UNIVERSITY OF WISCONSIN-LACROSSE

Graduate Studies

THE EFFECTS OF DIETARY METHYLMERCURY ON ZEBRAFISH
(*Danio rerio*) REPRODUCTIVE GENE TRANSCRIPTION

A Manuscript Style Thesis Submitted in Partial Fulfillment of the
Requirements for the Degree of Master of Science in Biology

Nathan D. Susnik

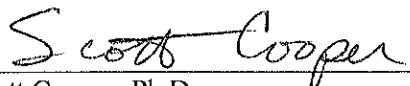College of Science and Health
Department of Biology

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THE EFFECTS OF DIETARY METHYLMERCURY ON ZEBRAFISH (*Danio rerio*)
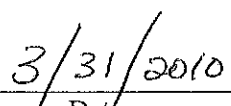REPRODUCTIVE GENE TRANSCRIPTION

By: Nathan Susnik

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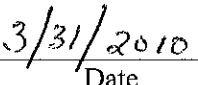The candidate has completed the oral defence of the thesis.

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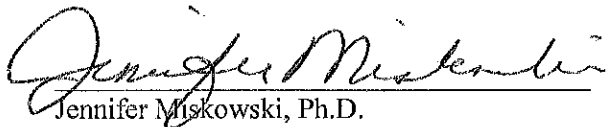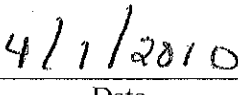
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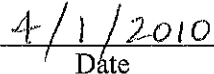
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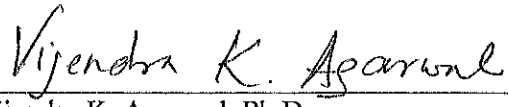
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ABSTRACT

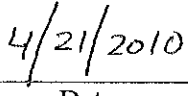Susnik, N.D. <u>The effects of dietary methylmercury on zebrafish (*Danio rerio*) reproductive gene transcription.</u> MS in biology, May 2010, 30. (C.T. Cooper)

Studies have revealed that environmentally realistic concentrations of dietary methylmercury have adverse effects on reproduction in many different fish species. Recent studies have used the fathead minnow (*Pimephales promelas*) to determine how methylmercury is affecting reproduction on a genomic level, and researchers have seen a disregulation of three reproductive genes: zona pellucida 2 (zp2), zona pellucida 3 (zp3) and vitellogenin (vtg). Unfortunately, the fathead minnow is not an optimal species for genomic and proteomic research because of the lack of genetic information available. The zebrafish (*Danio rerio*) is a major developmental model species that has most of its genome sequenced, yet there are no published studies exposing zebrafish to environmentally realistic concentrations of methylmercury. I fed groups of zebrafish a low (0.89 μg Hg g^{-1}) or a medium concentration (3.4 μg Hg g^{-1}) concentration of methylmercury for a total of 60 days. The expressions of gonadal zp2, zp3 and hepatic vtg were measured with quantitative reverse transcriptase polymerase chain reaction (QRT-PRC). Both zp2 and zp3 were down-regulated in females, while there was no significant change in males. The results were not consistent with studies in other species. Further studies will be required to determine if zebrafish will make a good model for methylmercury research.

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INTRODUCTION

Since the beginning of the industrial revolution, anthropogenic pollution has greatly increased globally. Measuring the effects of this pollution on organisms in the environment can be difficult for researchers, as many variables can influence the health of an animal. One heavy metal pollutant that has increased in the environment dramatically in the past 100 years is mercury (Lindberg et al., 2007). Mercury is deposited into the environment from both natural and anthropogenic sources. Natural events that lead to high levels of atmospheric mercury include forest fires and volcanic eruptions. The largest source of anthropogenic mercury released into the atmosphere is from combustion of fossil fuels, which accounts for about 60% of atmospheric mercury (Pacyna et al., 2000). Mercury is released into the atmosphere in its gaseous elemental form (Hg^o) and as reactive gaseous Hg (RGM), which includes oxidized gaseous Hg(II), and particle-bound Hg. These RGMs are then deposited into the environment regionally (Nater & Grigal, 1992) and globally (Keating et al., 1997).

In addition to being present in the atmosphere, mercury can enter aquatic environments from the atmosphere in precipitation or attached to solid particles (wet fall and dry fall) and from terrestrial sources through run-off (Lindberg et al., 2007). Once in an aquatic environment, oxidized forms of mercury can be methylated by microbes to form methylmercury. Methylmercury is lipid soluble and moves through aquatic food webs, with the greatest bioaccumulation in piscivorous animals. The most efficient method of methylmercury uptake is through the gastrointestinal tract (Hall et al., 1997).

Up to 95% of mercury entering the gastrointestinal tract is absorbed into the bloodstream (Keating et al., 1997). Dermal uptake of methylmercury also occurs but at a lower efficiency. Human exposure to methylmercury occurs mainly through consumption of fish or marine mammals. Methylmercury has a high affinity for sulfhydryl groups and will bind to the amino acid cysteine and can then be carried across the blood-brain and placental barriers via an amino acid transport protein (Kajiwara et al., 1996). Methylmercury was historically recognized for its effects on brain and neural tissue, and acute poisoning leads to ataxia, dysarthria, tremors, constriction of the visual field and auditory disturbances (Elhassani, 1982). However, there are other adverse effects of methylmercury exposure, particularly during development. Links between methylmercury and development were discovered during a large industrial contamination in Minimata, Japan (Harada, 1978). Women exposed to methylmercury showed little or no symptoms of mercury exposure, but infants born to those women had many problems including: mental retardation, cerebellar ataxia, slow reflexes, and spontaneous abortion of male fetuses (Harada, 1978; Sakatomoto et al., 2001). Methylmercury has effects on humans at high concentrations, but recent studies have shown effects on fish at much lower concentrations.

Links between reproduction and methylmercury exposure have been observed in teleost fish, which is a concern for the multi-billion dollar commercial fishing industry. Friedmann et al. (1996) first reported links between methylmercury and reproduction in walleyes (*Sander vitreus*). This study found that ecologically relevant exposure to methylmercury led to a reduced gonadal somatic index (GSI, total weight of the fish's gonad divided by the total wet weight of the fish) and testicular atrophy. Recent studies

in fathead minnows (*Pimephales promelas*) have shown that at dietary doses as low as 0.88 μg Hg g^{-1} dry weight, spawning success decreased 29%, and at 8.46 μg Hg g^{-1} dry weight, there was a 39% decrease in spawning success compared to controls (Hammerschmidt et al., 2002). Additional studies on the reproductive endocrinology of fathead minnows have shown that male fish exposed to low levels of methylmercury had a 50% decrease in testosterone and exposed females had an 80% decline in 17β-estradiol (Drevnick & Sandheinrich, 2003). The same study also noted a 40% decrease in the GSI of female fish fed low levels of methylmercury.

Ecotoxicogenomics is a new field where researchers measure changes in gene transcription in response to chronic exposure to a toxin. Researchers can accurately measure the amount of transcription of a single gene with a microarray or quantitative reverse transcriptase polymerase chain reaction (QRT-PCR). Microarrays are used to approximate the relative amount of transcription of many genes at once, whereas QRT-PCR is used to very accurately quantify the transcription of relatively few genes. The results from these assays indicate how gene expression is affected by exposure to a toxicant. While most toxicological research in aquatic systems is based on death as an endpoint, toxicogenomics provides a measure of milder cellular responses and thus could provide more accurate risk assessment methods based on low-dose chronic exposure.

In depth toxicogenomic studies have been performed on fathead minnows with microarrays to examine endocrine function at the transcriptional level. The regulation of three major genes involved in reproduction was affected by methylmercury. Vitellogenin (vtg) expression was up-regulated 142-fold in male fish exposed to low levels of methylmercury. Zona pellucida 2 (zp2) and zona pellucida 3 (zp3) also showed

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significant transcriptional up-regulation in male fish, 15-fold and 3-fold respectively. However, in female fish vtg was down-regulated 0.6-fold, and zp2 and zp3 were up-regulated 9.5-fold and 11.1-fold respectively (Klaper et al., 2006).

The proteins zp2, zp3 and vtg all have well characterized functions in the oocyte. All vertebrate species have an acellular envelope that surrounds the oocyte. In fish the envelope is referred to as the chorion and in mammals it is the zona pellucida. The zona pellucida varies slightly from species to species but it is normally comprised of three or four glycoproteins. The four known glycoproteins in mammals are named zona pellucida 1 to zona pellucida 4 (zp1-4). Zp1 provides structural support to the acellular envelope. Zp2 may serve as a secondary sperm receptor and will only bind sperm after the sperm has undergone the acrosome reaction. (Bleil et al., 1988). Zp3 is the primary sperm receptor in mammals and it is known to help induce the acrosome response in sperm (Caballero-Campo et al., 2006). Zp4 has recently been discovered only in mammals and the function is unknown. In teleost fish less is known about the functions of the zp glycoproteins but it is assumed that they have relatively the same functions as in mammals because of sequence homology. However, this has not been confirmed experimentally. While zp3 in fish is homologous to mammalian zp3 in zebrafish (*Danio rerio*), zp3 may also be involved with hardening of the chorion after fertilization (Del Giacco et al., 2000). Normally zp2 and zp3 are expressed in the ovaries of zebrafish, but hepatic expression of these two proteins has been seen in the Atlantic salmon (*Salmo salar*) (Celius & Walther, 1998). During dietary exposure to methylmercury, zp2 and zp3 expression has been detected in the gonads of male fathead minnows, but the effects of this transcription on the reproductive health of the minnows are unknown (Klaper et al.,

2006).

Vitellogenin (vtg) is another reproductive gene with disrupted expression in fathead minnows exposed to methylmercury (Klaper et al., 2006). Vitellogenin is a major female yolk-sac precursor glycolipoprotein produced in the liver of teleost fish and oviparous animals (Danilchik & Gerhart, 1987). During vitellogenesis, vtg is produced and released into the bloodstream. The protein travels to the oocyte and enters through receptor-mediated endocytosis where it then forms yolk granules with help from enzymes (Danilchik & Gerhart, 1987). Vitellogenin expression in male fish is a common biomarker for xenoestrogen pollutants in aquatic systems, and it could be used as a biomarker for high levels of methylmercury in aquatic systems. However, methylmercury is considered an endocrine disrupting chemical in fathead minnows (Drevnick & Sandheinrich, 2003) and not a xenoestrogen. Furthermore, altered regulation of vtg is a known biomarker for endocrine disruption in zebrafish (Rose et al., 2002, Martyniuk et al., 2007). Expression of vtg in male fish is thought to be harmful because males cannot remove it from their bodies through egg production, and the accumulation of vtg may result in renal failure (Folmar et al., 2001). Recent research has found that plasma vtg concentrations greater than 1 mg/mL induced by male exposure to estrogens led to a 33% increase in mortality. Decreased vtg expression in female fish also decrease reproductive success (Thorpe et al., 2007). A stressor that causes a 25% decrease in vtg production in female fathead minnows will cause a projected 35% decrease in population size (Miller et al., 2007).

Although vtg is a major yolk-sac precursor in many species, new research suggests that vtg may have a second function. Recent studies in honey bees (*Apis*

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mellifer) suggest that vtg may promote longevity, and have endocrine function that affects foraging behavior (Seehuus et al., 2006, Corona et al. 2007, Nelson et al., 2007). The suggested mechanism of action for the promotion of longevity is that vtg is an antioxidant (Seehuus et al., 2006). Although secondary functions of vtg have not yet been discovered in fish and mammals, it is possible that changes in vtg expression in fish exposed to methylmercury may also have non-reproductive repercussions.

Reproduction and reproductive endocrinology of fathead minnows are disrupted after exposure to dietary methylmercury. Vitellogenin is sensitive to estrogen induction, and is up-regulated in male and down-regulated in female fathead minnows during chronic exposure to methylmercury (Klaper et al., 2006). *Zp2* and *zp3* are genes that are insensitive to estrogen induction in the gonad (Liu et al., 2006) and are up-regulated in female and male fish by chronic exposure to methylmercury (Klaper et al., 2006). Whether the changes in expression of these genes occur by the same mechanism, or by multiple independent mechanisms, has not been determined.

Research on gene expression using fathead minnows as a model species is not optimal because of the lack of comprehensive genomic and proteomic data. An alternative model fish species used in many different types of genetic and developmental biology research is the zebrafish. It is a valuable model vertebrate species, in part, because of the recent progress in sequencing its entire genome. Information about the zebrafish genome, transcriptome, and proteome can be found at several online resources. Zebrafish are also small vertebrate teleost fish with a relatively short life cycle, which makes them an optimal species for toxicogenomic research. Toxicogenomic research in zebrafish has already revealed that methylmercury induced changes in gene expression in

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the liver and skeletal muscle, with the largest changes seen after 21 days in skeletal muscle (Gonzalez et al., 2005). However, the methylmercury concentrations in this study were too high to be considered environmentally realistic outside of areas of goldmine contamination (Durrieu et al., 2005; Wiener et al., 2003). There are currently no published studies where zebrafish were exposed to environmentally relevant levels of dietary methylmercury and the impact on gene expression was measured. Zebrafish are not only good for genomic research, but also for reproductive and developmental research. They are very easy to culture, lay a fairly large clutch of eggs, and have good fertilization rates. Zebrafish have a transparent chorion and the embryo can be seen throughout development. Zebrafish also develop very quickly and hatch about 72 hours after the eggs have been fertilized. The embryonic development of zebrafish has been studied extensively, making them a preferred model for studying vertebrate development (Hisaoka & Battle, 1958, Hisaoka & Firlit, 1960).

In this current study, two groups of zebrafish were fed different environmentally relevant concentrations of methylmercury for a total of 60 days to determine if similar changes in gene transcription could be observed in zebrafish as previously observed in fathead minnows (Klaper et al., 2006). Livers and gonads were removed from the fish, GSI were calculated and zp2, zp3 and vtg mRNA levels were quantified with QRT-PCR.

METHODS AND MATERIALS

Exposure

Adult zebrafish of the ZDF wild-type strain (Aquatica Tropicals, Plant City, Florida) were used for this study. All fish were allowed to acclimate for five days after arrival and were then sorted into their respective tanks. The zebrafish were randomly sorted into 15 separate 38-L tanks arranged with a flow-through system. The tanks were cleaned by siphon every five to six days by removing one-third of the water in each tank, which was then slowly replaced. The tanks were kept at 28°C for the duration of the study. There were three separate diet groups: control, low methylmercury, and medium methylmercury. There were five tanks per group, two that housed 16 male fish per tank and three that housed eight female fish per tank. The zebrafish were fed twice daily with a diet that was equal to about five percent of their wet body weight. The diets were designed to represent environmental conditions. The control diet was normal food sent from the manufacturer, and contained 0.034 μg Hg g^{-1} dry weight. The low methylmercury diet was contaminated with 0.890 μg Hg g^{-1} dry weight, and the medium methylmercury diet was contaminated with 3.40 μg Hg g^{-1} dry weight.

Tanks were maintained for 60 days on a photoperiod of 14 hours of light and 10 hours of dark per day. At the end of the 60th day the remaining fish were removed, sacrificed, weighed, and livers and gonads were removed, flash frozen in liquid nitrogen, and stored in a -80°C freezer until RNA isolation. Individual gonads were weighed before they were frozen for calculation of the GSI. The remains of the fish were saved

for methylmercury analysis.

Contamination of Diets

Fish food (TetraColor -- small granule, Tetra Products, Melle, Germany) was mixed with ethanol (Fisher Scientific, Walham, MA) containing dissolved methylmercuric chloride (Alpha Chemical, Stoughten, MA) in an acid washed glass pan. Control diet was mixed with only ethanol. The diets were then placed under a fume hood until dry, and then stored in a refrigerator until use.

Quantitative RT-PCR (QRT-PCR)

All liver and gonad tissue samples were processed to quantify the amount of zp2, zp3, and vtg mRNAs. Total RNAs were isolated using the Absolutely RNA Miniprep Kit® (Stratagene, La Jolla, CA) following manufacturer's protocol for isolation from animal tissues. The RNA isolations included treatment of the RNA with DNAse to prevent genomic DNA contamination. QRT-PCR was performed in two steps. The cDNA was made with AffinityScript QPCR cDNA Synthesis Kit® (Stratagene, La Jolla, CA) and random hexamers as primers. The reactions were made according to manufacturer's protocol, and incubated for five minutes at 25°C followed by a 45 minute incubation at 42°C and finally a five-minute incubation at 95°C to terminate cDNA synthesis. The cDNA was quantified with a Brilliant SYBR Green Q-PCR, AffinityScript Master Mix Kit® (Stratagene, La Jolla, CA) using a light cycler (Roche, Basel, Switzerland). cDNAs were quantified using the following primers: 5'- GAAATGCAGTCACTGTCCAG-3' (forward) and 5'-GAAACCTCAAACTGGCTGTCT-3' (reverse) for zp2; 5'-

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TGTGGTGATGATGACTGA-3', (forward) and 5'-GACCAGTCATCAGTCATGAG

(reverse) for zp3 in accordance with Xingjun *et al*. (2007); 5'-

ATCATGAAGGATGTTGGCTTGG-3' (forward) and 5'-

CTTCCATCATTGCAGCACCA-3', (reverse) for vtg1; 5'-

AGGATCTGTCTCTGCATGAC-3' (forward) and 5'-GACACACAAATTCCTCCAGC-3'

(reverse) for actin as previously published by Gonzalez *et al.* (2005). QRT-PCR was

performed with 10-minute denaturation at 95 °C and then 40 cycles of amplification as

follows: 95°C denaturation for 30 seconds, 53°C annealing for one minute, and 72°C

elongation for 1.5 minutes. This was followed by an analysis of melting curves in

accordance with the manufacturer's instructions. The amplification curves were

measured and normalized to actin mRNA to quantify the relative amount of transcription.

Statistical Analysis

Normalization to Actin mRNA

All calculated mRNA concentrations were normalized relative to actin mRNA

with the following formula $E_A{}^{ACP}/E_G{}^{GCP}$. E_A represents the efficacy of the actin

reaction; which was calculated by performing QRT-PCR on a serial dilution of a sample,

and the slope of the $\log^{-1}$ of the crossing points was calculated. A crossing point is the

point at which the extrapolated amplification curves cross the x-axis. ACP represents the

actin crossing point of the unknown sample. E_G represents the efficacy of the gene of

interest (either *zp2, zp3* or *vtg*) for the reaction; and was calculated from the slope of the

$\log^{-1}$ of the crossing points of a serial dilution of each gene's cDNA. GCP represents the

gene of interest's crossing point of the unknown sample. Averages and standard

deviations were calculated for the normalized cDNA levels.

Mortality rates were compared between sexes of fish and among treatment groups with a χ^2 test. The effects of mercury on growth of the fish, the gonadal somatic index, and expression of zp2, zp3, and vtg were tested with a one-way analysis of variance (ANOVA). A separate ANOVA was conducted for male and female fish. Individual tanks were considered as the experimental unit for purposes of statistical analysis and null hypotheses were rejected at $p < 0.05$. Correlation coefficients between gene transcription and body weight or GSI were calculated with Pearson's correlations.

RESULTS

Mortality

Through out duration of the exposure period mortality was monitored, and there was no significant difference in mortality among exposure groups of fish compared to the control diet (df=1 χ^2= 0.26 and 3.10 for the low and medium exposure groups). During the first 30 days of exposure, 32 fish died (nine from the control group, 10 from the low methylmercury group and 13 from the medium methylmercury group). During the last 30 days of exposure only nine fish died, two from the control group, two from the low methylmercury group, and five from the high methylmercury group. The mortality rate during the first 30 days was 3.6-times higher than the last 30 days.

Effects of dietary methylmercury on growth and gonad size

After exposure periods fish were sacrificed, weighed and tissues were removed, dietary methylmercury did not alter the growth of the zebrafish. There was no significant effect of mercury on body size (F=0.012, df= 2,11, p=0.99), gonad size (F=0.08, df=2,11, p=0.92) or the GSI (F=0.24, df=2,11, p=0.80) of female fish (Table 1).

Table 1. Effects of dietary methylmercury on growth and gonad size of female zebrafish after 60 days of exposure.

Methylmercury in diet	Wet weight (g)	Gonad weight (g)	GSI
Control	0.88 ± 0.14	0.11 ± 0.01	12.13 ± 1.48
Low	0.91 ± 0.13	0.12 ± 0.04	12.74 ± 3.32
Medium	0.82 ± 0.18	0.09 ± 0.03	10.47 ± 1.34

N=3. Error given in standard deviation

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Similarly, there were no significant differences between treatments in wet body weight (F=1.05, df=2,3, p=0.45), gonad weight (F=0.37, df=2,3, p=0.72) of male fish. Although the GSI of male zebra fish was 16% lower in fish receiving low and medium dietary methylmercury relative to control fish, these differences were not statistically significant (F=0.16, df=2,3, p=0.86) (Table 2).

Table 2. Effects of dietary methylmercury on growth and gonad size of male zebrafish after 60 days of exposure.

Methylmercury in diet	Wet weight (g)	Gonad weight (g)	GSI
Control	0.56 ± 0.08	0.001 ± 0.004	1.83 ± 0.80
Low	0.46 ± 0.19	0.007 ± 0.004	1.54 ± 0.98
Medium	0.51 ± 0.10	0.008 ± 0.002	1.53 ± 0.31

N=3. Error given in standard deviation

QRT-PCR Data

To determine if dietary methylmercury exposure affected the expression of specific reproductive genes, tissues were isolated from control and experimental groups and QRT-PCR was performed. Dietary methylmercury resulted in altered transcription of zp3 in female zebrafish (F=6.33, df=2,11, p=0.015, Table 3). In comparison to fish fed control diets, transcription of zp3 was 4.2-fold lower in fish fed diets with 3.40 μg Hg g^{-1} dry weight (Tukey's HSD q=3.34, q$_{crit}$=3.11). Dietary methylmercury also significantly altered transcription of Zp2 by the female fish (F=3.99, df=11, p=0.049). In female fish receiving the medium methylmercury diet, transcription was 1.7-fold lower than in female fish receiving the control diet, but this difference was not statistically significant (Tukey's HSD q= 1.94 q$_{crit}$=3.11, (Figure 1). Expression of hepatic vtg by the female

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zebrafish was highly variable, regardless of group or tank, frequently varying by more than 1000s-of-fold among fish from the same tank. There was no correlation between dietary methylmercury and vtg in female fish (Table 3).

Table 3. Amount of normalized transcription of zp2, zp3, and vtg in male zebrafish after 30 days of dietary methylmercury exposure.

Methylmercury in diet	Zp2	Zp3	Vtg
Control	0.39 ± 0.50	0.37 ± 0.29	0.03 ± 0.01
Low	0.16 ± 0.12	0.06 ± 0.08	0.42 ± 0.59
Medium	0.31 ± 0.34	0.003 ± 0.004	0.11 ± 0.15

N=2. Error given in standard deviation. Values are unitless and normalized to actin.
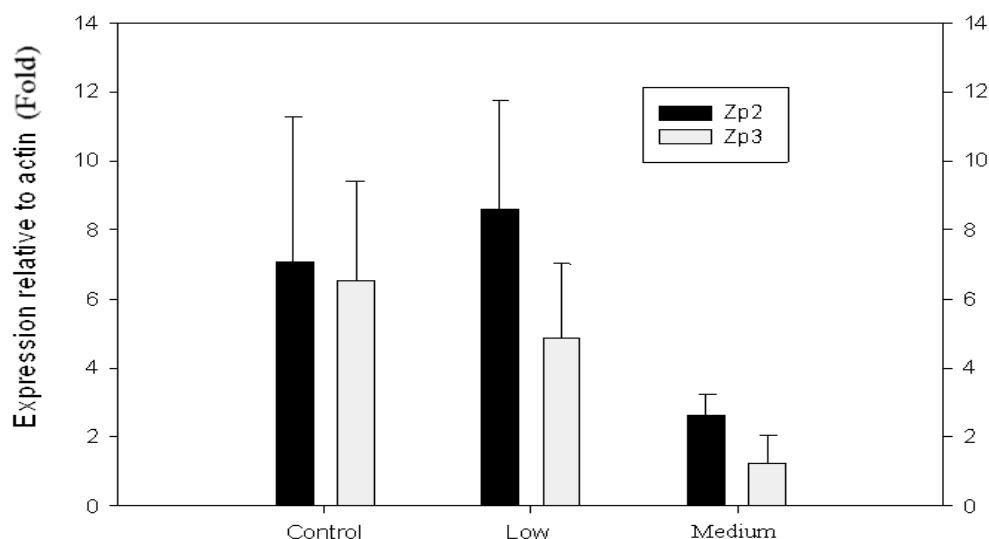

Figure 1. Female zp2 and zp3 expression after 60 days of exposure to dietary methylmercury. Error represented by standard deviation.

Expression of zp2 and zp3 was highly variable among male fish in all dietary treatments. There were no significant differences in transcription of zp3 by male fish among different dietary treatments after 30 days (F=0.52 df=2,3 p=0.64, Table 3) or 60

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days of exposure (F=2.88, df=3 p=2.00, Table 4). Although transcription of zp3 did

decrease in male fish receiving medium dietary methylmercury after 30 and 60 days of

exposure, the level of expression of zp3 was very low, even in the control fish, and the

difference in expression among groups was too small to be significant. Transcription of

zp2 by male fish was also very low and there was no significant difference in

transcription among dietary groups in the expression after 30 days (F= 0.56, df=2,3

p=0.62, Table 3) and 60 days of exposure (F=0.28, df=2,3, p=0.77, Table 4).

Table 4. Amount of normalized transcription of zp2, zp3, and vtg in male zebrafish after
60 days of dietary methylmercury exposure.

Methylmercury in diet	Zp2	Zp3	Vtg
Control	0.028 ± 0.02	0.031 ± 0.02	0.52 ± 0.14
Low	0.083 ± 0.10	0.002 ± 0.0002	0.62 ± 0.46
Medium	0.084 ± 0.11	0.004 ± 0.004	0.24 ± 0.028

N=2. Error given in standard deviation. Values are unitless and normalized to actin.

Transcription of vtg in liver of male zebrafish did not differ among diet groups

after 60 days (F=0.30, df=2,5, p=0.75). Relative to male fish receiving control diets,

transcription was 0.6-fold lower in fish receiving low methylmercury diets and 0.3-fold

lower in fish receiving medium methylmercury diets after 60 days, but variation in

expression was extremely high in all groups and the results cannot be considered

statistically or biologically significant. There was also no correlation between gene

transcription and GSI or body weight in any of the genes tested (For female body weight;

zp2 r=-0.16 p=0.32, *zp3* r=0.09 p=0.55, *vtg* r=0.008 p=0.97. For female GSI; *zp2* r=0.09

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p=0.54, *zp3* r=-0.02 p=0.91, *vtg* r=-0.18 p=0.33. For male body weight; *zp2* r=-0.18

p=0.52, *zp3* r=0.24 p=0.389, *vtg* r=-0.08 P=0.78. For male GSI *zp2* r=-0.18 p=0.52, *zp3*

r=-0.03 p=0.92, *vtg* r=-0.26 p=0.35).

DISCUSSION

There was substantial mortality in the first 30 days of exposure in all treatment groups, but there were no significant differences among groups. The increased mortality may have been caused by stress on the fish from shipment. If the experiment is repeated, it would be advisable to raise zebrafish from embryos and start exposure during juvenile stages, or plan to allow a longer acclimation period. It would also be advisable to use a longer period of exposure to methylmercury, better reflecting the conditions fish would encounter in the environment.

There were no significant differences in wet weight, gonad weight or GSI among exposure groups after 60 days. This is contrary to results found in similar studies of other fish species, but this study included only adult fish for 60 days of exposure. In studies of fish that had a reduced GSI, fish were fed methylmercury contaminated diets from juvenile stages into adulthood (Friedmann et al., 1996, Drevnick & Sandheinrich, 2003). This suggests that methylmercury may impair gonadal development in juvenile fish, but may have relatively little effect on GSI once fish have reached sexual maturity. Reduced GSI may be a developmental effect that occurs only with exposure during juvenile development of secondary sexual characteristics. This is consistent with the observation that methylmercury has teratogenic effects on zebrafish embryos, causing developmental defects such as fin fold abnormalities and tail flexures (Samson & Shenker, 2000).

The only significant change in transcription was a 4.2-fold drop in zp3 in female zebrafish after 60 days of exposure. There was also a 1.7-fold drop in zp2 transcript in

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females after 60 days, but these results were not significant. These results are the opposite of those seen in fathead minnows exposed to dietary methylmercury for 200 days, where there were 9.5-fold and 11.1-fold increases in transcription of zp2 and zp3 mRNA respectively (Klaper et al., 2006). The results from this study indicate that the changes in zp2 and zp3 are either species-specific or result from changes caused by methylmercury exposure during juvenile stages of the life cycle.

A decrease in zp3 expression may have an effect on fertilization rates of oocytes, as zp3 is the primary sperm binding protein. After sperm binds, zp3 helps to induce the cortical response. If the cortical response is not induced, zp2 will not be able to bind as a secondary sperm binding protein and fertilization may not occur (Bleil et al., 1988). Methylmercury is an endocrine disrupting chemical that can induce estrogenic responses (Drevnick & Sandheinrich, 2003; Klaper et al., 2006). Zona pellucida proteins are estrogen-independent, therefore any changes in zp2 or zp3 transcription could be induced by another pathway triggered by methylmercury exposure.

In male zebrafish, zp3 was also down-regulated by dietary exposure to methylmercury. Although the change was not significant, the average down-regulation of zp3 transcription was 14.5-fold and 7.8-fold in low and medium groups, respectively, after 60 days. In control groups, zp2 and zp3 expression were only 5-10% that of actin. This low level of expression was decreased further in exposure groups, and may be approaching the threshold of detection for our method and instrument. These results are opposite of those seen by Klaper et al. (2006) who found that zp3 was 14.7-fold higher in fathead minnow males that were fed 3.9 μg Hg g^{-1} dry weight. The primary function, if any, of zp3 in male fish is unknown and the significance of this discrepancy in results is

unclear.

Zp2 transcription in male zebrafish was 2-fold higher in both exposure groups after 60 days, but standard deviations were too high for the results to be significant. Klaper et al. (2006) found that zp2 increased 2.8 fold in male zebrafish exposed to 3.9 µg Hg g^{-1}. Both the current data and that from Klaper et al. (2006) report relatively small changes in male zp2 transcription with a high amount of deviation in exposure groups. The change in zp2 may be too small and fluctuate too much to have an effect on the fish or be biologically significant.

In this study there was no change in hepatic transcription of vtg in male or female fish in the low or medium exposure groups. This lack of change is consistent with the findings of Gonzelaz et al. (2005), who did not measure transcription of vtg, but did measure the transcription of 13 other genes in the liver. Zebrafish were fed food contaminated with 5.0 and 13.5 µg Hg g^{-1} and there were only minimal change in transcription after 7, 21, and 63 days. The largest changes were after 63 days of exposure to 13.5 µg Hg g^{-1}. However, 13.5 µg Hg g^{-1} is an exceptionally high methylmercury concentration and representative of areas contaminated by point source mercury pollution (Durrieu *et al.*, 2005). Vtg transcription was higher in male fish exposed to methylmercury, but the standard deviations were almost equal to the amount of transcription. Similarly, although Klaper et al. (2006) reported that transcription of vtg in fathead minnows increased an average of 142-fold, the deviations were almost as high as the relative values obtained. Zebrafish vtg transcription in males displayed too small of a difference between the control and exposure groups to be considered a reliable biomarker for adult methylmercury exposure. A possible explanation for the high variations in vtg

mRNA among fish, even in the same exposure group, is that zebrafish spawn all year long. The reproductive cycle was not controlled in this experiment, and the fish could have been in different staged of their reproductive cycles. One fish may have been undergoing vitellogenesis, while another may be ready to lay a clutch of eggs and was not expressing vtg. This may have been why such high variations of vtg were present in female fish at both 30 and 60 days exposure.

This study shows that environmentally realistic doses of methylmercury do variably affect zp2, zp3 and vtg transcription in zebrafish. There was a reduction of the amount of zp2 and zp3 transcript in both female and male fish, the largest and only significant change being a 4.2-fold down regulation in female zp3 exposed to 3.4 μg Hg g^{-1} dry weight for 60 days. It is yet to be determined if these changes are biologically relevant and affect fish reproduction.

The different findings in the current and previous studies may be due to experimental limitations mentioned previously, especially that zebrafish were exposed to methylmercury only in the adult stages of their lives. Endocrine disruption that occurs in juvenile stages of the fish's life may have effects on development of secondary sex characteristics that last throughout the fish's entire life. Further studies could include methylmercury exposure during the juvenile and early adult stages of the fish's life cycle. If reproductive effects of methylmercury exposure are, in fact, linked to developmental effects, zebrafish could be an appropriate species for further research as their embryonic development is well described. Zebrafish would allow for easier access to powerful techniques such as and immunohistochemistry, western blotting, transgenic and knockout animals, and morfilino (RNA interference) technology in the field of aquatic

environmental research. However, more research is needed to determine if zebrafish will

be a good model species for methylmercury toxicology.

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