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Environmental Toxicology and Chemistry, Vol. 36, No. 4, pp. 1077–1084, 2017

Printed in the USA

EFFECTS OF DIETARY METHYLMERCURY ON THE DOPAMINERGIC SYSTEM OF ADULT FATHEAD MINNOWS AND THEIR OFFSPRING

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(Submitted 11 June 2016; Returned for Revision 4 September 2016; Accepted 27 September 2016)

Abstract: Mercury (Hg) is a ubiquitous environmental contaminant and potent neurotoxin, which may be transformed by bacteria in aquatic ecosystems to methylmercury (MeHg), an organic form which bioaccumulates and biomagnifies. Consequently, long-lived organisms at the top of the food web are at risk of dietary MeHg exposure, which can be actively transferred from mother to offspring. Exposure during neurodevelopment can lead to serious, irreversible neurological dysfunction, associated with a variety of cognitive and motor abnormalities. At low dietary concentrations, MeHg exposure has been associated with deficits in attention and hyperactivity in multiple species. Pathways associated with cognitive function and motor activity are primarily associated with the dopaminergic system. The present study used a model fish species, *Pimephales promelas*, to examine the effects of MeHg exposure on dopamine concentrations and monoamine oxidase activity in embryos and adult brains. Adult fatheads were exposed for 30 d to either a control or a treated diet (0.72 ppm Hg). Embryonic and larval exposures were a result of maternal transfer of dietary MeHg. The authors confirmed hyperactive behaviors in embryos and detected significant changes in embryonic dopamine concentrations. Similar effects on dopamine concentrations were seen in the telencephalon of adult brains. Exposure to MeHg also corresponded with a significant decrease in monoamine oxidase activity in both embryos and brain tissue. Collectively, these results suggest that current exposure scenarios in North America are sufficient to induce alterations to this highly conserved neurochemical pathway in offspring, which may have adverse effects on fish behavior and cognition. *Environ Toxicol Chem* 2017;36:1077–1084. © 2016 SETAC

Keywords: Methylmercury Developmental toxicity Neurotoxicity Behavioral toxicology

INTRODUCTION

Mercury (Hg) is a ubiquitous environmental contaminant released through a variety of anthropogenic activities and natural processes. Following volatilization into the atmosphere, Hg can undergo long-range transport. Once oxidized by atmospheric components, wet or dry deposition can introduce Hg into aquatic ecosystems, where it partitions into sediments. In sediment, inorganic forms of Hg may be transformed by bacteria into organic forms [1]. Organic monomethylmercury (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 MeHg across the blood–brain barrier, from adult fish to eggs during oogenesis, and from maternal circulation to fetus across the placenta in humans [4–6].

Though neurotoxicity as a result of MeHg exposure occurs in adults, it is of particular concern to developing organisms, undergoing neurogenesis [5,7]. The developing nervous system is especially susceptible to the toxic effects of MeHg, resulting in a range of neurological defects at much lower exposure concentrations [8]. Effects of maternally derived MeHg in fish are understudied, and the exact mechanisms by which neurological insult occurs in offspring are not fully understood, though it is known that MeHg affects the regulation of several different neurotransmitters [9–15]. The dopaminergic system is among those neurotransmitters dysregulated in the presence of MeHg [11,16–18]. Dopamine is associated with cognition, attention, reward-motivated behaviors, and motor function; and dopaminergic drugs have been shown to affect locomotion and behavior in teleosts [19–21]. Dopamine is also a key neuroendocrine inhibitor of reproduction in adult teleosts [22,23]. Concentrations of neurotransmitters at the synapse are mediated by several mechanisms working in concert to control postsynaptic cellular responses. Regulatory mechanisms include vesicular release, transporters, receptors, and degradation [24–26]. Enzymatic degradation of dopamine by monoamine oxidase (MAO) has been shown to be significantly reduced in adult walking catfish exposed to 40 ppb MeHg for 4 mo [27]. Similarly, adult salmon exposed to 10 ppm dietary MeHg for 4 mo showed significant reductions in brain MAO activity [28]. It is worth noting that these studies are not representative of environmentally relevant exposure routes or concentrations; however, given that MAO has been shown to be strongly inhibited by sulfur-binding chemicals, such as MeHg, effects at relevant exposure scenarios are possible [29,30]. Spontaneous release of dopamine in larval mummichogs and adult walking catfish has also been shown to increase in the presence of waterborne MeHg, and vesicular release of dopamine has also been shown to increase in the presence of MeHg in the rat hippocampus [12,24,27]. Modulation of neurotransmitter control mechanisms has implications for neurotransmission, and given the sensitivity of the developing nervous system, it appears likely that pathways regulated by dopamine in developing fish would be affected by the presence of maternally transferred dietary MeHg [6,11,24].

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Published online 28 September 2016 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.3630

We previously observed significant hyperactivity at 2 d postfertilization (dpf) in fathead minnow embryos maternally exposed to MeHg, using comparable exposure scenarios to those of the present study [31]. Consistent with these behavioral changes, Mora-Zamorano et al. [32] found increased locomotor output in larval zebrafish exposed to maternally derived MeHg, indicating that hyperactivity as a result of developmental MeHg exposure can be expected to persist. A mechanism for the MeHg-induced hyperactivity in embryonic and larval fish 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 receptor–mediated motor activity [16,24]. Given the highly conserved nature of this system of neurotransmission, it is possible that fish embryos in the present study may also have displayed increased dopamine receptor–mediated motor activity. Further research is needed to establish if there is a shared mechanism by which hyperactivity is elicited in each of these species.

Significant increases in embryonic movement were correlated with decreased time to hatch in a study by Bridges et al. [31], attributed to increased distribution of hatching enzymes [33]. Changes in time to hatch can permanently impact fitness of early–life stage fish as a consequence of reduced development time [34,35]. For example, zebrafish offspring maternally exposed to benzo-[*a*]-pyrene displaying significant reductions in time to hatch also had significantly reduced body length and developmental deformities [35].

We confirm previous findings of embryonic hyperactivity 2 dpf, evaluate the effects of maternally transferred dietary MeHg on dopamine concentrations in fathead minnow offspring, and investigate the inhibition of embryonic MAO activity by MeHg in an in vitro assay. Both dopamine and MAO are highly conserved components of neurotransmission across species, and the goal of the present study was to determine if current MeHg exposure scenarios are sufficient to alter the dopaminergic system of embryonic fish [29,36]. We also characterized dopamine concentrations in various brain regions of adult fathead minnows. Studies examining MeHg exposure and changes in brain dopamine concentrations in these fish have typically provided whole-brain measurements, which may obscure potentially significant regional changes because both dopamine and MAO are key modulators in neuroendocrine control of reproduction [37,38]. To assess impacts of developmental exposure of MeHg on larval fitness, we measured length of larvae immediately posthatch. Fathead minnows were selected for use in the present study because of favorable attributes which make them ideal for reproductive studies. These include the ease and affordability of maintaining fathead cultures, as well as their asynchronous spawning pattern, which provides continuous, year-round production of offspring [39].

MATERIALS AND METHODS

Animal care

All procedures using fish were approved by the University of North Texas Institutional Animal Care and Use Committee under protocol 1303-3. Reproductively active, adult fathead minnows were obtained from Aquatic Biosystems and divided among 10 21-L glass aquaria. Each tank contained 3 female and 1 male fish, as well as 2 breeding tiles constructed from halved polyvinyl chloride pipe sections. Mean wet weights of female fish were 0.94 ± 0.18 g (controls) and 0.97 ± 0.30 g (treated). Mean wet weights of male fish were 2.19 ± 0.43 g (control) and 2.56 ± 0.15 g (treated). All tanks were equipped with bio sponge foam filters and heaters and were on a 16:8-h light:dark photoperiod. Tanks were filled with reconstituted moderately hard water (24 ± 1 °C, pH 7.2–7.8, dissolved oxygen 5 mg/L, hardness 80–100 mg/L $CaCO_3$) and maintained by daily siphoning of waste and debris and 25% water changes every 2 d. 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) for 2 wk to establish baseline reproduction. At the conclusion of the present study, adult fatheads were euthanized using buffered tricaine methanesulfonate at a concentration of 250 ppm, according to the Institutional Animal Care and Use Committee protocol.

Experimental design

Following the 2-wk acclimation period, aquaria were randomly assigned either a control diet containing 0.02 ± 0.0005 ppm Hg dry weight or an MeHg-spiked experimental diet containing 0.72 ± 0.013 ppm Hg dry weight. The concentration of the MeHg-spiked diet was selected because 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 to ensure environmentally relevant exposure scenarios [5,40,41]. All aquaria were administered similar quantities of food 2 times per day for 30 d. Diets were prepared by mixing fish food with MeHg 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. [40]. Samples of each diet were analyzed for Hg as described below (*Hg determination*). Waterborne MeHg from unconsumed food pellets was not considered a significant route of Hg exposure for adult fathead minnows or their offspring because other studies have shown very little dissociation of MeHg from food in comparable exposure scenarios [31,40]. In addition, all unconsumed food was siphoned following each feeding, and all clutches were placed in clean water 0 dpf.

Fathead minnows spawn asynchronously, and ovaries of sexually mature fathead minnow ovaries contain oocytes of all developmental stages, with a total maturation time of approximately 3 d per oocyte [42]. To ensure that all eggs used in the present study were exposed to maternally transferred MeHg throughout all stages of oogenesis, eggs produced during the first 7 d 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 reconstituted moderately hard water and methylene blue (to discourage fungal growth) 0 dpf, and gently aerated with an air stone. To avoid fluctuations in environmental factors, all clutches were cultured at 23 °C in an environmental chamber on a 16:8-h photoperiod. Every fifth day, whole clutches from each treatment were dried and analyzed for Hg content using a DMA-80 Direct Mercury

Analyzer (Milestone) to monitor Hg concentrations in eggs for the duration of the study.

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 Bridges et al. [31]. The spontaneous movements exhibited by embryonic fish are analogous to startle responses in adult fish and involve flexion of the trunk of the embryo into a *C* shape [33,43]. Mean movements per minute for each diet were calculated using the means of each individual clutch (control diet, $n = 9$; Hg diet, $n = 9$).

A subsample of embryos were kept through hatch, and newly hatched larvae were immediately photographed under an Olympus dissecting scope equipped with a Canon Vixia HF G30 video camera. A ruler was captured in the image to scale larval lengths, followed by analysis using ImageJ (National Institutes of Health). Length was calculated using a line originating at the beginning of the head of the larvae, which continued along the spine and ended at the termination of the tail for 10 fish per clutch. The 10 lengths were then used to find a clutch mean, and clutch means (control diet, $n = 6$; Hg diet, $n = 6$) were further averaged to determine the mean length for each diet.

Hg determination

Skretting starter crumble, experimental diets, embryos, larvae, and adult tissues (muscle, gonad, and brain) were analyzed for total Hg using the methods and quality assurance protocols described in Bridges et al. [31]. The method detection limit, determined as the standard deviation (SD) of 7 replicates multiplied by 3, was estimated to be 0.0015 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 sample materials from the National Research Council of Canada (MESS-3, TORT, DORM, and DOLT-4) were analyzed every 10 samples with mean percent recoveries as follows: MESS-3 $102.6 \pm 5.77\%$ ($n = 8$), TORT $105.28 \pm 3.12\%$ ($n = 6$), DORM $104.38 \pm 2.49\%$ ($n = 4$), and DOLT-4 $102.64 \pm 5.86\%$ ($n = 8$).

Dopamine extraction

Clutches of eggs were collected for analysis of dopamine concentrations either 1 dpf, 2 dpf, or 3 dpf. Fertilized eggs from each clutch were transferred from crystallizing dishes into individual, preweighed microcentrifuge tubes and immediately frozen at –80 °C. 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 min 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 as an internal standard, 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 [44,45].

Brains were immediately dissected on ice into 3 sections. The olfactory bulb/telencephalon (forebrain) and cerebellum/pons/medulla (hindbrain) were removed from the brains, and the remaining tissue was categorized as midbrain. Dissected brain regions were transferred to microcentrifuge tubes, weighed, and snap-frozen in liquid nitrogen. Samples were then stored in a –80 °C freezer to avoid degradation of analytes. A subsample of brain tissue from each diet was used for DMA-80 analysis, and all remaining samples were either analyzed for dopamine concentrations or used to determine MAO activity.

Brain tissue samples used for analysis of dopamine were weighed, spiked with appropriate volumes of 25 ppm internal standard to reach a final sample concentration of 0.5 ppm, and homogenized using a motorized pellet pestle. Cold acetonitrile was added to the microcentrifuge tubes at a 5:1 volume to mass ratio, followed by incubation in a –80 °C freezer for approximately 45 min. Samples were centrifuged for 5 min (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 °C freezer until liquid chromatography–tandem mass spectrometric (LC-MS/MS) analysis. Extraction methods for all tissues were adapted from the methods of Sari et al. [44] and Wang et al. [45].

LC-MS/MS analysis of dopamine

Concentrations of dopamine in extracts were obtained using a Waters model 2695 high-performance liquid chromatograph with a Micromass Quattro Ultima triple quadrupole mass spectrometer at the University of North Texas, fitted with a Phenomenex Hydro-RP column (150 mm × 3.0 mm). The binary mobile phase (0.2 mL/min) consisted of an ultrapure water (A) and methanol (B) gradient, with 0.1% formic acid. The gradient was held at 100% A until dopamine eluted at 5 min and 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 microliter of extract was injected, and the following transitions were monitored: D4-dopamine $158 > 147$ and dopamine $154 > 137$. For analysis of embryos, a 9-point standard curve ranging from 6 ppb to 1600 ppb dopamine ($R^2 = 0.999$) was run prior to sample analysis, and a separate 6-point curve ranging from 50 ppb to 1600 ppb was run for brain tissue. The D4-dopamine internal standard was held at 500 ppb for both curves. No dopamine was detected in any unspiked method blanks ($n = 15$), and method blanks spiked with 500 ppb dopamine ($n = 8$) had a mean percent recovery of 97.38 ± 23.704 (range = 138.15–70.12%). Methods used in LC-MS/MS analysis of dopamine were adapted from the methods of Sari et al. [44] and Wang et al. [45] for all tissues.

MAO activity determination

Inhibition of MAO activity in 3 dpf fathead minnow eggs and adult brain tissue was determined with a MAO assay kit (Sigma-Aldrich) using a range of MeHg concentrations as an inhibitor. Concentrations of MeHg 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 *g* for 10 min to remove unwanted debris. Egg homogenate was diluted with assay buffer and pipetted into individual wells on a 96-well plate which contained either ultrapure water or 1 of 5 concentrations of MeHg (range = 0–0.38 ppm) in ultrapure water and incubated for 10 min.

Brains from control fish were homogenized using a disposable pestle and diluted with assay buffer before

centrifugation at 14 000 *g* for 10 min. 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 or 0.19 ppm, 0.38 ppm, or 0.76 ppm MeHg ($n = 6$) and incubated in the dark for 10 min.

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

Statistical analyses

All data were analyzed using JMP 11.1 (SAS Institute). Normality of all data was determined using the Shapiro-Wilk test, and all data were determined to be normally distributed unless otherwise stated. Total Hg 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, were analyzed using a single-factor ANOVA, by dietary Hg concentration. Length differences in larvae were evaluated using a single-factor ANOVA by adult dietary Hg concentration. An α of 0.05 was used to determine statistical significance for all tests. Differences in embryonic activity between treatments by days postfertilization were determined using a repeated measures ANOVA, followed by a Tukey's post hoc test. Mean dopamine concentrations in clutches from each treatment by days postfertilization were analyzed using a 2-factor ANOVA (using diet and days postfertilization as factors), followed by a Tukey's post hoc test. Dopamine data from brain regions were determined to be nonnormal, and a Kruskal-Wallis test was performed. Data from MAO activity assays were analyzed using a single-factor ANOVA. An α of 0.05 was used to determine statistical significance for all tests.

RESULTS AND DISCUSSION

Accumulation of Hg

The primary route of embryonic exposure to MeHg is 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 because 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 that 67% of fish egg mass is attributed to moisture [46]. Control clutch concentrations ($n = 8$) had a mean dry weight concentration of 0.03 ± 0.02 ppm Hg (wet wt equivalent = 0.0096 ppm). Mean dry weight Hg concentrations in eggs were significantly different between diets (ANOVA degrees of freedom [*df*] = 1, $F = 92.27$, $p < 0.001$). Larval concentrations of Hg (Table 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 0.064 ppm. 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.

Wet weight gonad Hg concentrations (Table 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 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 to meet the energetic demands of egg production because MeHg is generally found associated with proteins in the muscle of fish [47,48]. 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 well below maximum reported tissue concentrations observed in wild-caught freshwater fish [49]. This suggests that the concentration and exposure routes in the present study represent environmentally relevant exposure scenarios. Analysis of brain tissue for total Hg (Table 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 ($n = 5$).

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 [33]. Patterns in movement frequency in embryos from both the control and MeHg-spiked treatments followed a similar trend for each observed time point postfertilization, with no movement at 0 dpf to 1 dpf ($n = 10$), followed by maximum activity at 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 at 2 dpf (Figure 1) compared with controls (ANOVA, $df = 5$, $F = 31.84$, $p < 0.01$). This confirms the findings of Bridges et al. [31], in which significantly increased movements

Table 1. Mean mercury (Hg) tissue concentrations[a,b]

	Control (ppm Hg wet wt)	Mercury (ppm Hg wet wt)
Female muscle	0.084 ± 0.02A ($n = 8$)	0.804 ± 0.08D ($n = 8$)
Male muscle	0.084 ± 0.02A ($n = 5$)	0.506 ± 0.04C ($n = 5$)
Female gonad	0.024 ± 0.007A ($n = 10$)	0.276 ± 0.071BC ($n = 8$)
Male gonad	0.028 ± 0.01A ($n = 5$)	0.204 ± 0.04AB ($n = 5$)
Brain tissue	0.07 ± 0.01 ($n = 5$)	0.46 ± 0.23 ($n = 5$)
Eggs	0.01 ± 0.006 ($n = 8$)	0.074 ± 0.02 ($n = 8$)
Larvae	0.01 ± 0.004 ($n = 6$)	0.064 ± 0.02 ($n = 6$)

[a] ± 1 standard deviation.
[b] Letters denote statistically different groups.

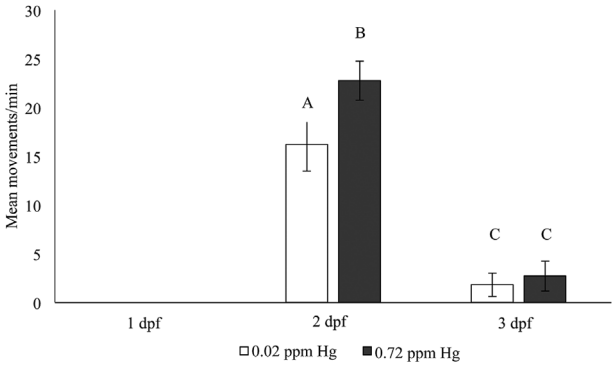

Figure 1. Mean number of observed embryo movements per minute (±1 standard error) for each diet by days postfertilization (dpf). Letters denotes statistically different groups ($p < 0.05$), $n = 10$ clutches for 0 dpf to 2 dpf and $n = 5$ for 3 dpf. Hg = mercury.

were observed at 2 dpf in offspring of adults fed a 0.87-ppm diet. In addition to embryonic hyperactivity at 2 dpf, increased distribution of hatching enzymes led to significantly accelerated hatching rates [31,33]. Mora-Zamorano et al. [32] also attributed hyperactive behaviors observed in zebrafish larvae to embryonic MeHg exposure and indicated that these behaviors may increase the vulnerability of larvae to predators.

Larval length

Larvae from adults administered the 0.72-ppm Hg diet were significantly shorter compared with control larvae (ANOVA $df = 1$, $F = 37.1$, $p < 0.001$). Mean length (±1 SD) of larvae from clutches spawned in treatment tanks were 3.66 ± 0.01 mm ($n = 6$ clutches), whereas larvae from control clutches ($n = 6$ clutches) displayed a mean length of 4.24 ± 0.04 mm. Differences in larval length may be a result of shorter developmental time because of MeHg-induced hyperactivity, disruption of cellular processes, or a combination of these factors [31,50]. 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 [51,52]. Recruitment of fish populations relies on small differences in individual fitness that increase the probability of survival to sexual maturity [51]. Given the apparent negative effects MeHg has on larval size at the low exposure concentrations used in the present study, current exposure scenarios may lead to similar consequences.

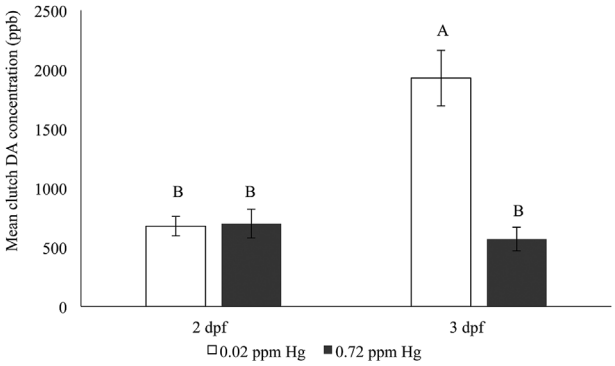

Figure 2. Mean dopamine concentrations in clutches (±1 standard error) for each diet by days postfertilization (dpf). Letters denote significantly different groups ($p < 0.05$), $n = 6$ for all groups. DA = dopamine.

Effects on dopamine and MAO in eggs

Significant differences in dopamine concentrations were observed between treatments (2-way ANOVA $df = 1$, $F = 11.48$, $p = 0.003$; Figure 2). Eggs from control tanks had significantly higher dopamine concentrations at 3 dpf (1.927 ± 0.526 ppm dopamine) compared with eggs exposed to maternally transferred MeHg at 3 dpf (0.568 ± 0.243 ppm dopamine). These results were not anticipated because dopamine is associated with motor activity and significantly more embryonic movement was observed in treated offspring at 2 dpf (though no significant differences in dopamine concentrations were detected at 2 dpf). As previously described, neurotransmitters such as dopamine 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 dopamine 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. Multiple studies have demonstrated increased presynaptic release of dopamine from brain tissue in rats, mice, and rat PC-12 cell lines following MeHg exposure [16,18,24,53,54]. In addition, the results of the present study show significant decreases in MAO activity in eggs incubated with MeHg concentrations as low as 0.048 ppm compared with controls (ANOVA, $df = 5$, $F = 9.43$, $p < 0.01$) [16]. 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). Enzymatic degradation of dopamine is catalyzed by MAO; consequently, significant reductions in MAO activity because of the presence of Hg have negative implications for the rate at which enzymatic degradation of dopamine at the synapse can occur [29]. Reduced MAO activity coupled with possible increased presynaptic release of dopamine may be associated with the hyperactivity seen in developing embryos and fish larvae.

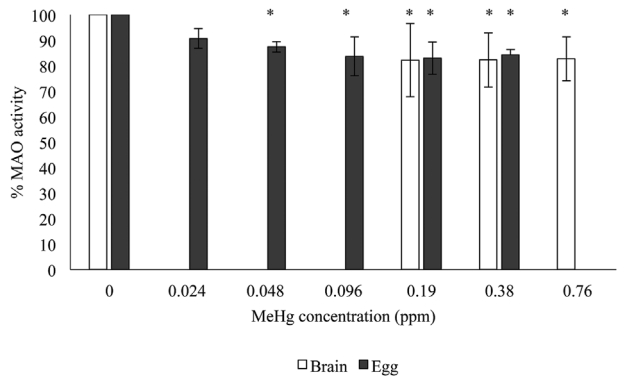

Figure 3. Mean percent monoamine oxidase (MAO) activity (±1 standard deviation) relative to controls measured in pooled clutches of 3 d postfertilization fathead minnow eggs ($n = 5$) and adult brain tissue ($n = 6$) following incubation with a range of relevant methylmercury (MeHg) concentrations. All 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 (*statistical significance).

Dopamine in adult brain tissue

Brain regions showed significant differences in dopamine concentrations regardless of diet (Kruskal-Wallis, $df = 5$, $\chi^2 = 17.89$, $p = 0.003$), with the highest concentrations found in the forebrain (Figure 4). Significant differences between treatment dopamine concentrations were also measured in the forebrain. Mean dopamine concentrations measured in the forebrain of control fish were determined to be 2.07 ± 0.456 ppm, whereas 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 were grouped by diet to represent whole-brain dopamine, no differences in concentrations were indicated. This suggests that whole-brain quantification of dopamine may indeed obscure regional changes that may be associated with alterations in biological function and behavior.

Studies examining the function of the teleost telencephalon (categorized as forebrain in the present study) have indicated that 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 avoidance behavior and learning, and lesions of the dopaminergic projection that connect lower brain centers with the telencephalon lead to reduced locomotor activity [19]. Results of the MAO assay, performed on control brain tissue incubated with a range of MeHg concentrations, showed that mean percent 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 $17 \pm 0.26\%$ (Figure 3). These results are not surprising because the active site of MAO has been shown to be inhibited by chemicals which bind to sulfhydryl groups [55]. Sequencing of human MAO-A and MAO-B has shown that each subunit contains 9 cysteine resides. Of these residues, 2 in MAO-A and 3 in MAO-B are essential for catalytic activity because covalent binding of flavin adenine dinucleotide is prevented in mutant forms of MAO which have serine substituted for cysteine [29,30]. Although teleost MAO is not identical to human isoforms, sequencing of trout MAO shows that in excess of 70% of the amino acid sequence is conserved.

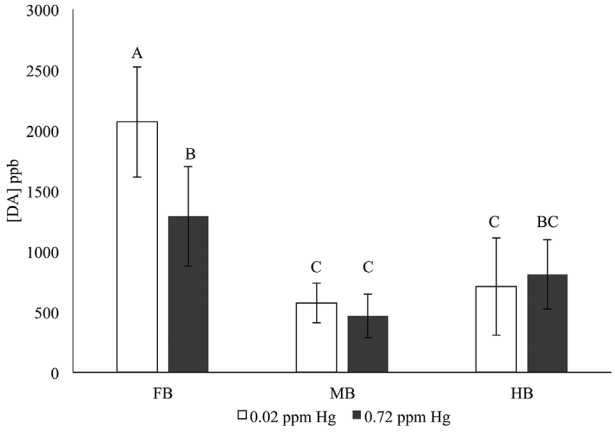

Figure 4. Mean dopamine concentrations in brain regions of adults from each diet (±1 standard deviation). Significant differences in dopamine between brain regions were detected, with the highest concentrations found in the forebrain. Forebrains of control fish were also significantly higher in dopamine concentrations than those of fish fed 0.72 ppm dietary Hg following the 30-d dietary study. Letters denote significantly different groups ($p < 0.05$), $n = 5$ for all groups. DA = dopamine; FB = forebrain; HB = hindbrain; MB = midbrain.

In addition, known inhibitors of human MAO isoforms inhibit trout MAO, suggesting conservation of critical residues in the active site [29,30]. This indicates that inhibition of MAO activity by MeHg may have serious implications for the developing nervous system in higher vertebrates, including humans. As mentioned previously, it is possible that decreased enzymatic degradation of dopamine by MAO may counteract overall decreases in dopamine concentrations, allowing for short-term maintenance of dopaminergic neuron activity in adult brains. It is also very possible that presynaptic release of dopamine is increased in brains of fish, similar to results obtained in studies using rat brain, mouse brain, and rat PC-12 cell lines [16,18,24,53,54].

Reproduction

No significant effects of dietary MeHg were observed on any reproductive endpoints measured in adults during the 30-d feeding study, including mean clutch size (ANOVA, $df = 1$, $F = 1.11$, $p = 0.31$) and spawning frequency (ANOVA, $df = 1$, $F = 0.96$, $p = 0.34$). 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 d did not lead to deleterious effects on reproduction [31,40].

Dopamine has been shown to have a powerful inhibitory effect on reproduction in teleosts, by opposing the stimulatory function of gonadotropin-releasing hormone [22,37,56]. Increases in dopaminergic activity and/or significant reductions in MAO activity can prevent final oocyte maturation, ovulation/spermiation, as well sexual maturation in some teleost species [37]. Total-brain dopamine concentrations were not significantly different between diets after 30 d, and the effects of regional differences in overall dopamine concentrations seen in the forebrain of adult fish are likely diluted by the significant reduction in MAO activity. As previously described, catecholamines (including dopamine) have complex cycling, and it is possible that other mechanisms (transporters, vesicular release) are able to maintain proper dopamine 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 d) exposure to low levels of dietary MeHg.

CONCLUSIONS

Effects of MeHg on fish health are well studied in adult animals, but few studies are available that examine the effects of maternally derived MeHg on early–life stage fish. This is despite the sensitivity of early–life stage fish to MeHg exposure, which can result in adverse impacts on development at very low exposure concentrations. Changes to overall dopamine concentrations in both exposed embryos as well as adult telencephalons were observed. Significant inhibition of MAO activity in the presence of MeHg concentrations measured in exposed eggs were also observed in in vitro assays. Given that MAO enzymatically degrades dopamine, it is reasonable to conclude that functions regulated by the dopaminergic system, including cognition, attention, reward-motivated behaviors, and motor function, may be altered in the presence of MeHg in embryos [12,57]. In support of this conclusion, significantly increased motor activity in MeHg-exposed offspring was confirmed by the present study, behavior which can be expected to persist into larval stages [32]. Hyperactivity has been associated with decreased time to hatch under similar exposure scenarios, and it is possible that reduced

development time may impact larval size [31]. Given that hyperactivity and reduced size are critical factors which greatly increase the likelihood of predation, these modified behaviors may contribute to reduced recruitment of young [52,58]. Further research is needed to determine the extent to which these changes in behavior and survival may affect individual fitness and population structure.

Acknowledgment—The authors thank B. Soulen for her contributions to the present study. All procedures were approved by the University of North Texas Institutional Animal Care and Use Committee under protocol 1303-3.

Data availability—Data, associated metadata, and calculation tools are available from the corresponding author (aproberts@unt.edu).

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