 ± 15.1 | 7 | 66.2 ± 27.2 | 2.08 | 0.02 |
| *glycerol | 7 | 250.1 ± 41.1 | 9 | 401.2 ± 156.3 | 1.60 | 0.02 |
| *stearic acid | 7 | 1126 ± 363.5 | 9 | 767.2 ± 193.9 | 0.68 | 0.04 |
| *palmitic acid | 7 | 860.6 ± 236 | 9 | 621.8 ± 170.8 | 0.72 | 0.05 |
| *oleic acid | 7 | 357.1 ± 158 | 9 | 197.2 ± 135.5 | 0.55 | 0.05 |
| cholesterol | 7 | 11931.4 ± 3790.7 | 9 | 8017.2 ± 5617.7 | 0.67 | 0.12 |
| 2-hydroxypyridine | 5 | 40.4 ± 20.9 | 8 | 56.2 ± 20.3 | 1.39 | 0.22 |
| L-mimosine | 5 | 19.3 ± 15.1 | 6 | 11.3 ± 5.1 | 0.59 | 0.31 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Sucrose | 5 | 14.5 ± 8.8 | 9 | 39.5 ± 69.6 | 2.73 | 0.32 |
| myristic acid | 7 | 965.9 ± 445.7 | 9 | 772.3 ± 349.1 | 0.80 | 0.36 |
| L-glutamic acid | 7 | 210.1 ± 84.6 | 9 | 263.1 ± 140.1 | 1.25 | 0.37 |
| Phenylalanine | 6 | 420.7 ± 214 | 9 | 316 ± 224.5 | 0.75 | 0.38 |
| D-malic acid | 7 | 88.2 ± 33.4 | 9 | 70.9 ± 45.4 | 0.80 | 0.40 |
| L-threonine | 5 | 35.9 ± 13.4 | 8 | 45.7 ± 27.8 | 1.27 | 0.41 |
| D-mannose | 6 | 122.1 ± 51.9 | 8 | 97.8 ± 55.2 | 0.80 | 0.42 |
| glycerol 1-phosphate | 6 | 36.8 ± 25.5 | 9 | 55.8 ± 60.4 | 1.52 | 0.42 |
| pyrophosphate | 7 | 220.5 ± 246 | 9 | 146.8 ± 194.4 | 0.66 | 0.52 |
| L-allothreonine | 5 | 150.1 ± 50.3 | 5 | 130.6 ± 50.8 | 0.87 | 0.56 |
| DL-isoleucine | 5 | 230.2 ± 155.1 | 7 | 188.6 ± 100.1 | 0.82 | 0.62 |
| Creatinine | 7 | 775.3 ± 551.9 | 9 | 656.6 ± 522.9 | 0.85 | 0.67 |
| L-(+) lactic acid | 7 | 566.6 ± 305.6 | 8 | 499.6 ± 311.1 | 0.88 | 0.68 |
| urea | 7 | 221.2 ± 206.7 | 8 | 248.9 ± 153.2 | 1.13 | 0.78 |
| succinic acid | 7 | 38.2 ± 27.5 | 7 | 34.3 ± 22.7 | 0.90 | 0.78 |
| L-pyroglutamic acid | 5 | 241.6 ± 122 | 6 | 280.3 ± 294.9 | 1.16 | 0.78 |
| glycine | 7 | 466 ± 250.8 | 9 | 434.7 ± 206.3 | 0.93 | 0.79 |
| L-(+) lactic acid | 7 | 566.6 ± 305.6 | 7 | 521.4 ± 329.4 | 0.92 | 0.79 |
| O-phosphocolamine | 6 | 118.7 ± 39.6 | 9 | 125.6 ± 71.4 | 1.06 | 0.81 |
| citric acid | 7 | 327.7 ± 155 | 9 | 346.9 ± 166.7 | 1.06 | 0.82 |
| phosphoric acid | 7 | 7765.7 ± 3142.8 | 9 | 7393.3 ± 3235.9 | 0.95 | 0.82 |
| N-acetyl-L-aspartic acid | 6 | 168.4 ± 132.4 | 9 | 157.7 ± 91 | 0.94 | 0.87 |
| L-valine | 6 | 197.8 ± 89.3 | 6 | 191.4 ± 119.3 | 0.97 | 0.92 |
| glycolic acid | 7 | 22.5 ± 11 | 8 | 22.1 ± 9.6 | 0.98 | 0.94 |
| L-valine | 6 | 197.8 ± 89.3 | 7 | 194.1 ± 109.1 | 0.98 | 0.95 |
| glycolic acid | 7 | 22.5 ± 11 | 7 | 22.8 ± 10.2 | 1.01 | 0.97 |

Larvae exposed to maternally transferred MeHg also displayed a two-fold increase (T-test, $p < 0.05$) in L-serine (Table 4.2), a non-essential amino acid which adult organisms derive from a combination of four sources. These sources include dietary intake, biosynthesis from intermediates of glycolysis, from glycine, or by protein/phospholipid degradation [35]. As larval fish were collected immediately after hatch, dietary intake is ruled out as a possible explanation for the differences in L-serine between treatments. The increased RF in MeHg exposed larvae suggests inhibition of biochemical pathways that require L-Serine as a substrate. It is widely accepted that one mechanism by which MeHg exerts toxicity is by inhibiting the activity of many enzymes by binding to sulfur and selenium containing residues [34]. L-Serine is known to play a critical role in cellular proliferation by acting as a precursor molecule in several different pathways. These include gluconeogenesis, neuromodulation, neurodevelopment, trans-sulphuration pathways, and lipid synthesis [35]. Sulfur containing enzymes, highly sensitive to binding by MeHg, are involved in conversion of L-Serine to other products in each of these pathways.

Gluconeogenesis using L-Serine as a substrate can be catalyzed by two different sulfur containing enzymes in teleosts; serine dehydratase, and serine racemase. This presents a link between serine homeostasis and cellular energetics [35, 36]. Serine racemase has been shown to be strongly inhibited by compounds which interact with sulfhydryl groups, such as MeHg [37]. In brain tissue, enzymatic production of pyruvate occurs concomitantly with conversion of L-Serine to the D-Serine enantiomer, via the serine racemase pathway [37-39]. D-Serine is an extremely powerful agonist of the *N*-methyl-D-aspartate (NMDA) receptor, which plays a key

role in proper brain function and neurodevelopment [37, 40]. Decreased enzymatic conversion of L-Serine to D-Serine, coupled with reduced pyruvate production during development has clear implications for neurodevelopment in offspring. Disruption of this pathway also may help explain the significantly elevated RF of L-serine (Table 4.2) seen in larvae exposed to maternally transferred MeHg.

The trans-sulphuration pathways using L-serine as a precursor include those by which taurine, sulphate, cystathionine, and cysteine are generated. Cysteine is a precursor for the synthesis of the antioxidant, glutathione, the most abundant low molecular weight thiol containing protein in the CNS [35]. Glutathione is a known molecular target of MeHg, and therefore plays an important role in the mediation of neurotoxicity [34]. Deficiencies in glutathione have been shown to cause hemolytic anemia, neurological abnormalities, and reduced cell survival *in vivo* [35]. Inhibition of the trans-sulphuration pathway associated with Taurine generation by MeHg exposure also has negative implications for CNS function. Taurine functions as an inhibitory neurotransmitter, and also plays an important role in osmoregulation [35]. Inhibition of these trans-sulphuration pathways by MeHg is highly likely, considering the extremely high affinity of MeHg for sulfur residues. It also provides yet another mechanism by which the increased L-Serine abundances may be occurring in the MeHg exposed larvae.

L-Serine is also involved in synthesis of some lipids, including glycerophospholipids, and sphingolipids [35]. Sphingolipids are of particular importance in the nervous system, playing an important role in cell signaling cascades which mediate apoptosis, proliferation, stress responses, necrosis, and differentiation [41]. Considering the overt toxicity of MeHg [42] to the nervous system, and its association with increased apoptosis and necrosis in the CNS of fish,

disruption of this pathway is likely [43]. Overabundance of L-Serine in MeHg exposed larvae due to inhibition of biosynthesis of some lipids is highly probable when considered alongside the significant changes in glycerol and important precursor fatty acids seen in Table 4.2.

Glycerol is a backbone molecule in both glycerophospholipids, and triglycerides. Additionally, glycerophospholipid synthesis is dependent on intermediates from the metabolism of glycine, threonine, and most importantly, serine. The doubled RF of both glycerol and L-serine (T-test, $p < 0.05$) seen in the Hg exposed clutches may be explained by inhibition of these biosynthetic pathways. This conclusion is further supported by the findings of Klaper et al. [44], in which transcription of phosphoethanolamine methyltransferase (PMT), an enzyme involved in glycerophospholipid metabolism, was significantly downregulated in adult fatheads exposed to 3.93 ppm dietary MeHg. This enzyme catalyzes the transfer of methyl groups using AdoMet as a co-factor, and as previously discussed, depletion of AdoMet (due to binding of S by MeHg) may cause further loss of enzyme function. Several reactions involved in metabolism of glycerophospholipids are catalyzed by PMT, including biosynthesis of phosphatidylcholines [45]. Phosphatidylcholines are the most abundant phospholipid in the membranes of eukaryotes, and a key molecule in cell cycle signaling, and apoptosis [46]. They are also precursors to other types of glycerophospholipids, including phosphatidylserine [47]. Phosphatidylserine has particularly important neuroprotective/antioxidant functions, and dietary supplementation in humans has been indicated in the prevention and treatment of attention deficit disorders in children, as well as memory/cognitive defects, both of which are major hallmarks of MeHg induced neurotoxicity [10, 42, 47, 48].

Taking into consideration the significant decreases (T-test, $p < 0.05$) in RF's of some fatty acids (FA) in Hg exposed larvae, it is possible that inhibition of triglyceride synthesis may also be contributing to the increased glycerol RF. Triglycerides are composed of a glycerol backbone with three FA tails, and significant reductions in the RF's of some FA were observed, including palmitic, stearic, and oleic acids. Disruption of ideal molar ratios of precursor molecules (e.g. FA) would almost certainly inhibit biosynthesis of triglycerides, resulting in excess levels of the backbone precursor, glycerol.

Methylmercury is known to exert toxicity by generation of reactive oxygen species, which can lead to oxidation of biomolecules, providing a mechanism by which decreased FA abundances may be occurring [34]. Decreased FA abundance due to oxidation by ROS is further supported by the findings of Klaper et al. [49], in which adult fatheads exposed to MeHg showed significantly upregulated transcription of acetyl-coenzyme-A carboxylase beta (ACACB). Acetyl-coenzyme A (ACC) is a multi-domain enzyme which catalyzes the first step in FA synthesis, which is the production of malonyl-coA from acetyl CoA [50]. The ACACB subunit functions to prevent beta-oxidation of FA, in support of biosynthesis carried out by the other ACC domains [51]. In mice deficient in ACACB, significantly increased fatty acid oxidation, low body fat, and kidney dysfunction (attributed to abnormal fatty acid metabolism) were indicated [52, 53]. Given the significant decreases in the RF's of several FA in the MeHg exposed offspring, upregulation of ACACB may indicate a cellular response to increased FA oxidation, due to MeHg mediated oxidative stress.

Fatty acid synthesis relies on Acetyl-CoA, a sulfur containing molecule generated from pyruvate [54]. In addition to potential reductions in bioavailability of Acetyl-CoA due to

irreversible binding of the thioester group, MeHg may also reduce the Acetyl CoA availability by inhibiting the synthesis of its precursor, pyruvate. As previously discussed, pyruvate synthesis in brain tissue is likely inhibited by MeHg, due to reduced activity of serine racemase [34, 35]. MeHg has also been shown to affect S containing enzymatic domains of the fatty acid synthase (FAS) protein complex itself, in addition to suppressing transcription of the FAS gene [49]. The FAS complex catalyzes synthesis of palmitic acid, by facilitating thioester linkages necessary for chain elongation [34, 55]. Palmitic acid is the precursor to longer fatty acids, and once converted to palmitoyl-CoA (by the addition of an acetyl-CoA), it may be further modified to produce other fatty acids [56]. The significant, and relatively proportionate reductions in RF's for other FA in the Hg exposed larvae are likely at least partially explained by the decreased availability of this precursor FA. However, synthesis of oleic acid is likely also inhibited by MeHg mediated gene suppression of stearoyl-CoA desaturase, an enzyme which catalyzes the synthesis of oleic acid from stearic acid [49, 57]. Maintenance of the proper ratios of these particular FA are important for cell growth and differentiation as they participate in cell membrane fluidity and cell signaling [58]. Disruptions to ideal FA ratios (as indicated by the significantly reduced RF) may help explain the decreased size of MeHg exposed larvae.

Deficiencies in FA's, particularly palmitic acid, have serious implications for the developing nervous system. Palmitic acid is a precursor for sphingolipids, important constituents of cell membranes and myelin sheaths, which participate in cell differentiation, proliferation, and apoptosis signaling [41]. In fact, breakdown of sphingolipids in both mice and humans causes progressive neurological deterioration [59]. Severe neurological damage attributed to MeHg exposure occurs through a number of other established pathways, making

the significant reductions in FA abundance resulting from approximate wet weight larval concentrations of only 0.06 ppm MeHg of even greater ecological concern.

### *Conclusions*

Methylmercury exposure via maternal transfer lead to significant alterations in several important metabolic pathways in larvae at environmentally relevant concentrations. In light of the data generated by the current study, it is possible that MeHg concentrations observed in fish in lakes and rivers in North America are sufficient to disrupt important cellular pathways in developing organisms. Putrescine, and the PAs generated from it, have been shown to be critical factors in cell proliferation. This combined with the observed disruption of L-Serine homeostasis presents potential explanations for growth differences between offspring. Inhibition of these enzymes would have adverse consequences for cellular energetics/gluconeogenesis, resulting in reduced energy available for growth. Evidence of disruptions in lipid metabolism due to MeHg are indicated in metabolic profiles of exposed larvae, the dysfunction of which also has serious implications for organism growth and development. Lipids participate in signaling cascades which regulate animal growth and development, in addition to acting as energy storage molecules [60]. Given that MeHg is known to accumulate in mitochondria, where it disrupts Ca2+ homeostasis, oxidative phosphorylation, and electron transport chain function, further disruptions to cellular energetic processes could have meaningful, additive effects on growth at even very low MeHg concentrations [61]. In light of the findings of the current study, and evidence from the available body of work regarding developmental exposure to maternally transferred MeHg at

realistic exposure concentrations, it is reasonable to conclude current exposure scenarios may lead to adverse consequences for individual fitness, reduced survival of wild larval fish, and potential disruptions to recruitment in wild fish populations.

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# CHAPTER 5

# DISCUSSION

## Implications for Survival

Using environmentally relevant exposure scenarios, methylmercury (MeHg) led to toxicity throughout all life stages of the fathead minnow. Transgenerational effects of MeHg were observed not only in embryonic fish, but continued after hatch as seen in the metabolic profiles of larvae from each treatment. All endpoints included in the studies had the potential to adversely affect biological fitness and survival in the wild. Behavioral changes, presumably due to disruption of dopamine cycling in the presence of maternally derived MeHg, led to hyperactive behaviors beginning 2 days post fertilization (DPF). The observed hyperactivity was also associated with premature hatch. Mora-Zamorano et al. [1] demonstrated hyperactive behaviors are expected to persist throughout larval stages, increasing the likelihood of predation. Predation is the major source of larval mortality, and a critical factor determining recruitment during early life stages. Small differences among individuals that positively or negatively affect predator avoidance can strongly affect the probability of survival for larval fish [2, 3]. In addition to hyperactivity, increased larval growth/size also increases the probability a larval fish will avoid predation [4]. Taking size into consideration, the observed decreases in mean larval length, coupled with premature hatch (resulting in smaller, less mature larvae) seen in the present studies, would almost certainly decrease the potential of MeHg exposed individuals to survive to sexual maturity. Additionally, metabolic profiles of larval fish exposed

to MeHg indicate current MeHg concentrations in North American lakes and rivers are sufficient to disrupt important cellular pathways in early life stage fish.

## Implications for Populations and Communities

The aforementioned results indicate dietary MeHg concentrations achieved in water bodies not directly affected by point-source Hg pollution (represented by the 0.87/0.76/0.72 ppm Hg dietary administrations) are sufficient to affect recruitment of young. This indicates disruptions to community structure are possible, given variability in species sensitivities to MeHg. The significant increases in larval mortality seen in the offspring of adults administered the diet spiked with 5.5 ppm Hg are representative of toxic effects on early life stage fish for more polluted ecosystems. These results also agree with results obtained by Latif et al. [5], which showed a decline in hatching success of embryonic walleye with increasing egg MeHg concentrations. The decreased survival and physiological abnormalities seen in offspring from the 5.5 ppm Hg diet may also be representative of outcomes for sensitive individuals/species at current environmental concentrations. More research is needed to determine the potential impacts these developmental abnormalities may have on community structure in aquatic ecosystems.

## Factors Influencing Mercury Toxicity

### *Selenium*

Recently, a great deal of attention has focused on the role selenium plays in mercury toxicity. Selenium is an essential trace element used to synthesize important selenoenzymes,

which also exerts toxicity when present in excess quantities [6]. Due to the extremely high affinity of mercury for selenium, and the irreversible nature with which they bind, the role selenium plays in mercury toxicity is undeniably important, though two divergent relationships have been proposed [7].

The first suggests that selenium ameliorates mercury toxicity, and interprets the high binding affinity between Se and Hg as a mechanism by which Hg is sequestered, and toxicity is prevented [8]. Selenium has been shown to potentially ameliorate Hg toxicity in adult fish, as long as Se:Hg molar ratios are in excess of 1 [9-11]. As a result, many scientists have proposed risk assessments and mercury tissue concentration guidelines for fish consumption should factor in Se:Hg molar ratios in order to gain a complete picture of the risk associated with dietary Hg exposure.

Proponents of the other side argue that much of the toxicity associated with MeHg exposure, is in fact, largely due to selenium deficiencies precipitated by irreversible binding of Se with Hg [12]. Consequently, free selenide available to synthesize selenoenzymes is depleted. Many selenoenzymes are critical to the proper functioning of various physiological systems, including somatic growth, metabolism, osmoregulation and reproduction [7, 9]. Consequently, depletions in free selenide may have serious consequences for organisms, particularly those in developmental stages.

Adult zebrafish coexposed to dietary selenium and mercury in varying molar ratios revealed several interesting conclusions. Coexposure to dietary Se and MeHg protected adult fish from reductions in growth and development, but not reductions in reproductive success [13]. The likely explanation for this finding, is that Se presence does not render MeHg

biologically inert in terms of selenoenzyme independent pathways of toxicity. Additionally, eggs from coexposed parents indicated the presence of Hg increased the transport of Se into eggs, leading to more than additive toxic outcomes in embryos, as well as decreased survival [13].

In light of recent evidence that oceanic Hg concentrations are on the rise due to anthropogenic emissions, current mercury emissions must be reduced in order to prevent serious ecological, and human health repercussions [14]. Furthermore, revisions of current dietary guidelines for humans, especially those for pregnant women and children, based on average molar ratios of Se:Hg is irresponsible. More research is needed to fully ascertain the complex biological interactions between Se and Hg, however current research indicates excess dietary Se is only partially protective against toxicity in adults, and may actually potentiate toxicity in more sensitive life stages [13].

## Human Health Implications

It is widely accepted that early life stage exposure to most toxicants can lead to irreversible, adverse impacts at much lower concentrations than those seen in adults. In spite of this well accepted knowledge, current FDA fish consumption guidelines for pregnant women list only 4 types of fish to avoid (shark, king mackerel, tilefish, and swordfish). Additionally, consumption guidelines recommend consuming on average 8-12 oz. of a variety of low mercury fish per week, [15, 16]. Included in the list of low Hg fish recommended by the FDA for pregnant women, is light tuna. On average light tuna contains lower Hg concentrations than Albacore, though labeling it a low Hg fish is misleading. Pregnant women and children can

easily exceed the mercury dietary guidelines set forth by the FDA and EPA by consuming only 5 oz. of light tuna per week, far below the recommended 8-12 oz. touted by the FDA as "safe [17]." Current FDA consumption guidelines for pregnant women and children regarding albacore tuna state 6 oz is a safe quantity to consume, however a recent survey of Hg content of Albacore tuna indicates pregnant women may exceed safe dietary concentrations by consuming only 2.5 oz of Albacore [17].

Drevnick et al [14] currently estimate tuna Hg concentrations are increasing at least 3% annually, in response to anthropogenic loading of Hg into oceans. Mercury concentrations in fish from unpolluted waters, including tuna, are typically less than 1 ppm. However, Karagas et al. [18] point out the growing body of literature suggesting that even low levels of MeHg consumption may be associated with adverse health effects in both adults and children. Unsurprisingly, the most profound effects are seen in the developing fetus. In the United States alone, Trasande et al. [19] estimates that 300,000 to 600,000 children are born annually with cord blood Hg concentrations sufficient to significantly decrease intelligence. Deleterious effects on cognitive abilities and behavioral defects, including attention deficit hyperactivity disorder (ADHD), have also been associated with low level MeHg exposure [18, 20-22].

Comparable behavioral defects were shown in embryonic fish in the present studies, and appear to be associated with disruption of the dopaminergic system. Given a similar relationship between mercury mediated disruption of dopamine cycling, and motor activity abnormalities in adult rats, the possibility this mechanism of toxicity may also apply to human children appears likely [23-25]. More research is needed to establish a link between ADHD in human children, and mercury mediated disruption of the dopaminergic system.

## Directions for Future Research

Non-mammalian model systems are becoming increasingly useful for studying mechanisms of toxicity in highly conserved physiological systems, including overlapping neurochemical profiles between teleosts and mammals [26]. In light of evidence suggesting studies on developmental neurotoxicity due to maternal MeHg exposure in humans tend to underestimate developmental neurotoxicity (by an estimated 50% or greater), the need for additional studies which investigate these highly preserved systems is evident [22]. Furthermore, sub-lethal effects of MeHg on reproduction, growth and survival of juvenile fish are widely unknown, in spite of the potential to alter community structure. Further research is needed to determine the extent to which these changes in behavior and survival may affect individual fitness, and population structure.

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