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EMBRYOTOXICITY OF MATERNALLY TRANSFERRED METHYLMERCURY TO FATHEAD MINNOWS (*PIMEPHALES PROMELAS*)

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Abstract: Mercury (Hg) is a ubiquitous environmental contaminant and potent neurotoxin. In aquatic environments, Hg can be transformed into methylmercury (MeHg), which bioaccumulates in aquatic food webs, including fish. Methylmercury has been shown to transfer from female fish to developing eggs; however, relatively little is known regarding the effects of maternally transferred MeHg on fish embryos. The present study evaluated the effects of maternally transferred MeHg on fathead minnow (*Pimephales promelas*) embryos. Embryos were collected from adult fatheads exposed for 30 d to 1 of 3 diets spiked with MeHg: a control diet (0.02 ppm Hg dry wt), a low diet (0.87 ppm Hg dry wt), or a high diet (5.5 ppm Hg dry wt). No effects on spawning frequency, clutch size, or total egg output were observed. In embryos, Hg concentration was a function of female diet and the duration (number of days) of female exposure. Compared with controls, embryos from the low-diet treatment displayed altered embryonic movement patterns (hyperactivity) and decreased time to hatch. Embryos from the high-diet treatment had delayed hatching and increased mortality compared with the other treatments. Collectively, these results suggest that maternally transferred Hg may impact survival, behavior, and developmental milestones of the embryo-larval stages of fish. *Environ Toxicol Chem* 2016;35:1436–1441. © 2015 SETAC

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INTRODUCTION

Mercury (Hg) is a widespread environmental contaminant released through a variety of natural processes and anthropogenic activities. Following deposition into aquatic systems, Hg can be transformed by microbes into methylmercury (MeHg), a form which bioaccumulates and biomagnifies through aquatic food webs. Methylmercury results in effects on fish health at concentrations exceeding 0.2 ppm to 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 and alter reproduction in some fish [2–4].

A feeding study examining the effects of MeHg-spiked diets (0.455 ppm Hg wet wt, 0.959 ppm Hg wet wt) on predator avoidance behavior of adult golden shiners (*Notemigonus crysoleucas*) concluded that several predator avoidance behaviors were altered after a 90-d 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 mates. A review by Depew et al. [1], which incorporated data from feeding studies using commercially prepared diets spiked with MeHg, MeHg injected prey items, and prey items naturally contaminated with environmental MeHg, concluded that fish consuming diets greater than approximately 0.2 ppm dry weight dietary Hg are at risk for reproductive effects. Such effects include decreases in gonadosomatic index, sex steroid production, spawning success, spawning behavior, fertilization success, and fecundity, and increases in ovary apoptosis. Drevnick et al. [6] reported that plasma testosterone in males and 17β-estradiol in female fathead minnows were significantly decreased in fish fed an MeHg-spiked diet containing 0.87 ppm (dry weight) Hg compared with low Hg 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 Hg concentrations 0.07 ppm to 0.10 ppm (wet wt) in eggs of rainbow trout led to increased embryo mortality. Transgenerational effects of maternally derived MeHg are understudied, but 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 Hg in adult fish and their eggs is MeHg [10]. Because Hg has a strong bonding affinity for reduced sulfur atoms, MeHg forms conjugates with sulfur containing biomolecules, resulting in compounds that resemble endogenous amino acids. This molecular mimicry provides a mechanism of transfer for dietary MeHg from adult female fish to eggs through amino acid transporters [11]. Previous research has shown that the diet of the maternal adult during oogenesis, rather than adult body burden, is the principal source of Hg in eggs [12].

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

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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 and divided among fifteen 21-L glass aquaria. Each tank contained 3 females (control, 2.01 ± 0.51 g; low, 1.89 ± 0.45 g; and high, 1.81 ± 0.49 g wet wt) and 1 male fish (control, 4.17 ± 1.8 g; low, 4.21 ± 1.2 g; and high, 3.97 ± 1.6 g wet wt), as well as 2 breeding tiles constructed from halved PVC pipe sections. All tanks were equipped with activated carbon filtration systems 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 were maintained by performing 25% water changes daily and 50% water changes every 3 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. Waste and food debris were siphoned daily from each aquarium. At the conclusion of the study, adult fatheads were euthanized using MS-222 according to IACUC protocol.

Experimental design

Following the 2-wk acclimation period, aquaria were assigned randomly to either a control diet containing 0.02 ± 0.002 ppm Hg dry weight ($n = 5$) or 1 of 2 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 because 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 2 times per day for 30 d. Diets were prepared by mixing fish food with MeHg chloride (Sigma-Aldrich) dissolved in reagent ethanol (Fisher Scientific) in an acid-washed glass dish. Ethanol was removed by evaporation in a fume hood. Food was prepared according to 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 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 occur 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, because they are asynchronous spawners. Total maturation time for each oocyte is approximately 3 d [14]. 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 5 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 spawning, clutch size, and total egg production for each diet were tabulated by first finding a mean for each replicate ($n = 5$) and then finding the mean of the replicates to obtain a treatment mean. Subsamples of 20 fertilized embryos (determined by microscopic examination) from each clutch were transferred to a crystallizing dish containing reconstituted moderately hard water 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.

The effects of maternally transferred MeHg on embryo neurodevelopment were assessed by comparing the mean number of movements per minute observed in subsamples of embryos daily for 7 d postfertilization (dpf). To attain the mean number of movements per minute, each clutch was transferred to a Petri dish containing reconstituted moderately hard water under an Olympus dissecting scope equipped with a Canon Vixia HF G30 video camera. After a 2-min acclimation period on the stage of the microscope, each clutch was recorded for 1.5 min. Movement counts were observed for all 20 embryos in each clutch subsample over a consistent timeframe within the recording. This was done to ensure that environmental factors affecting startle responses were standardized. 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 5:00 PM each day until 7 dpf, the number of hatched embryos in each clutch was 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 Hg with a DMA-80 Direct Mercury Analyzer (Milestone) according to US Environmental Protection Agency method 7473 [15]. Calibration curves were generated using 3 reference materials from the National Research Council of Canada: MESS-3 (marine sediment; certified value = 0.091 ± 0.009 ppm dry wt), 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. Blanks (empty boats) were analyzed every 20 samples, and concentrations for all blanks ($n = 38$) were below the method detection limit. The method detection limit, determined as the standard deviation of 7 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 as follows: MESS-3, $101.7 \pm 5.2\%$ ($n = 6$); TORT-2, $109.6 \pm 3.2\%$ ($n = 7$); and DOLT-4, $105.1 \pm 1.3\%$ ($n = 7$).

Statistical analyses

Data were analyzed using JMP 11.1. Normality of the data was confirmed using the Shapiro–Wilk test. Adult muscle and gonad total Hg concentrations were evaluated for differences using a 2-factor analysis of variance (ANOVA) followed by a Tukey's post hoc test, with gender and food Hg concentration as factors. Single factor ANOVA models were used to compare data for spawning frequency, mean clutch size, and mean egg

output using food Hg 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 (ET50) 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 Hg in adults and eggs

Gonad and muscle Hg concentrations in both genders were significantly different among all 3 diets (ANOVA, $F_{2,13} = 68.6$, $p < 0.01$ and ANOVA, $F_{2,13} = 322.2$, $p < 0.01$, respectively). Mean gonad Hg concentrations (Table 1) were significantly different between genders in fish administered the 5.5-ppm diet (ANOVA, $F_{5,52} = 77.2$, $p < 0.01$). Males had significantly higher gonad concentrations than females, which may be attributed to losses of contaminants due to maternal transfer of dietary MeHg to eggs [16,17]. Mean muscle Hg concentrations (Table 1) did not vary significantly between genders (ANOVA, $F_{5,52} = 197.6$, $p > 0.5$), 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 more than 5000 sites in Canada reported mean wet weight Hg concentrations in 104 fish species, including Walleye, Northern Pike, and Lake trout, which are 3 important sport fish in Canada. The median Hg concentrations for these 3 species were 0.41 ppm, 0.38 ppm, and 0.28 ppm, with maximum concentrations of 10.4 ppm, 10.9 ppm, and 10 ppm, respectively [18]. 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.

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 through 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, whereas 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 standard deviation associated with the treatment mean. The proportion of Hg mobilized to eggs increased with increasing dietary concentrations (Figure 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.

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 1). Because 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 [16,17,19], a plateau in egg Hg concentrations would be anticipated in all treatments if maternal transfer depended 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 indicate that egg Hg 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 Hg concentration (ANOVA, $F_{2,13} = 220.9$, $p < 0.01$).

A study by Stefansson et al. [16] employed the use of stable MeHg isotopes to investigate the sources of Hg transferred to eggs. Adult sheepshead minnows (*Cyprinodon variegatus*) were exposed to 1 of 3 MeHg-spiked diets containing 1 ppm, 5 ppm, 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 us to characterize the proportion of Hg in eggs derived from maternal body burden versus maternal diet during oogenesis. The results indicate that a constant percentage of maternal body burden was transferred to eggs across all treatments; however, the majority

Table 1. Mean ± 1 standard deviation total mercury (Hg) concentrations in muscle and gonad from adult fathead minnows fed 1 of 3 experimental diets

Diet	Gender	*n*	Mean muscle Hg (ppm dry wt)	Mean gonad Hg (ppm dry wt)
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 statistical significance ($p < 0.05$).

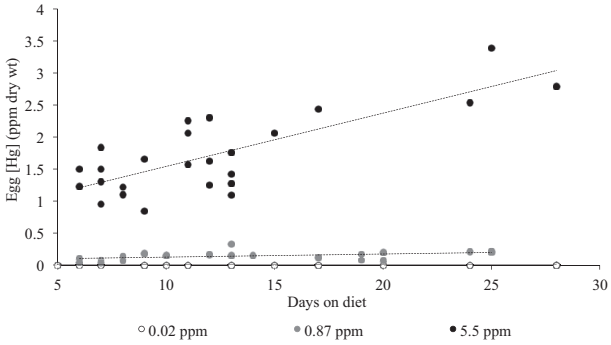

Figure 1. Concentration of total mercury (Hg) in eggs from adult fathead minnows fed 1 of 3 methylmercury-spiked diets for 30 d. Each data point is representative of mean Hg concentration measured in an individual clutch.

(range = 84% to 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 d administration of MeHg-spiked diets.

Reproduction

No significant effects of dietary MeHg were observed on any reproductive endpoints measured in adults during the 30-d feeding study (Figure 2), including mean clutch size (ANOVA, $F_{2,14} = 3.2$, $p = 0.08$), mean egg output (ANOVA, $F_{2,13} = 0.54$, $p = 0.59$), and spawning frequency (ANOVA, $F_{2,15} = 1.0$, $p = 0.41$). Although muscle Hg concentrations resulting from experimental diets in the present study exceeded the threshold of 0.2 ppm wet weight thought to be protective against changes in reproduction, this lack of effect was anticipated [1,4]. Penglase et al. [17] demonstrated that fish fed MeHg-spiked diets (12 ppm Hg dry wt) displayed increased mating (+66%) and overall reproductive success (+100%) when compared with controls during the first 100 d of the feeding study. Thereafter, 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 that in controls, whereas reproductive success was 37% lower when compared with 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 1 of 3 MeHg-spiked diets (0.88 ppm Hg, 4.11 ppm Hg, or 8.46 ppm Hg dry wt) 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 despite increasing body burdens during the 136-d exposure period. All fish used in the present study were sexually mature prior to dietary exposure, and exposure duration was significantly shorter (30 d). 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].

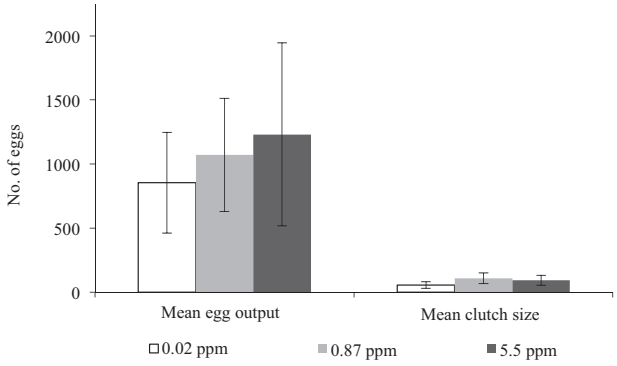

Figure 2. Reproductive metrics from adult fathead minnow fed 1 of 3 methylmercury-spiked experimental diets. No significant effects on mean clutch size ($p = 0.10$) or mean egg output ($p = 0.59$) were observed among the diets. Mean egg output and mean clutch size were both calculated using means from 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 Hg diet treatments (Figure 3). This consisted of little to no movement 0 to 1 dpf, followed by a spike to the maximum number of movements per minute 2 dpf, and then decreasing movement each day until hatch. However, the 0.87-ppm Hg treatment embryos had greater number of movements 2 dpf (Figure 3) than the other 2 treatments (ANOVA $F_{21,61} = 34.85$, $p < 0.01$). At 3 dpf, the 0.87-ppm treatment eggs still displayed a higher number of movements than the other 2 treatments (similar number of movements to the maximum for the other 2 treatments that occurred 2 dpf).

A study conducted by Webber [5] found a 90-d exposure to environmentally relevant concentrations of dietary MeHg (range = 0.46–0.96 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 ET50 (± 95% confidence interval) 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 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 caused by increased embryonic movement [19]. Premature hatch was also observed in zebrafish (*Danio rerio*) embryonically exposed to waterborne concentrations as low as 0.010 ppm MeHg [20]. Data in the present study suggest that maternally transferred Hg, a more ecologically relevant mode of exposure compared with waterborne dosing, also affects embryonic development in fish.

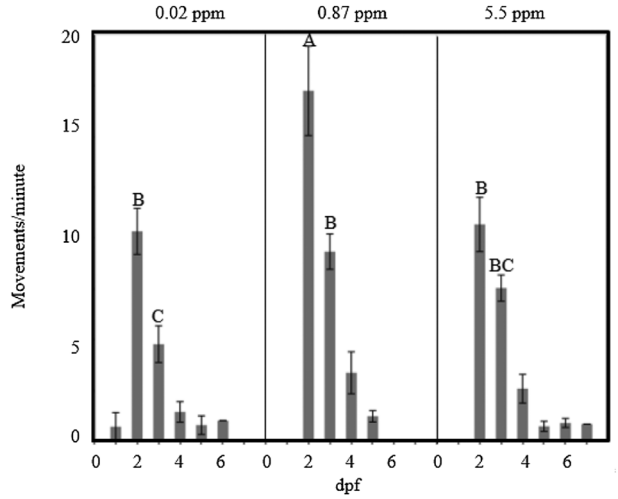

Figure 3. Mean embryonic movement per minute each day postfertilization (dpf), by treatment ± 1 standard error. Letters denote statistically different groups. Mean movements per minute for each treatment were calculated using means of individual clutches (control, $n = 5$; low, $n = 5$; high, $n = 6$).

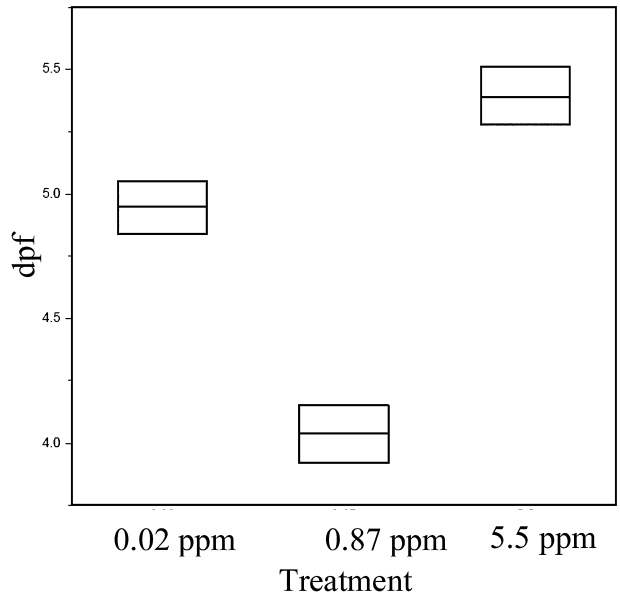

Figure 4. Median time to 50% hatch for egg clutches from adult fathead minnows fed 1 of 3 experimental mercury diets. Boxes represent 95% confidence intervals. dpf = days postfertilization.

Embryos from the 5.5-ppm diet experienced a delayed hatch, as demonstrated by the ET50 values (Figure 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 honest significant difference $p < 0.04$) when compared with the offspring from the 0.87-ppm (21.5% ± 2.3) and control diets (24.6% ± 9.0; ANOVA, $F_{2,12} = 5.7$, $p = 0.02$). Anecdotally, a large number of surviving offspring from the 5.5-ppm treatment also displayed spinal deformities 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 embryolarval stages, and dose–dependent decreases in hatching success were observed in eggs from walleye in Clay Lake, Ontario, following exposure to increasing environmentally concentrations of waterborne MeHg (0.1–7.8 ppm) [8]. In contrast to the results of the present study, however, 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 h postfertilization [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.

CONCLUSION

Assessments concerning the effects of MeHg on fish health have focused largely 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]. In the present study, 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 d exposure. Consequently, significant effects on hatch and survival were observed. These results indicate that 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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Data availability—Data, associated metadata, and calculation tools are available from the authors (aproberts@unt.edu).

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