FEMALE REPRODUCTIVE IMPACTS OF DIETARY METHYLMERCURY IN YELLOW PERCH (*Perca flavescens*) AND ZEBRAFISH (*Danio rerio*)

by

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ABSTRACT
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The University of Wisconsin-Milwaukee, 2015
Under the Supervision of Professor Michael J. Carvan, III

This study sought to evaluate the effects of dietary MeHg exposure on female teleost reproduction and phenotypically-anchor gene dysregulation in adult yellow perch (*Perca flavescens*) and zebrafish (*Danio rerio*) in order to establish relevant biomarkers relating exposure and subsequent reproductive effects. Yellow perch were used in the study for their socioeconomic importance within the Great Lakes basin, and their significance to the food web. Utilization of zebrafish allowed for a detailed analysis of the molecular effects of MeHg and established its relevance as a model for other fish species. MeHg exposures at environmentally relevant levels were done in zebrafish for a full life cycle and in adult yellow perch for twenty weeks, capturing early seasonal ovarian development. RNA-seq elucidated the effects of exposure on gene expression and determined putative molecular mechanisms for negative reproductive impacts in zebrafish. In zebrafish, several genes involved in reproductive processes were shown to be dysregulated by RNA-seq and QPCR, but no significant phenotypic or physiological changes were observed with ovarian staging, fecundity, or embryo mortality. Yellow perch did not appear to be

affected by MeHg, either at a molecular level, as assessed by QPCR of eight genes in the pituitary, liver, and ovary tissue, or a physiological level, as seen with ovarian somatic index and circulating estradiol. Lack of impact in yellow perch hinders the characterization of a biomarker, limits the usefulness of zebrafish as a model, and suggests that the reproductive sensitivity to environmentally relevant levels of MeHg differs between yellow perch and zebrafish.

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1. Introduction

Methylmercury (MeHg) is a pervasive contaminant in aquatic ecosystems, particularly within the Great Lakes region. Historically, industrial processes introduced a man-made source of mercury to the Great Lakes ecosystem around 1850, and continuing industrial practices have contributed to persistent, although slowly decreasing, levels of mercury in the region (Wiener et al., 2012a). While natural biogeochemical processes such as volcanic activity release inorganic mercury into the atmosphere, anthropogenic emissions greatly increase the amount of mercury present in the environment, with coal-fired power plants being the largest emitters of mercury in the Great Lakes region today (Wiener et al., 2012a).

Inorganic mercury enters the water after atmospheric deposition, at which point anaerobic microbial processes transform the mercury to MeHg. MeHg (the dominant form of mercury in fish tissue in the Great Lakes region) can then bioaccumulate and biomagnify up through higher order trophic levels to potentially harmful concentrations (Benoit et al., 2003, Wiener et al., 2012a). Several environmental conditions contribute to a greater degree of biotransformation by these microbes, including increased temperature, low pH, and low alkalinity (Schultz and Newman, 1997). These factors will have increasing relevance as climate change and other anthropogenic influences modify fundamental biogeochemical features of aquatic ecosystems. High levels of mercury pollution and subsequent biotransformation have resulted in fish consumption advisories (defined as MeHg concentrations at or above 0.3 ppm

wet weight [w.w.] in fish) in almost every body of water in the Great Lakes region (Wiener et al., 2012a).

Mercury is a harmful neurotoxicant, and exposure to the developing nervous system can lead to a continuum of consequences ranging from very subtle behavioral changes to overt neurological damage and death; however, knowledge is currently limited on population-level effects of chronic, sub-lethal exposure of fish to MeHg (Gilbert and Grant-Webster, 1995, Klaper et al., 2006). MeHg exposure could impact recruitment, or the number of new fish that enter the population each year, in a number of ways, from different spawning behaviors to decreased larval survival to altered physiology (Alvarez et al., 2006, Drevnick et al., 2006, Weber et al., 2008). The reproductive repercussions of chronic MeHg exposure in fish populations could potentially impact spawning, consequently reducing population levels (Hammerschmidt et al., 2002). A comparative analysis of several MeHg exposure studies determined that some fish species begin experiencing adverse reproductive effects from MeHg at a tissue concentration of 0.2 ppm w.w. (Depew et al., 2012).

Beyond its classification as a neurotoxicant, MeHg has also been identified as a possible endocrine disrupting chemical, and as such, could modulate expression levels of reproductively-associated genes in both male and female fish, minimizing reproductive success (Klaper et al., 2006, Crump and Trudeau, 2009). Endocrine disrupting chemicals alter normal bodily functions by mimicking or interfering with hormone functions, ultimately leading to reproductive disorders such as feminization of populations and reduced

spawning capacity (Hachfi et al., 2012). In female fish, endocrine disruptors can modify expression of necessary genes for reproduction, resulting in severe consequences on hormone regulation, egg yolk production, and other processes that are critical to successful reproduction (Jobling et al., 1998, Skolness et al., 2011, He et al., 2012).

Knowledge is currently limited on the direct effects of chronic MeHg exposure directly on female fish reproduction. In oviparous organisms, the brain, pituitary, liver, and gonad are interconnected to influence overall reproductive ability; understanding how a contaminant impacts reproductive functions along this axis is necessary for a complete assessment of overall reproductive effects (Fig. 1; Villaneuve et al. 2007). Hormonal cues from the brain and pituitary signal the transfer of 17β-estradiol (E2) from the ovary to the liver, signaling the liver to produce vitellogenin—a phospholipoglycoprotein yolk precursor that is produced in the liver and transported to the ovary for yolk deposition (Ng and Idler, 1983, Sumpter and Jobling, 1995). More specifically, gonadotropin-releasing hormone (GnRH) is produced in GnRH neurons in the hypothalamus and binds to GnRH receptors on gonadotropes in the pituitary. This stimulates release of gonadotropins, which are comprised of a glycoprotein-hormone alpha subunit (GTHα) and a specific beta subunit (FSHβ and LHβ) (Yaron et al., 2003). Follicle stimulating hormone (FSHβ) is mainly involved in regulating early follicle maturation and signaling follicle cells to produce E2, while luteinizing hormone (LHβ) is primarily involved in later gamete maturation and resulting ovulation;

these genes are up-regulated during spawning in fish (Yaron et al., 2003, Campbell et al., 2006, Levavi-Sivan et al., 2010, Goetz et al., 2011).

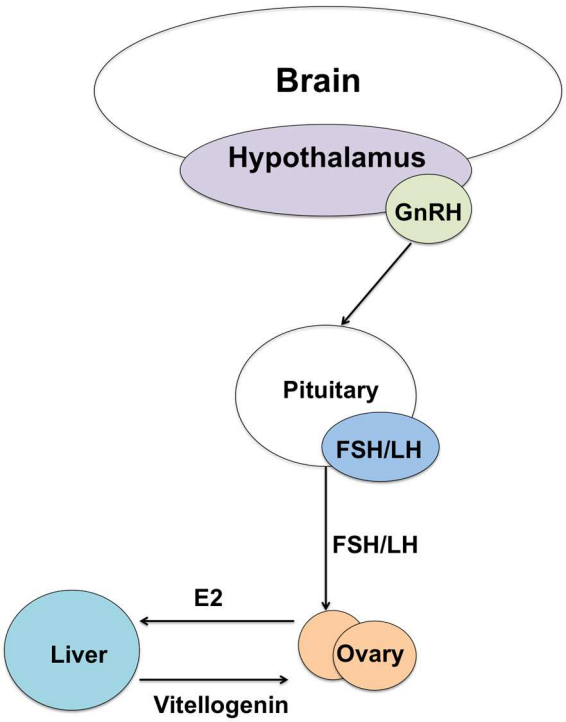


Figure 1. Schematic of the HPLG axis. GnRH causes the release of FSH/LH, which in turn triggers estradiol release from the ovarian follicles. Estradiol signals the synthesis of vitellogenin proteins in the liver, which are then incorporated as yolk in the oocytes. Adapted from Yaron et al. (2003).

While the capacity to which MeHg modulates the expression of certain genes along this axis and particularly in the ovary is relatively unknown, exposure has been linked with specific physiological changes in female fish. In female teleost fish, reproductive success is dependent upon proper development of gonads, thus individuals with more developed gonads have a greater capacity to spawn (Hammerschmidt et al., 2002). At environmentally relevant doses low enough to not inhibit survival or growth, dietary MeHg exposure reduces gonadal development in females, thereby reducing the ovarian somatic index (OSI), or the percentage of total body weight contributed by the gonads (Hammerschmidt et al., 2002, Drevnick and Sandheinrich, 2003). A larger value for OSI is positively

correlated with the number of eggs laid per gram of female carcass, implying that MeHg inhibits egg development (Hammerschmidt et al., 2002).

MeHg exposure has also been implicated in decreasing vitellogenin gene expression in the liver concurrent with reduced circulating E2 levels (Baldigo et al., 2006, Klaper et al., 2006, Crump and Trudeau, 2009). Furthermore, MeHg exposure up-regulates *apolipoprotein E* (*apoe*) expression in female livers; *apoe* is involved with processes such as cholesterol and lipid transport (Klaper et al., 2006). To date, the majority of studies evaluating the impact of MeHg on female reproduction have been performed using fathead minnows (*Pimephales promelas*) (Depew et al., 2012); sensitivity to MeHg may be species- or subspecies-dependent, and fathead minnows may not be representative of how all fishes react to MeHg (Meyer and Di Giulio, 2003, Heinz et al., 2009). Additionally, exposure schemes have not been consistent with duration of exposures nor with dosing concentrations (Depew et al., 2012), limiting the efficacy of cross-study comparisons. One compound may yield very different results if timing and acuteness of the exposure are adjusted (Crump and Trudeau, 2009). Due to the nature of MeHg contamination in the Great Lakes region, fish are most likely to experience low-level chronic exposure throughout their lifespan (Wiener et al., 2012a), not a short-term acute exposure.

Pervasive and historical contamination of MeHg in the Great Lakes has paralleled declines in native fish populations. While several factors, including the introduction of the alewife (*Alosa pseudoharengus*), the zebra mussel (*Dreissena polymorpha*), and various contaminants have been implicated in these declines;

none appear to be acting in isolation (Marsden and Robillard, 2004, Drevnick et al., 2006). At the moment, little is understood about the effects of historic and chronic mercury pollution on population dynamics, particularly in the manner in which female reproduction may be impacted in native, piscivorous fish species.

Yellow perch are an abundant economically and culturally important fish species native to the Great Lakes region, and as such, are an important intermediate of the trophic transfer of MeHg to other wildlife as well as humans (Wiener et al., 2012b). Furthermore, total mercury concentrations in yellow perch found in the Great Lakes are analogous to other piscivorous fish species (Wiener et al., 2012b).

In order to address the effects of MeHg on yellow perch, zebrafish were used for comparative analysis, with the intention of determining the efficacy of using zebrafish as a laboratory model for yellow perch. Zebrafish are a powerful laboratory organism for assessing the impact of MeHg on female reproduction. These fish have the advantage of a short generation time, becoming sexually mature at three to six months of age (Harper and Lawrence, 2012). Zebrafish also have numerous resources for gene expression analysis, including a sequenced genome. This makes it possible to perform high-throughput transcriptomic analyses such as RNA-sequencing (RNA-seq) to determine gene expression profiles and discover candidate biomarkers of exposure. Because of their short generation times, exposing zebrafish for a whole-life cycle, from parental exposure through adulthood, is possible, mimicking a realistic environmental exposure scenario. Female yellow perch do not reach sexual

maturity until two to four years of age (Schneider, 1984), and the genome is not yet entirely sequenced (Pierron et al., 2011). Both of these factors are considerable limitations to the duration of an exposure experiment and the strength of molecular analyses in elucidating subtle changes in yellow perch.

Potential drawbacks for using zebrafish as a model for yellow perch include the following: zebrafish are year-round spawners, while yellow perch and most other Great Lakes species spawn once per season (Krieger et al., 1984), and zebrafish naturally inhabit a different ecosystem (Spence et al., 2008). Female fish deposit MeHg into their eggs (Hammerschmidt and Sandheinrich, 2005), therefore zebrafish may have an additional route of regular MeHg elimination that is not present in yellow perch. While spawning phenology and ecosystem preferences differ between the two species, the sensitivity to environmental toxicants may be conserved in physiological pathways controlling reproduction (Heiden et al., 2008), suggesting that MeHg exposure should have similar effects across teleosts. Of note, the two species are separated by 250 million years of evolution (Hedges et al., 2006); this may result in reproductive differences between the two species or highlight fundamental reproductive similarities shared among teleost fish.

The objective of this study was to evaluate the effects of dietary MeHg exposure on female reproductive physiology, providing evidence to phenotypically-anchor gene dysregulation in adult zebrafish ovaries and yellow perch livers, pituitaries, and ovaries. Taking advantage of their short generation time, zebrafish were subjected to whole-life cycle MeHg exposure, from parental

deposition into eggs, through dietary exposure for the entirety of this experiment, simulating realistic exposure conditions in the Great Lakes. Yellow perch were exposed to dietary MeHg for up to 20 weeks, encompassing a part of seasonal ovarian development. If MeHg were to have an effect on yellow perch ovarian development, it should become apparent within that time frame. Ovarian staging, fecundity, embryo mortality, RNA-seq and quantitative real-time polymerase chain reaction (QPCR) in zebrafish, and Ovarian Somatic Index (OSI), hormone analysis, and QPCR in yellow perch provided evidence to address the overt reproductive impairment caused by dietary MeHg exposure. RNA-seq was used to elucidate the effects of exposure on gene expression and determine putative molecular mechanisms for negative reproductive impacts in zebrafish. Alteration anywhere along the Hypothalamus-Pituitary-Liver-Gonadal (HPLG) axis should impact ovarian gene expression and morphology and change the number of eggs that are produced, hatch, and survive to adulthood (Devlin and Nagahama, 2002, Hammerschmidt et al., 2002); therefore, adult ovaries were the primary focus of this study. Taken together, this data was used to evaluate the effects of MeHg on female fish reproduction and attempt to establish relevant biomarkers relating exposure and subsequent reproductive effects.

2. Materials and Methods

As zebrafish and yellow perch were two separate experiments, the methods will be outlined in two separate sections. Whole-life cycle exposure scenarios are not possible in yellow perch due to their longevity and reproductive limitations, and mimicking the exposure window from the yellow perch in zebrafish would not hold the same degree of ecological relevance. Additionally, many molecular tools to assess transcriptomic changes are available in zebrafish, but are lacking in the yellow perch. Therefore, these experiments were carried out in the zebrafish with the hope of making inferences to the yellow perch. Both species bolster the findings of this thesis and enhance our understanding of the effects of MeHg on female fish reproduction. A graphical representation of the parallel nature of this study is shown below (Fig. 2). Unless otherwise noted, all statistical analyses were performed in SigmaPlot 11.0 (SYSTAT Software, Inc., San Jose, CA).

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Milwaukee.

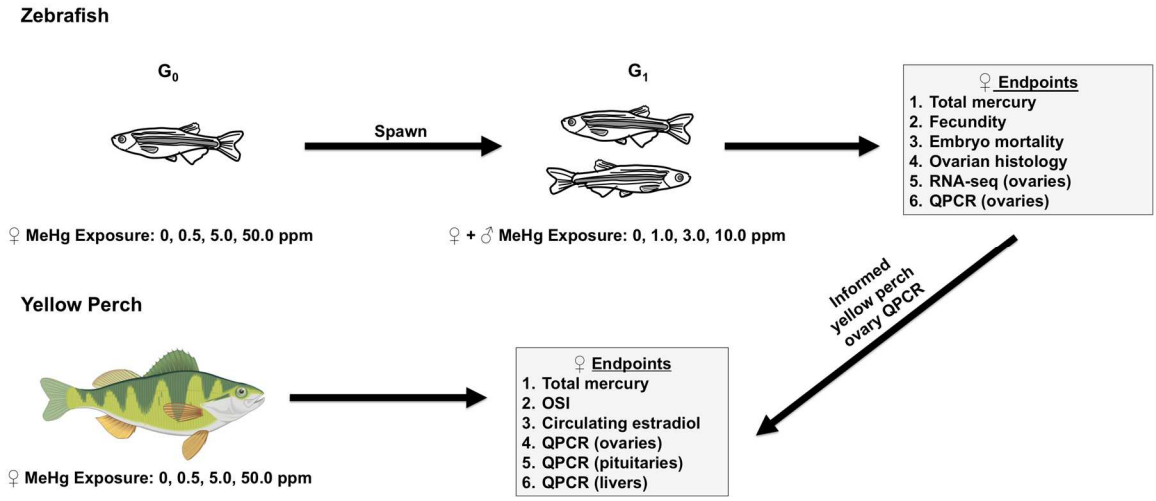


Figure 2. Graphical representation of research scheme. The zebrafish experimental design represents whole-life exposure conditions to MeHg, where the body burden of MeHg in maternal G_0 impacts the levels in G_1 embryos. Exposure continued throughout experiment. Yellow perch exposure was completed during a limited time frame, incorporating a portion of ovarian development. Endpoints for both zebrafish and yellow perch are listed. Zebrafish RNA-seq and QPCR results informed ovarian QPCR in yellow perch. Image sources: personal communication, Francisco Mora, Jan. 9, 2015; http://www.search-best-cartoon.com/cartoon-fish/cartoon-fish-perch.jpg (accessed: Jan. 18, 2015).

2.1 Zebrafish

MeHg Exposure of G_0 Fish

Zebrafish (*Danio rerio*) used in this study were from the EK strain (originally obtained from EkkWill Waterlife Resources [Ruskin, Florida, USA] and maintained in laboratory for over 15 generations) and were raised in the NIEHS Children's Environmental Health Core Center (Milwaukee WI, USA). For this exposure, adult females were reared using standard husbandry conditions, which include flow-through de-chlorinated municipal water at 26-28°C with a 14 h light : 10 h dark cycle.

At approximately five months of age, females were separated from males and maintained in 12 3-L tanks, at a density of 12 fish per tank. Each tank was

randomly assigned to one of four dietary MeHg exposure groups (0.0, 0.5, 5.0, and 50.0 ppm). The experimental diet consisted of Biodiet starter (Bio-Oregon, Longview, WA, 4% body weight per day) containing MeHg at 0.0, 0.5, 5.0, or 50.0 ppm (ethanol was used as the vehicle in all groups, including 0.0 ppm). Actual total mercury concentrations in the food (Supplementary Table 1) were determined by atomic absorption spectrophotometry as described below. To prevent mercury waste from escaping the tanks in wastewater, fish were fed under static conditions and each tank was siphoned each afternoon. Filters were placed in the outflows of the tanks to capture any mercury-containing particulate waste that may have remained. It was previously determined that using this setup the water was free of mercury, and that 100% of the applied mercury was in the fish, uneaten food, and fish waste. This diet, along with supplementary *Artemia* (platinum grade Argentimia, Argent Laboratories, Redmont, WA), was maintained for two months, at which point these fish were spawned with unexposed male EK fish.

After each spawning event, 200 embryos per spawning group (n = 3 clutches per dose) were analyzed for total mercury as described below. This occurred approximately every two to three weeks to ensure that embryos had accumulated sufficient levels of mercury from maternal burden so as to be separated into environmentally relevant low, medium, and high exposure groups. Approximately 180 juvenile fish per replicate per dose were raised to adulthood (G_1 population).

MeHg Exposure of G_1 Fish

G_1 zebrafish larvae were given micro-encapsulated food in a range of sizes to account for growth (Golden Pearl, Brine Shrimp Direct, Ogden, UT) until large enough to eat adult flake food (Aquatox, Pentair Aquatic Ecosystems, Apopka, FL). Both foods were supplemented with MeHg at 0.0, 1.0, 3.0, or 10.0 ppm, and were fed to individuals derived from G_0 females, fed 0.0, 0.5, 5.0, and 50.0 ppm MeHg, respectively. This diet, along with supplemental *Artemia* (platinum grade Argentimia, Argent Laboratories, Redmont, WA) to ensure proper growth, was maintained for approximately seven months. Fish were fed until satiation. Mercury analysis was performed to confirm foods contained the proper concentration of total mercury (Supplementary Table 1). Upon development of visible sexual characteristics, the sexes were separated with twelve female fish per 3-L tank and six male fish per 1.5-L tank. Control of hazardous mercury waste was maintained as described above with the G_0 fish. Overall, each exposure group had three individual tanks of each sex.

Fecundity and Embryo Mortality

Each tank of twelve female G_1 fish was spawned with six males from the same exposure group; spawning of the three tanks within each exposure group was separated by one week. Fish were spawned weekly prior to final spawning to maintain a constant cycle. Total number of eggs from each tank was counted, and mortality of embryos was assessed 24 hours post-fertilization (hpf). The number of embryos per female was assessed by dividing the number of eggs per

tank by the number of females in that tank. This provided a fecundity estimate for each treatment group, showing the effect of MeHg on the number of eggs laid per female. Survival of these embryos also provided an overt endpoint to illustrate the impact of maternal MeHg exposure, where MeHg might affect egg quality. Mortality was calculated as a percent of dead embryos relative to the number of eggs laid. Both eggs per female and mortality were statistically analyzed using a one-way analysis of variance (ANOVA).

Tissue Collection

Approximately 24 hours post-spawning, nine of the available twelve G_1 females per replicate tank per dose were euthanized by an overdose of neutral-buffered MS-222 (Western Chemical, Inc., Ferndale, WA) and ovaries were collected. Of the nine ovaries, three were used for total mercury analysis and six were used for RNA isolation. The three remaining zebrafish were euthanized six days following the previous spawning event for histological analysis.

In preparation for RNA isolation, G_1 ovaries were dissected from fish immediately after euthanasia, placed individually in 1.7-ml microcentrifuge tubes (MidSci, St. Louis, MO) containing 200µL RNA*later* (Qiagen, Hilden, Germany), flash frozen in liquid nitrogen, and placed on dry ice until proper long-term storage at -80°C.

Mercury Analysis

Total mercury content in zebrafish ovary tissues was determined by atomic absorption spectrophotometry using a DMA-80 (Milestone, Inc., Shelton, CT) based on the methods of Nam and Basu (2011). All concentrations are reported as ppm (mg/kg) wet-weight. All zebrafish foods were analyzed for total mercury concentration. Total mercury levels were measured in 200 embryos per spawning group approximately every two weeks to confirm accumulation of mercury. Tissue residue levels in these embryos were statistically analyzed using a one-way ANOVA followed by a Holm-Sidak post-hoc test for comparisons of log-transformed total mercury values relative to parental exposure. Total mercury was also measured in nine zebrafish ovaries for each exposure group from G_1 zebrafish. To compare differences in mercury in ovary tissue among exposure groups, log-transformed mercury values were analyzed using a Kruskal-Wallis one-way ANOVA on ranks followed by a Dunn's post-hoc test.

Histology Analysis

Fish were euthanized as described above, and an incision was made along the ventral surface. Whole fish were preserved individually in cassettes in 10% neutral buffered formalin. Histology slide preparations and scanning were completed at the Medical College of Wisconsin in the Histology Core. These fish were embedded in paraffin and sectioned along the sagittal plane down the midline and at two lateral sections to the right and left of the midline. Samples were stained with hematoxylin and eosin, and the slides were scanned for

analysis in NanoZoomer Digital Pathology (Deroulers et al., 2013). To determine MeHg effects on the ovary, two sections from five different females were analyzed from both 0.0 ppm and 10.0 ppm MeHg (highest) exposure groups with the group identity blinded to the observer. Area and perimeter of the ovary were measured for each slide, and follicles were classified as pre-vitellogenic, mid-vitellogenic, and late-vitellogenic based on Nagahama (1983), Selman et al. (1993), Miranda et al. (1999), and Johnson et al. (2009). Pre-vitellogenic oocytes comprised of perinucleolar and cortical alveolar oocytes, mid-vitellogenic oocytes were defined as early vitellogenic oocytes, and late-vitellogenic oocytes also included mature/spawning oocytes. To normalize variation in ovary size, individual follicle types were calculated as a proportion of total follicles in an ovary. Statistical analyses were performed with an unpaired t-test, comparing control and 10.0 ppm fish for each follicle classification.

RNA Isolation

RNA was isolated from individual ovaries using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irving, CA), according to manufacturer's instructions, and including the DNase treatment step. Tissues were homogenized on ice in approximately 500 μL of Direct-zol™ (Zymo Research) in 1.7-ml microcentrifuge tubes, using a sterile micropestle (MidSci) and running the homogenate through a 21-gauge needle (BD Biosciences, Franklin Lakes, NJ). RNA quantity and quality were assessed using a NanoDrop ND1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE), and an Experion

Automated Electrophoresis System (Bio-Rad, Hercules, CA). Only samples with an RNA Quality Indicator (RQI) value of 8.0 or greater were used for downstream analysis (Supplementary Table 4). Equal amounts of RNA were pooled from six zebrafish ovaries from each tank of 0.0, 1.0, and 3.0 ppm exposure groups. Equal amounts of RNA were pooled from the available ovaries in the 10.0 ppm exposure group—this resulted in pooled RNA from five zebrafish for two tanks and four zebrafish for the third tank. RNA was pooled to reduce individual biological variation. From each tank, 2.0 µg of pooled RNA from up to six ovaries were sent to the University of Wisconsin-Madison Biotechnology Center for analysis of the transcriptome by RNA-sequencing (RNA-seq), using an Illumina HiSeq 2000 (Illumina, Inc., San Diego, CA).

At the University of Wisconsin-Madison Biotechnology Center, each RNA library was generated using a paired-end approach following the Illumina "TruSeq RNA Sample Preparation Guide" and the Illumina TruSeq RNA Sample Preparation Kit. Samples were run with 12 samples per lane, with 100 base pair, paired-end reads. Sequencing depth was 14-32 million reads per sample.

RNA-Seq Bioinformatics

All bioinformatics procedures and analyses were performed by the University of Wisconsin-Milwaukee Laboratory for Public Health Informatics and Genomics (LPHIG). Adapters and low quality bases were removed from the initial 2x101bp Illumina TruSeq and trimmed using Cutadapt (Martin, 2011). Illumina TruSeq Adapters were removed as prescribed by the Cutadapt manual, using an

error rate of 10% and a minimum overlap between the read and the adapter of five nucleotide bases. To alleviate sequencing-related GC biases at the 5' end of each read, the first seven bases were removed from all forward and reverse strand reads. FastQC was used to ensure that cleaned reads were of higher quality than initial raw reads supplied by the sequencer; per-base GC% and over-represented sequence statistics also confirmed adapter contamination was minimized.

The cleaned reads for each sample were independently aligned to the reference zebrafish genome (Zv9, UCSC) using TopHat (v. 2.0.11) (Trapnell et al., 2010, Kim et al., 2013). The alignment output from TopHat was converted into a transcriptome using Cufflinks (v. 2.2.1), with the Zv9 Gene Transfer Format (GTF) as a guide; a mate-pair-distance of 0 and a maximum of 2 mismatches bases per alignment was used. Alignment data was confirmed using RNAseQC (DeLuca et al., 2012) against the Zv9 reference transcriptome. Using these alignments, an ovary-specific transcriptome was assembled using Cufflinks (Trapnell et al., 2010), with the Zv9 transcriptome as a reference to correct fragment biases by better identifying the start/end point of each exon (Roberts et al., 2011). The transcriptome from each sample was then merged together into a single ovary transcriptome using Cuffmerge. Differential expression was conducted with Cuffdiff using pooled dispersion, geometric normalization, and the merged ovary transcriptome; TopHat alignments were grouped using MeHg exposure levels.

Gene ontology was determined using WEB-based GEne SeT AnaLysis Toolkit (WebGESTALT; Zhang et al. 2005). In order to visualize differentially expressed genes, a heat map was generated using GenePattern (Broad Institute; Reich et al. 2006), with hierarchical clustering of genes based on Pearson Correlations.

Identification of QPCR Biomarkers

Quantitative real-time polymerase chain reaction (QPCR) was used to identify putative biomarkers that could be analyzed in yellow perch. Normalizer genes were selected based on previous literature (Tang et al., 2007) and stable expression in this study based on RNA-seq data. Target genes were selected based on RNA-seq results from the zebrafish experiment described here, relevance to fish reproduction, and availability of transcripts in yellow perch (a species without an entirely sequenced genome). Primers were designed using Primer-BLAST, with each primer pair containing a GC clamp, 50-60% GC content, and spanning an exon-exon junction. Primers were 18-24 base pairs (bp) in length and amplicons were 100-200 bp.

Prior to complementary DNA (cDNA) synthesis, RNA quantity was confirmed using a Qubit® RNA assay kit (Life Technologies, Carlsbad, CA), according to manufacturer's instructions. cDNA was synthesized using EasyScript™ Reverse Transcriptase and Master Mix (MidSci), which contains a mixture of oligo(dT) and random primers, according to manufacturer's instructions, from approximately 1,000 ng of total RNA. Reverse Transcription

reactions were performed in duplicate, producing approximately 2,000 ng of cDNA per sample, and was then diluted to a final concentration of 8.0 ng/μL. For each primer pair, a PCR reaction was performed at three different annealing temperatures, and products were run on a 1.5% agarose gel in order to confirm PCR product size and visualize any potential off-target results; this also allowed for confirmation of an appropriate annealing temperature across all primer pairs.

QPCR was performed using a StepOne Plus™ system (Applied Biosystems, Foster City, CA) and was run with 10μL reactions that each contained: 1 μL of 8.0 ng cDNA, 5.0 μL EvaGreen® qPCR Master Mix, and 2 μL of both the forward and reverse primers (see Supplementary Table 2 for a list of primer concentrations). Efficiency curves were performed for each gene primer pair. Reaction efficiencies ranged from 90-110% based on a four-fold dilution series of the cDNA reactions with five dilution points and all samples run in triplicate (Supplementary Table 2). Genomic DNA contamination was assessed in each RNA sample by running a no reverse transcriptase (RT) control. RNA samples containing genomic DNA were re-treated with DNase (Promega Corporation, Madison, WI), and cDNA was re-synthesized.

Relative quantification of gene expression was measured in 12 fish per exposure group, with each sample run in triplicate and each plate containing all three normalizer genes (*actin, beta 1* [*actb1*], *ribosomal protein L13a* [*rpl13a*], and *small nuclear ribonucleoprotein D1 polypeptide* [*snrpd1*]) and a no-template control for each gene. Each reaction was run with the following cycles: one cycle of 95°C for 9 minutes, 40 cycles of 95° C for 15s, 56°C for 30s, and 72°C for 45s,

followed by 1 cycle of 95°C for 30s, 55°C for 30s, and 95°C for 30s. Melting curves were generated for each amplification product to ensure targeted amplification of a single product and no primer-dimer formation.

Analysis was completed using the StepOnePlus™ software. A normalized quantity mean was calculated by accounting for primer efficiencies and three normalizer genes (Pfaffl 2001, Hellemans et al., 2007). The normalized relative quantity (NRQ) was calculated using the individual sample with the lowest expression level, based on the lowest normalized quantity mean, as the calibrator (NRQ=1; Liu et al., 2013). Outliers were removed from each exposure group for each target gene using a Grubbs' test (GraphPad QuickCalcs, La Jolla, CA). A *Cq'* was calculated for each sample, using the following equation to reduce heterogeneity of variance: $Cq' = log_2(NRQ)$ (Rieu and Powers, 2009). A one-way ANOVA, followed by a Holm-Sidak test, was used to determine any effects of MeHg exposure on the expression of each target gene. In data sets that failed the Shapiro–Wilk normality test or Equal Variance Test, a Kruskal–Wallis One Way Analysis of Variance on Ranks was used. Significant differences between treatments and control were evaluated using a Dunn's multiple comparison test. To calculate fold change relative to the control group, the average NRQ value of each exposure group was divided by the average NRQ of the control group. As this calculated a fold-up value, the inverse of this value was taken to present the fold-down value for exposure groups where gene expression was down-regulated.

2.2 Yellow Perch

MeHg Exposure of Yellow Perch

In preparation for MeHg exposure, sexually mature, female yellow perch (average mass at start of experiment = 463.6 g), raised with standard husbandry procedures, were transferred from the aquaculture facility at the University of Wisconsin-Milwaukee School of Freshwater Sciences to twelve 55-gallon polytanks with flow-through water, at a density of 12 female fish per tank. Fish were acclimated for 14 days at 20°C, with photoperiods coinciding with natural light cycles; fish were fed enough untreated perch diet (Finfish Perch 45-12 5.0 mm Slow Sinking food [Ziegler Bros., Inc. Gardners, PA]) three times per day to reach satiation. Upon initiation of MeHg exposures, adults were exposed to one of four different concentrations of dietary MeHg (0.0, 0.5, 5.0, or 50.0 ppm) (Sigma-Aldrich Co., St. Louis, MO) added to the perch diet (0.35% body weight per day), using ethanol as the vehicle in all groups, including the control group. Actual mercury in the food was determined by atomic absorption spectrophotometry (Supplementary Table 1).

Fish were fed under static conditions and tanks were siphoned daily to remove any residual MeHg in each tank that could escape with wastewater. To simulate conditions in which gonadal maturation and vitellogenesis would occur in the fall in Cambridge, MD (38°34'N, 76°05'W), where this line of fish originated, temperature was maintained at approximately 20°C for 12 weeks and then gradually decreased in the flow-through water until reaching approximately 11°C in the final three weeks (Dabrowski et al., 1996). To maintain consistency with

natural lighting events, an AstroDial Suntracker™ (Paragon® Electrical Products, Albuquerque, NM) was used to mimic the natural changing of the photoperiod. Overall, three tanks of 12 fish were exposed to each exposure condition.

Measurements and Tissue Collection

Four females per tank were euthanized at three separate time points after commencement of the experiment (12, 16, and 20 weeks) in order to capture different time points in ovarian development. Yellow perch were euthanized by an overdose of neutral-buffered MS-222. Ovary weight, whole fish weight, and fish length were measured. Ovarian Somatic Index (OSI) was calculated using the following equation: $OSI = \frac{WO}{WT} \times 100$, where WO is the ovary weight, and WT is the total fish weight. OSI was analyzed with a two-way ANOVA (factors = exposure and sampling date), followed by Holm-Sidak post hoc test.

Ovaries, livers, pituitaries, and blood were collected from each fish, flash frozen in liquid nitrogen, and stored at -80°C. Approximately 0.5 ml of blood was collected with a 21-gauge needle from the caudal vein of each fish. Ovaries, livers, and pituitaries were collected to assess the impact of MeHg on gene expression, and blood samples were collected to measure the amount of circulating estradiol.

Mercury Analysis

Total mercury content in yellow perch muscle tissues was determined as described with zebrafish. Tissue residue levels were statistically analyzed using a

one-way ANOVA followed by a Holm-Sidak post-hoc test to compare log-transformed total mercury values to exposure concentrations within each sampling date. A Pearson Moment Correlation was used to correlate the amount of total mercury in the ovary and the muscle, using unpublished data from previous work with identical exposure scenarios in yellow perch (personal communication, Jessica Head, March 9, 2011).

Estradiol Analysis[1]

As a key component to female reproductive signaling, circulating estradiol was measured using a radioimmunoassay, following the recommended procedures of the U.S. EPA (Jensen et al., 2001). Estradiol levels were analyzed with two-way ANOVA (factors = exposure and sampling date), followed by Holm-Sidak post-hoc test.

Ovary and Liver RNA Isolation

RNA was isolated from approximately 50 mg pieces from six individual ovaries per exposure group per sampling date using the Direct-zol™ RNA MiniPrep kit (Zymo Research), following the manufacturer's instructions, including the DNase treatment. Ovary tissues were homogenized on ice in 1.0 ml of Direct-zol™ in 5 ml polystyrene Falcon™ tubes (BD Biosciences) using a Dremel® MultiPro Model 395 Type 5 (Dremel, Racine, WI) at full speed. Carryover RNA on the homogenizer was hydrolyzed with 0.5 M NaOH, and

[1] Brandon Armstrong (Michigan State University) measured circulating estradiol

multiple sterile water rinses were employed between uses. RNA was isolated from each ovary in duplicate and samples were subsequently combined after elution. RNA quantity and quality were assessed on a NanoDrop ND1000 spectrophotometer and a 2100 Bioanalyzer Instrument (Agilent Technologies, Santa Clara, CA). Samples with RNA Integrity Numbers (RINs) below 7.0 were not used for downstream analyses (Supplementary Table 5).

RNA was isolated from approximately 50 mg of liver tissue using TRI-Reagent® (Zymo Research), according to manufacturer's instructions. Four to six livers per control and 50.0 ppm exposure groups were used per sampling date. While the RNA extraction method differed from yellow perch ovaries, liver tissues were homogenized in the same fashion. All samples were DNase treated prior to cDNA synthesis. RNA quantity and quality were assessed as described with yellow perch ovary tissues, although all samples were used for downstream analysis (Supplementary Table 6).

Ovary and Liver QPCR

Genes for QPCR were selected based on the zebrafish RNA-seq and QPCR results and the availability of sequence information for these specific genes in yellow perch. Primers were selected using previously published information where available. If no information was available, primers were designed using Primer-BLAST, querying unpublished 454 sequencing data from perch provided by the ARS/USDA-UWM Perch Program, and ensuring that each primer pair contained a GC clamp and 50-60% GC content. Primers were 18-24

bp in length and amplicons were 100-200 bp. For each primer pair, PCR products were run on a 1.5% agarose gel as described with zebrafish.

Prior to cDNA synthesis, RNA quantity was confirmed using a Qubit® RNA assay kit. cDNA was synthesized as described with the zebrafish, and reactions were diluted to a final concentration of 8.0 ng/µL for ovary QPCR and 1.0 ng/µL for liver QPCR.

Relative quantification of gene expression was measured in four to six fish per exposure group per sampling date, with each sample run in triplicate and each plate containing all three normalizer genes: *elongation factor 1a* (*ef1a*), *elongation factor 2* (*ef2*), and *ribosomal protein L13a* (*l31a*), and a no-template control for each gene. QPCR was performed using a StepOne Plus™ system and was run with 10 µL reactions that each contained: 1 µL of 8.0 ng cDNA for ovary or 1.0 ng cDNA for liver, 5.0 µL EvaGreen® qPCR Master Mix and 2 µL of both the forward and reverse primers (Supplementary Table 2). As with the zebrafish, efficiency curves determined the efficiency of each primer pair for each gene (Supplementary Table 2). Genomic DNA contamination was assessed and managed in each RNA sample as performed with zebrafish.

Relative quantification of gene expression was measured relative to the three normalizer genes. All three genes showed stable expression across individuals. Each reaction was run with the following cycles: one cycle of 95°C for 9 minutes, 40 cycles of 95° C for 15s, 56.2°C for 30s, and 72°C for 45s, followed by 1 cycle of 95°C for 30s, 55°C for 30s, and 95°C for 30s. Melting curves were

generated on each plate for each amplification product to ensure targeted amplification of a single product and no primer-dimer formation.

Analysis was completed using the StepOnePlus™ software, as described above. Outliers were removed from each exposure group for each target gene using a Grubbs' test. A *Cq'* was calculated for each sample to reduce heterogeneity of variance as with the zebrafish (Rieu and Powers, 2009). To determine any effects of MeHg exposure on the expression of each target gene, a two-way ANOVA (factors = exposure and sampling date), followed by a Holm-Sidak test, was used. Fold change was calculated as described with zebrafish.

Pituitary RNA Isolation[2]

RNA was extracted from yellow perch pituitaries by using TRI-Reagent® (Sigma-Aldrich Co.) and the illustra RNAspin 96 RNA isolation kit (GE Healthcare, Milwaukee, WI), according to Goetz et al. (2011). The concentration of RNA in each sample was obtained using a NanoDrop ND1000 spectrophotometer. cDNA was synthesized from approximately 500 ng RNA using the Promega ImProm-II RT system, which contains a combination of oligo(dT) and random primers, according to manufacture's instructions.

Pituitary QPCR

Genes for QPCR were selected based on their importance to fish reproduction. Both *follicle stimulating hormone, beta polypeptide* (*fshb*) or

[2]Dr. Frederick Goetz completed pituitary RNA isolation and QPCR

luteinizing hormone, beta polypeptide (*lhb*) were cloned from the yellow perch pituitary, using primers from fugu (*Takifugu rubripes*), and then primers for QPCR were created based on those amplicons (Supplementary Table 2; Goetz et al. 2011). To ensure a single band of the correct size, PCR products from primer amplification of each primer set were run on an agarose gel.

From the pituitaries, QPCR was used for the analysis of *fshb* or *lhb* transcript levels in twelve fish per exposure level per sampling date. All QPCR reactions contained 25 µL made up of the following: 2.5 µL of 1:10 dilution of cDNA, 5 µL each of the forward and reverse gene primers (Supplementary Table 2), and 12.5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems). Each plate also contained the normalizer gene, *actin, beta*, and a no template control for each primer set. Cycling and fluorescence measurements were performed in an Mx3000P qPCR system (Stratagene, La Jolla, CA) with the following cycling parameters: 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15s and 58°C for 1 min. Melting curves were generated on each plate for each amplification product, and no-template controls were included for each primer pair on each plate as well. Raw data were processed with Real-Time PCR Miner (Zhao and Fernald, 2005).

The relative messenger RNA concentration (R_0) was calculated for each gene per individual sample (Goetz et al., 2011). This was done using the following equation:

$$R_0 = \frac{1}{(1+E)^{C_t}}$$

E is the gene efficiency (calculated as the average of all individual sample

efficiencies across all reactions for a given gene per QPCR plate) and C_t is the cycle threshold (Zhao and Fernald, 2005). From each sample, *actin, beta* acted as the normalizer gene to calculate R_0 for each gene. The total amount of *fshb* or *lhb* transcript per pituitary was calculated as follows:

$$Total\left(GTH_{pituitary}\right) = \frac{QPCR_{GTH} \times RNA_{pituitary}}{0.5}$$

This adjusted for the total amount of RNA extracted for each sample. Total $GTH_{pituitary}$ is the total amount of *fshb* or *lhb* in the pituitary, $QPCR_{GTH}$ is the normalized QPCR value of *fshb* or *lhb*, $RNA_{pituitary}$ is the total amount of RNA (µg) extracted from the pituitary, and 0.5 is the amount of RNA (µg) used to synthesize cDNA. The *fshb* or *lhb* levels were analyzed with two-way ANOVA (factors = exposure and sampling date), followed by Holm-Sidak post hoc test.

3. Results

Because the design of this experiment involved two separate fish species with differing MeHg exposure conditions and methodology, the results will discuss each species separately. Selection of genes for QPCR of yellow perch ovaries was dependent upon zebrafish RNA-seq and QPCR results; therefore all zebrafish results will be presented first, followed by yellow perch. Prior to publication, all transcriptomics data will be submitted to Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/, accessed Jan. 15, 2015).

3.1 Zebrafish

Mercury Accumulation in G_1

The zebrafish component of this experiment depended upon accumulation of MeHg in the eggs of females exposed through their diet in order to simulate whole-life cycle exposure, thus it was necessary to test the amount of mercury in the eggs every few weeks during maternal exposure. Exposure of the G_0 zebrafish to MeHg resulted in accumulation of mercury in the G_1 eggs in a dose- and time-dependent manner (Fig. 3). By week 9, all pairwise comparisons of exposure levels were significantly different ($p < 0.001$) and the levels mimicked environmental exposures; these eggs were reared to adulthood.

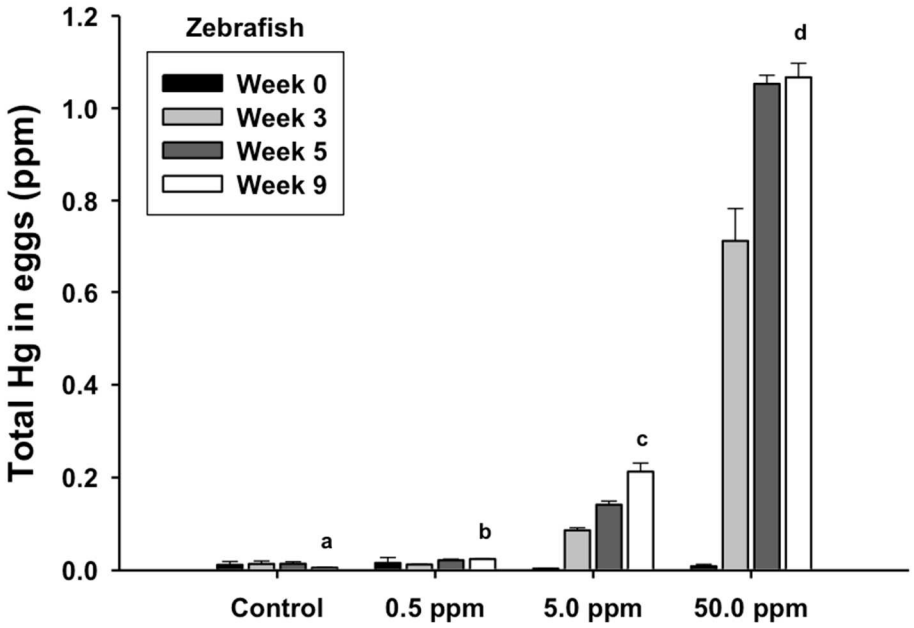


Figure 3. Total mercury (Hg) accumulation in zebrafish eggs over nine weeks of parental exposure to dietary MeHg. By week nine, eggs were separated into four distinct groups of mercury accumulation, dependent upon maternal dietary MeHg exposure (n = 3, each sample consisted of 200 eggs). Log-transformed Hg values in the embryos were statistically analyzed using a one-way ANOVA for comparisons relative to parental exposure. Lower case letters in the final sampling date indicate significant differences ($p < 0.001$).

To strengthen any conclusions about biological changes in zebrafish between exposure groups, it was necessary to assess the actual accumulation of mercury in the ovarian tissue in the G_1 zebrafish. Whole-life cycle dietary exposure to MeHg resulted in accumulation of mercury in the ovary consistent with exposure levels (Table 1). Control fish were significantly different from 3.0 ppm and 10.0 ppm, but not 1.0 ppm fish, and 1.0 ppm fish were significantly different from 10.0 ppm, but not 3.0 ppm fish ($p < 0.001$).

Fecundity

In order to relate changes in gene expression to phenotypic endpoints, fecundity and embryo mortality were measured. Fecundity in G_1 zebrafish was

not significantly affected by whole-life cycle exposure to MeHg (p = 0.691). Embryo mortality was also not significantly impacted (p = 0.692; Table 1).

Table 1. Average ovarian mercury (Hg) levels, fecundity, and embryo mortality in zebrafish following whole-life cycle exposure to MeHg. All values expressed as the mean ± SEM. For total Hg, n = 8-9 individuals/treatment group. Fecundity was calculated as the number of eggs per female in each replicate tank (n = 3). For embryo mortality n = 3 replicate tanks assessed at 24 hpf. Lower case letters indicate significant differences among treatment groups (p < 0.001).

Exposure	Total Ovary Hg (ppm)	Fecundity (eggs/female)	Embryo Mortality (%)
0 ppm	0.09 ± 0.02^{a}	170.92 ± 60	19.09 ± 2.98
1 ppm	0.83 ± 0.05^{ab}	235.75 ± 40.97	15.98 ± 2.83
3 ppm	2.14 ± 0.14^{bc}	206.89 ± 70.32	16.81 ± 2.27
10 ppm	6.53 ± 0.28^{c}	272.38 ± 66.84	24.44 ± 9.68

Ovary Staging

Staging of ovarian development was used as another measure to correlate changes in gene expression with altered morphology. At the highest exposure concentration, MeHg did not overtly impact the structure of the ovary (Fig. 4). In both exposed and control fish, the majority of follicles were pre-vitellogenic, and the least present follicles were mid-vitellogenic. Control fish did have a higher proportion of pre-vitellogenic follicles, while ovaries at the highest concentration of MeHg exposure had a higher proportion of mid- and late-vitellogenic follicles; this is consistent with fecundity data, in which fish at the highest exposure concentration laid more eggs per fish. However, only the proportion of mid-vitellogenic follicles was statistically different (p = 0.015).

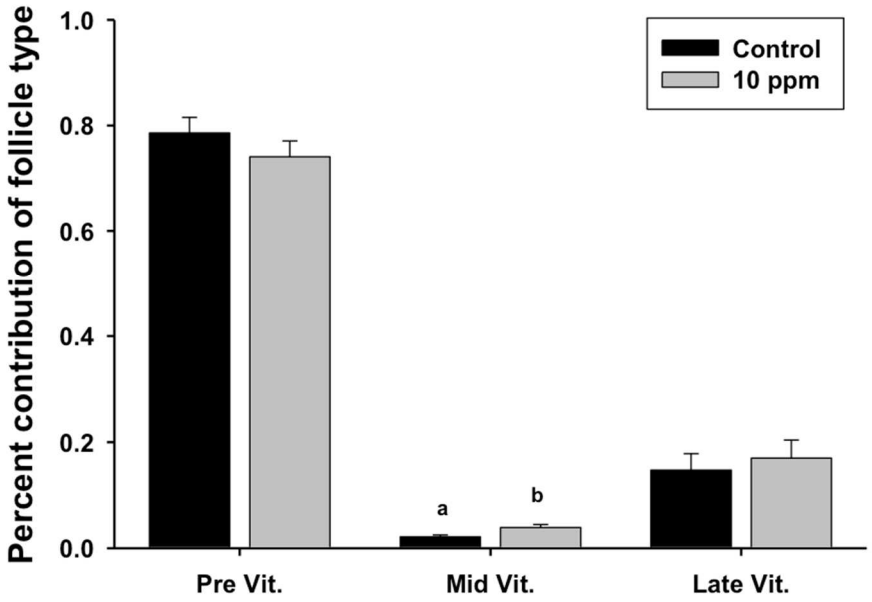


Figure 4. Proportion of follicles by stage (error bars = SEM). Pre-vitellogenic oocytes comprised of perinucleolar and cortical alveolar oocytes, mid-vitellogenic oocytes were defined as early vitellogenic oocytes, and late-vitellogenic oocytes also included mature/spawning oocytes. To normalize variation in ovary size, individual follicle types were calculated as a proportion of total follicles in an ovary. Statistical analyses were performed with an unpaired t-test, comparing control and 10.0 ppm exposure groups for each follicle classification. Lower case letters denote statistically significant differences (n = 10-11 sections per exposure; $p < 0.05$).

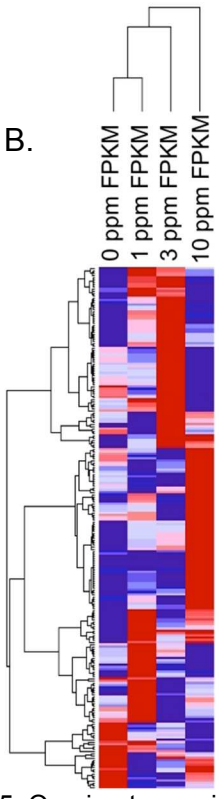
RNA-Seq

RNA-seq is a powerful tool for assessing subtle changes in the transcriptome. Rather than performing any relative quantitation, this technique quantifies the actual number of times a transcript sequence is present relative to the total number of reads, providing a value of Fragments Per Kilobase of exon per Million fragments mapped (FPKM) for each gene for each exposure group. RNA-seq was performed on three samples per exposure group. To minimize the effect of individual biological variation in these zebrafish, each sample run contained RNA that was pooled from up to six individuals, giving a truer read of the overarching impact of MeHg exposure.

Following whole-life cycle dietary exposure, transcriptomic analysis by RNA-seq revealed a total of 117 independent genes that were significantly dysregulated in treated zebrafish ovaries relative to untreated fish (False Discovery Rate (FDR) < 0.05; Fold-Change ≥ 2; Fig. 5). The top 10 significantly enriched gene ontology (GO) terms for biological processes, molecular functions, and cellular components ($p < 0.05$) are shown in Table 2. Several of these biological processes are directly involved with normal ovarian function, including lipid transport, lipid localization, response to estradiol stimulus, and response to estrogen stimulus. See Supplementary Table 7 for a complete list of significantly dysregulated genes.

A.

Exposure	Total Dysregulated Genes	Up-Regulated	Down-Regulated
1ppm MeHg	53	33 ↑	20↓
3ppm MeHg	45	37↑	8↓
10ppm MeHg	56	36↑	20↓



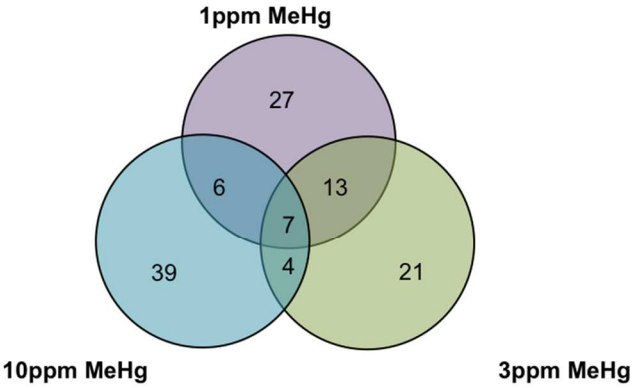


Figure 5. Ovarian transcriptomic analysis by RNA-seq. Each exposure group is comprised of three samples of equal amounts of pooled RNA from four to six individuals. (A.) The number of genes at least two-fold up- or down-regulated in treated zebrafish relative to control fish is shown (FDR < 0.05). (B.) Hierarchical clustering analysis of Fragments Per Kilobase of exon per Million (FPKM) fragments. Blue indicates the exposure group with the lowest FPKM value, and red signifies the exposure group with the highest FPKM value for each given gene. (C.) Overlap of significantly dysregulated genes among treatment groups is shown in the Venn diagram.

Table 2. Gene ontology annotations for significantly dysregulated genes ($p < 0.05$) in MeHg-treated zebrafish ovary as determined by RNA-seq. Top ten biological processes, molecular functions, and cellular components are shown.

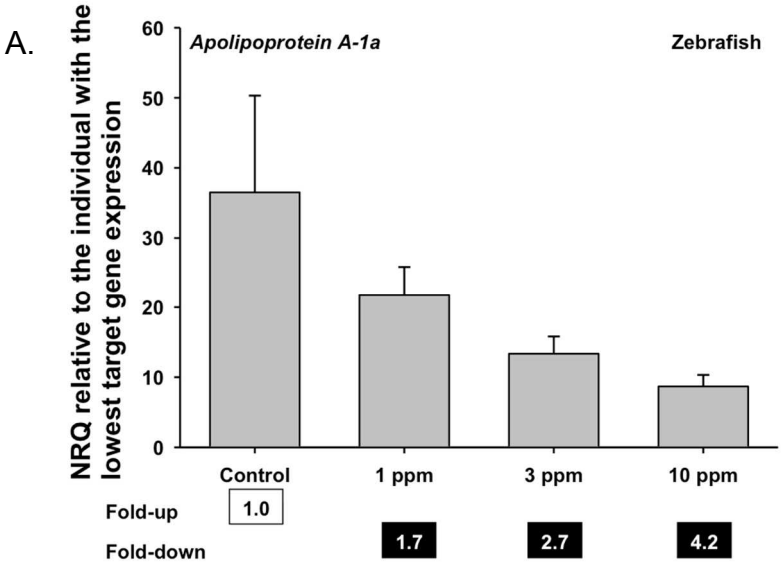
Biological Processes
Proteolysis
Lipid transport
Lipid localization
Response to estradiol stimulus
Response to estrogen stimulus
Single-organism metabolic process
Small molecule metabolic process
Protein metabolic process
Metabolic process
Cellular iron ion homeostasis
Molecular Function
Peptidase activity, acting on L-amino acid peptides
Serine-type endopeptidase activity
Endopeptidase activity
Peptidase activity
Serine hydrolase activity
Serine-type peptidase activity
Structural molecule activity
Phospholipase inhibitor activity
Lipase inhibitor activity
Lipid transporter activity
Cellular Component
Intermediate filament cytoskeleton
Keratin filament
Intermediate filament
Cytoskeletal part
Non-membrane-bounded organelle
Intracellular non-membrane-bounded organelle
Cytoskeleton
Extracellular region
Extracellular region part
Extracellular space

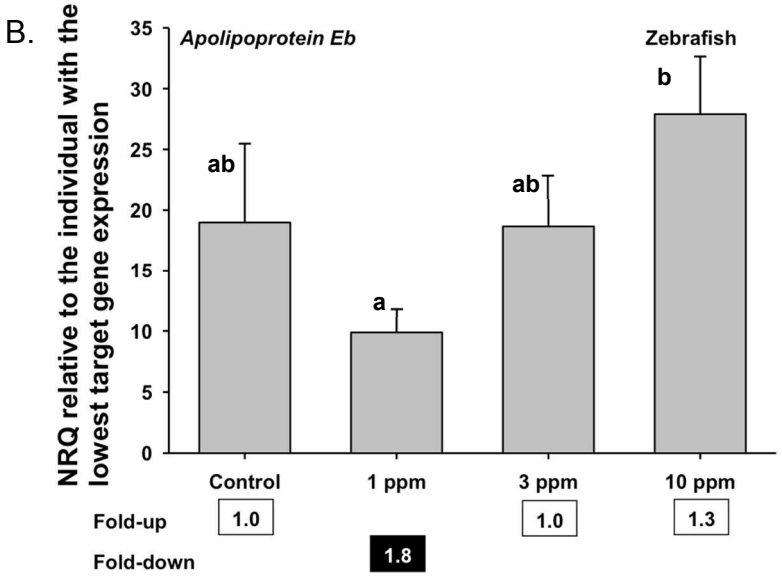
Zebrafish QPCR

In order to identify putative biomarkers for exploration in the yellow perch, genes were selected from pooled zebrafish RNA-seq data for QPCR analysis in individual zebrafish. Selected genes were based on relevance to reproduction and/or dysregulation across all exposure groups relative to the control group, as well as sequence information available in yellow perch. Seven genes were chosen that fit these criteria: *apolipoprotein A-1a* (*apoa1a*), *apolipoprotein Eb* (*apoeb*), *cytochrome P450, family 19, subfamily A, polypeptide 1a* (*cyp19a1a*), *elastase 3 like* (*ela3l*), *protease, serine, 59, tandem duplicate 1* (*prss59.1*), *vitellogenin 2* (*vtg2*), and *vitellogenin 3* (*vtg3*).

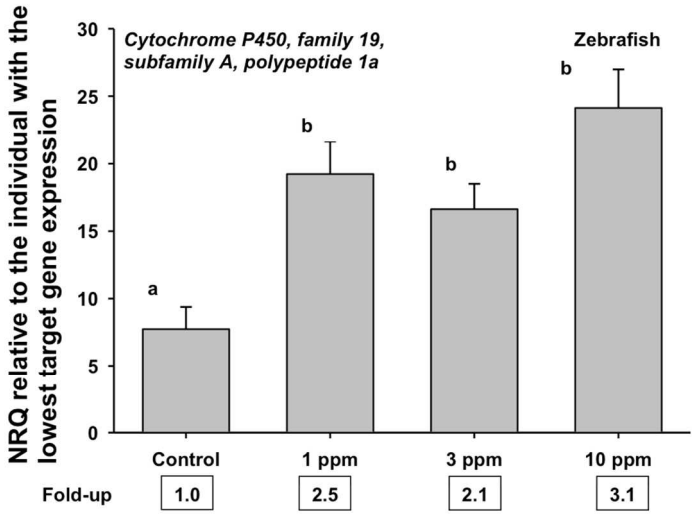
While not statistically significant ($p = 0.110$), *apoa1a* was down-regulated in a dose-dependent manner, with the 10.0 ppm exposure group down-regulated 4.2x relative to the control (Fig. 6A). Additionally, the zebrafish exposed to 10.0 ppm MeHg expressed *apoeb* significantly greater than the zebrafish exposed to 1.0 ppm MeHg ($p = 0.034$; Fig. 6B). Several genes showed patterns of gene dysregulation in ovaries, but only *cyp19a1a* was significantly up-regulated in all zebrafish exposed to MeHg relative to control fish ($p < 0.001$; Fig. 6C). Both *ela3l* (Fig. 6D) and *prss59.1* (Fig. 6E) were expressed in almost identical manners at different magnitudes. Expression of these genes was extremely variable among individual zebrafish, but MeHg did not impact the expression of either gene (*ela3l*: $p = 0.792$ and *prss59.1*: $p = 0.683$). In fish exposed to MeHg, *vtg2* expression was also reduced, although the high degree of variation among fish accounted for a lack of significant results ($p = 0.085$; Fig. 6F). Even with outliers

removed, several fish expressed *ela3l, prss59.1, vtg2,* and *vtg3* (p = 0.625; Fig. 6G), at least one order of magnitude greater than other fish in the same treatment group. Based on these results, *cyp19a1a* had the greatest potential to become a biomarker for MeHg impact on ovarian function.

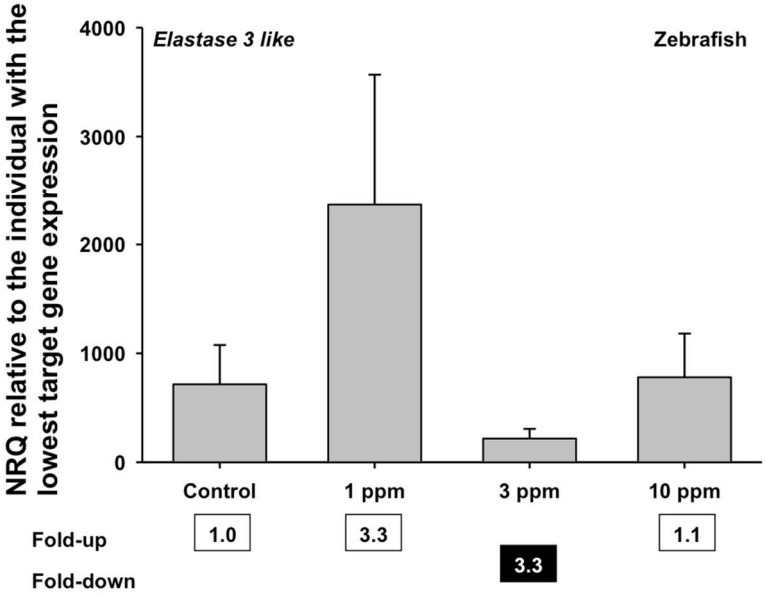




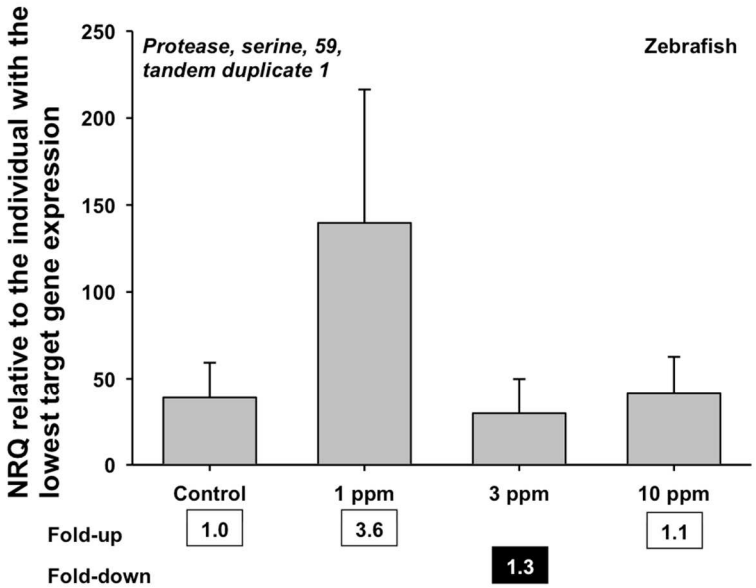
C.


Cytochrome P450, family 19, subfamily A, polypeptide 1a
Zebrafish
NRQ relative to the individual with the lowest target gene expression
30
25
20
15
10
5
0
a
b
b
b
Control
1 ppm
3 ppm
10 ppm
Fold-up
1.0
2.5
2.1
3.1

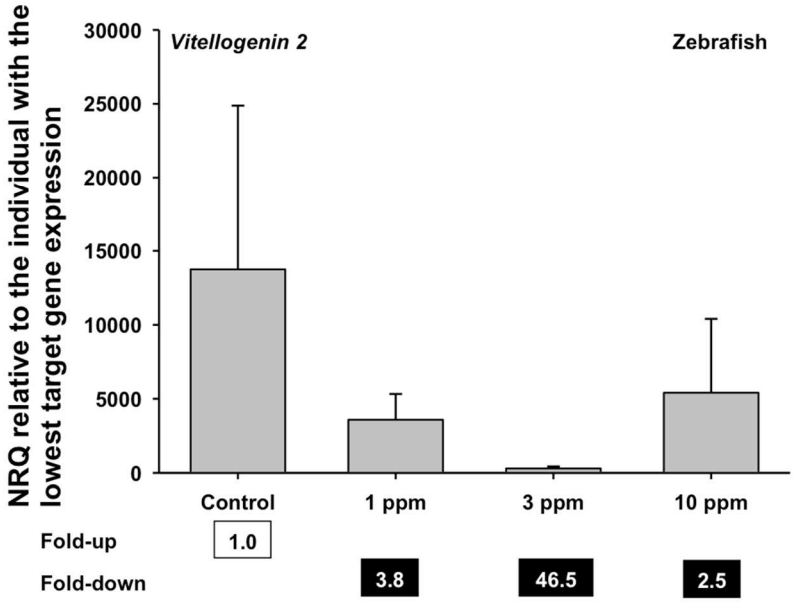
D.


Elastase 3 like
Zebrafish
NRQ relative to the individual with the lowest target gene expression
4000
3000
2000
1000
0
Control
1 ppm
3 ppm
10 ppm
Fold-up
1.0
3.3
1.1
Fold-down
3.3

E.


Protease, serine, 59, tandem duplicate 1
Zebrafish
NRQ relative to the individual with the lowest target gene expression
250
200
150
100
50
0
Control
1 ppm
3 ppm
10 ppm
Fold-up
1.0
3.6
1.1
Fold-down
1.3

F.


Vitellogenin 2
Zebrafish
NRQ relative to the individual with the lowest target gene expression
30000
25000
20000
15000
10000
5000
0
Control
1 ppm
3 ppm
10 ppm
Fold-up
1.0
Fold-down
3.8
46.5
2.5

G.

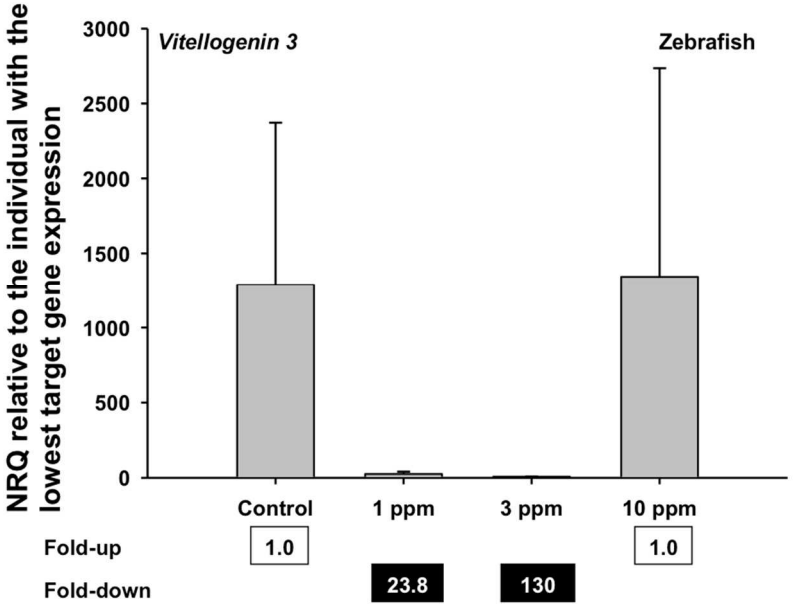


Figure 6. QPCR analysis of relative transcript abundance in zebrafish ovaries (error bars = SEM; *n* = 10-11 individuals per treatment group, outliers removed). Genes included are: (A.) *apolipoprotein A-1a*, (B.) *apolipoprotein Eb*, (C.) *cytochrome P450, family 19, subfamily A, polypeptide 1a*, (D.) *elastase 3l*, (E.) *protease, serine, 59, tandem duplicate 1*, (F.) *vitellogenin 2*, (G.) *vitellogenin 3*. Data is shown as mean normalized relative quantity (NRQ) per treatment group; each individual sample was normalized to *small nuclear ribonucleoprotein D1 polypeptide* (*snrpd1*), *ribosomal protein L13a* (*rpl13a*), and *actin, beta 1* (*actb1*). NRQ was calibrated to the individual with the lowest normalized quantity mean value. Overall fold change per exposure group is shown in the boxes below each exposure group. To calculate fold change, the average NRQ value of each exposure group was divided by the average NRQ of the control group. As this calculated a fold-up value, the inverse of this value was taken to present the fold-down value for exposure groups where gene expression was down-regulated. Lower case letters denote statistically significant changes in expression levels, as calculated using a one-way ANOVA ($p < 0.05$).

3.2 Yellow Perch

Mercury Accumulation

Adult female yellow perch were exposed to MeHg (0.0, 0.5, 5.0, or 50.0 ppm) for up to 20 weeks, with sampling occurring at three separate dates: 12, 16, and 20 weeks after commencement of the exposure. This sampling scheme captured different time points in ovarian development during a reproductive season. To confirm that MeHg was different among exposure groups, total

mercury was measured in muscle tissue. Yellow perch accumulated total mercury in the muscle tissue consistent with dietary exposure. At each date, all pairwise comparisons were significantly different ($p < 0.001$; Table 3). Additional unpublished work with identical exposure scenarios in yellow perch (personal communication, Jessica Head, March 9, 2011) showed a statistically significant correlation between ovary mercury and muscle mercury (Supplementary Table 3; $r = 0.921$, $p < 0.05$).

Table 3. Average total mercury (ppm) in muscle tissue of yellow perch in each exposure group (± SEM; n = 11-12 individuals) at each sampling point after exposure to dietary MeHg. Statistically significant differences are noted by lower case letters ($p < 0.05$).

Exposure	Week 12	Week 16	Week 18
0 ppm	0.05 ± 0.00[a]	0.050 ± 0.00[a]	0.05 ± 0.00[a]
0.5 ppm	0.19 ± 0.01[b]	0.23 ± 0.01[b]	0.18 ± 0.01[b]
5.0 ppm	1.39 ± 0.12[c]	1.56 ± 0.12[c]	1.56 ± 0.11[c]
50.0 ppm	8.66 ± 0.61[d]	9.30 ± 0.73[d]	9.34 ± 0.60[d]

OSI and Estradiol

Ovarian somatic index (OSI), or the proportion of total body weight contributed by the ovary, is a common metric used to determine overt changes in ovarian development, and can be used to correlate those results with circulating hormone and gene expression analyses (Drevnick and Sandheinrich, 2003, Goetz et al., 2011). In this study, circulating estradiol was measured, as this hormone is essential to fish reproduction and is involved in the feedback mechanisms that were assessed with QPCR. These measurements provide ways to anchor changes in gene expression to altered physiology. The OSI increased throughout the time course of the experiment, as the ovary developed (Fig. 7A), and estradiol peaked at the second sampling date, likely indicating the

beginning of vitellogenesis (Fig. 7B) (Dabrowski et al, 1996). The OSI was significantly greater in the 50.0 ppm exposure group relative to the 5.0 ppm group in the third sampling date (p = 0.004), but MeHg did not affect the OSI at any other time point. MeHg had no affect on estradiol concentration within any sampling date (p = 0.999).

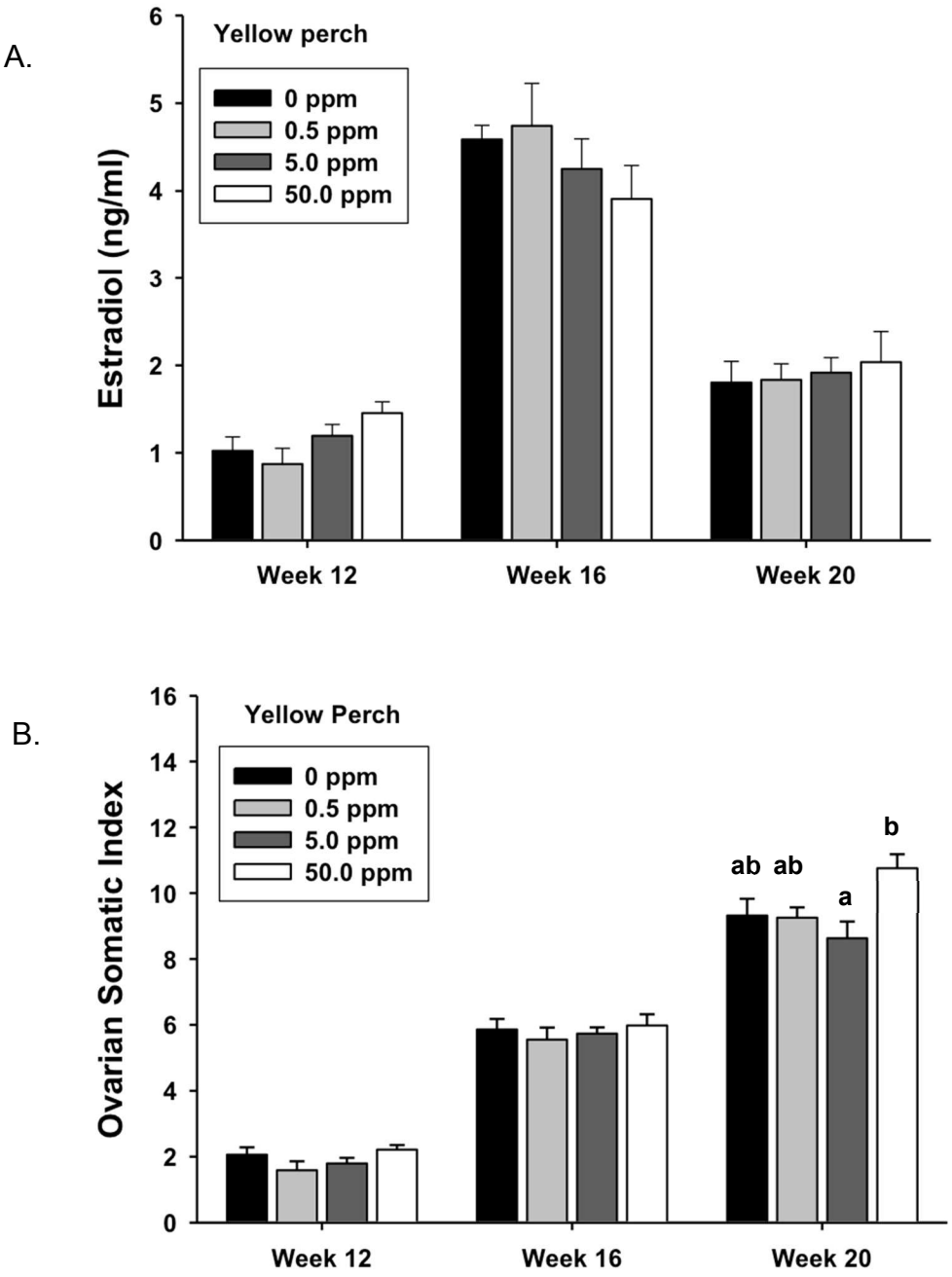


Figure 7. Effects of varying concentrations of MeHg on yellow perch estradiol concentration and Ovarian Somatic Index over a 20 week period (error bars = SEM). (A.) Estradiol levels (ng/ml) in yellow perch blood samples (B.) OSI in yellow perch, as calculated by the ovary weight (WO) divided by the total fish weight (WT) multiplied by 100. Estradiol and OSI were analyzed by two-way ANOVA (factors = sampling date and exposure; n = 11-12 individuals). Lower case letters denote statistically significant changes ($p < 0.05$).

Yellow Perch QPCR

QPCR was used to compare effects on transcript abundance between zebrafish and yellow perch, assess the effects of MeHg exposure on reproduction at a molecular level in yellow perch, and determine putative molecular biomarkers of MeHg exposure. As expected, ovarian maturation did impact the expression of several genes across sampling dates. However, MeHg exposure did not significantly alter the expression of any target genes in yellow perch ovaries, livers, or pituitaries at all sampling dates.

Transcript levels in the pituitary show that *follicle stimulating hormone, beta polypeptide* (*fshb)* was relatively high in abundance for the first two sampling dates, when early follicle maturation may have occurred, then declined in the final sampling date (Fig. 8A). Additionally, *luteinizing hormone, beta polypeptide* (*lhb)* peaked at week 16, but was otherwise expressed at a relatively low level (Fig. 8B); this pattern mimicked circulation estradiol concentrations. MeHg exposure did not show any significant statistical influence on expression of these genes (*fshb*: $p = 0.756$, *lhb*: $p = 0.669$).

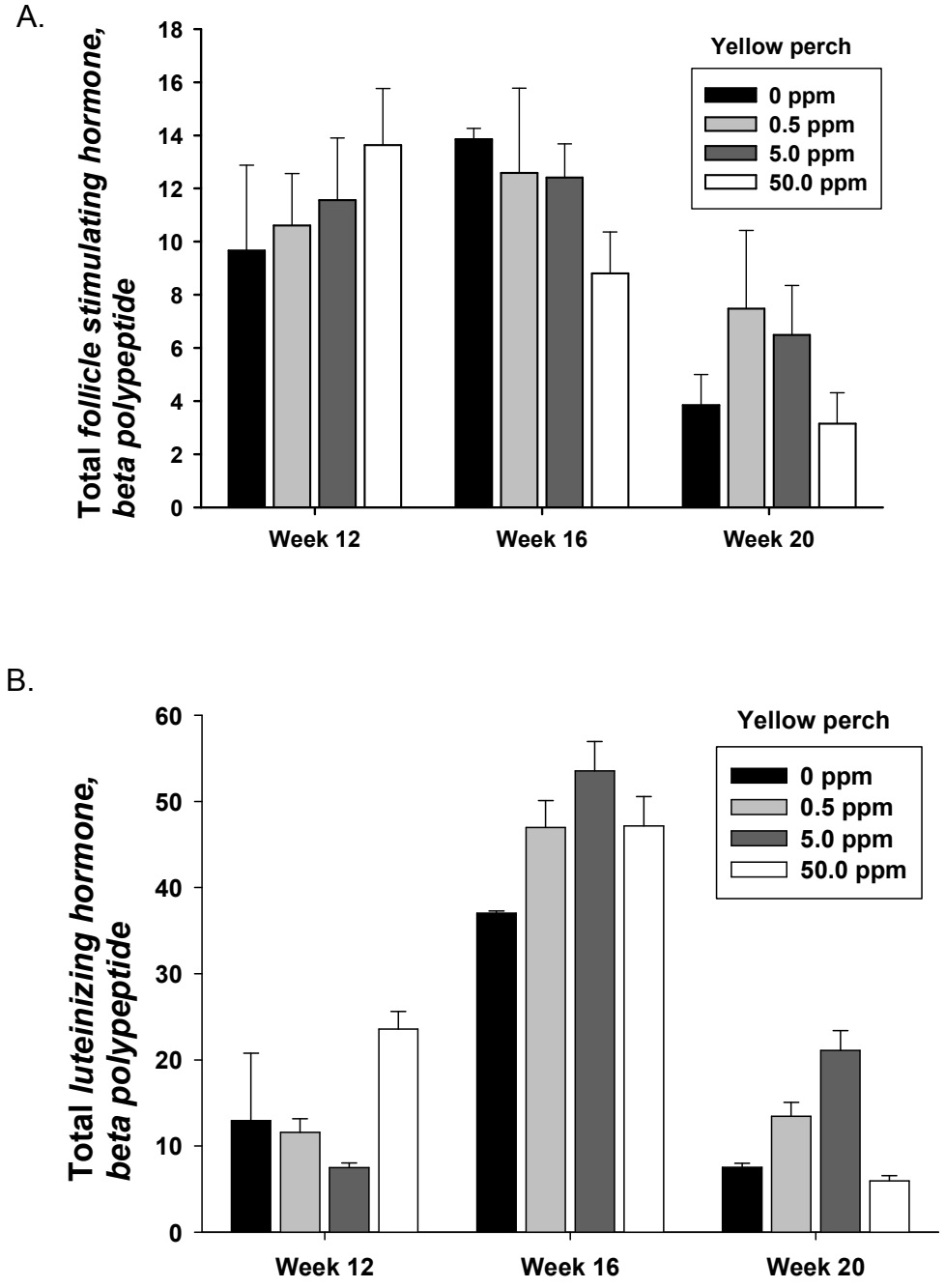


Figure 8. QPCR analysis of relative transcript abundance in yellow perch pituitaries (error bars = SEM; *n* = 9-12 individuals per treatment group per sampling date). (A.) Total *follicle stimulating hormone, beta polypeptide* (*fshb*) and (B.) *luteinizing hormone, beta polypeptide* (*lhb*). *Actin, beta* acted as the normalizing gene. Data is shown as mean total *fshb*/*lhb* per pituitary. The *fshb/lhb* levels were analyzed with two-way ANOVA (factors = sampling date and exposure).

Livers were analyzed for vitellogenin expression at the control and highest dose (50.0 ppm) of MeHg at each sampling point. Had a significant difference been observed, samples from each of the other exposure groups would have been analyzed. Based on this analysis, MeHg did not significantly affect expression of either *vitellogenin Ab* (*vtgab*) (p = 0.237; Fig. 9A) or *vitellogenin C* (*vtgc*) (p = 0.245; Fig. 9B), although *vtgab* was slightly down-regulated at each sampling date.

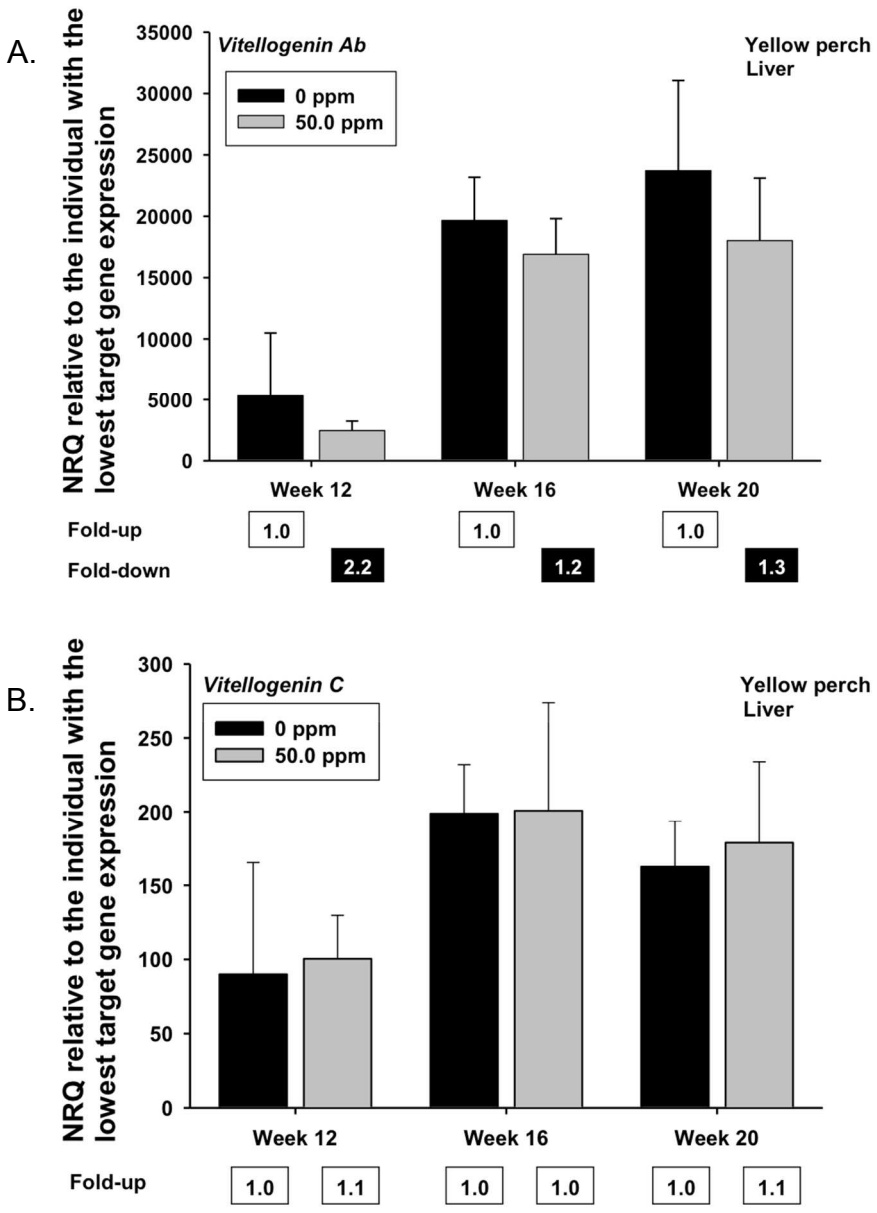
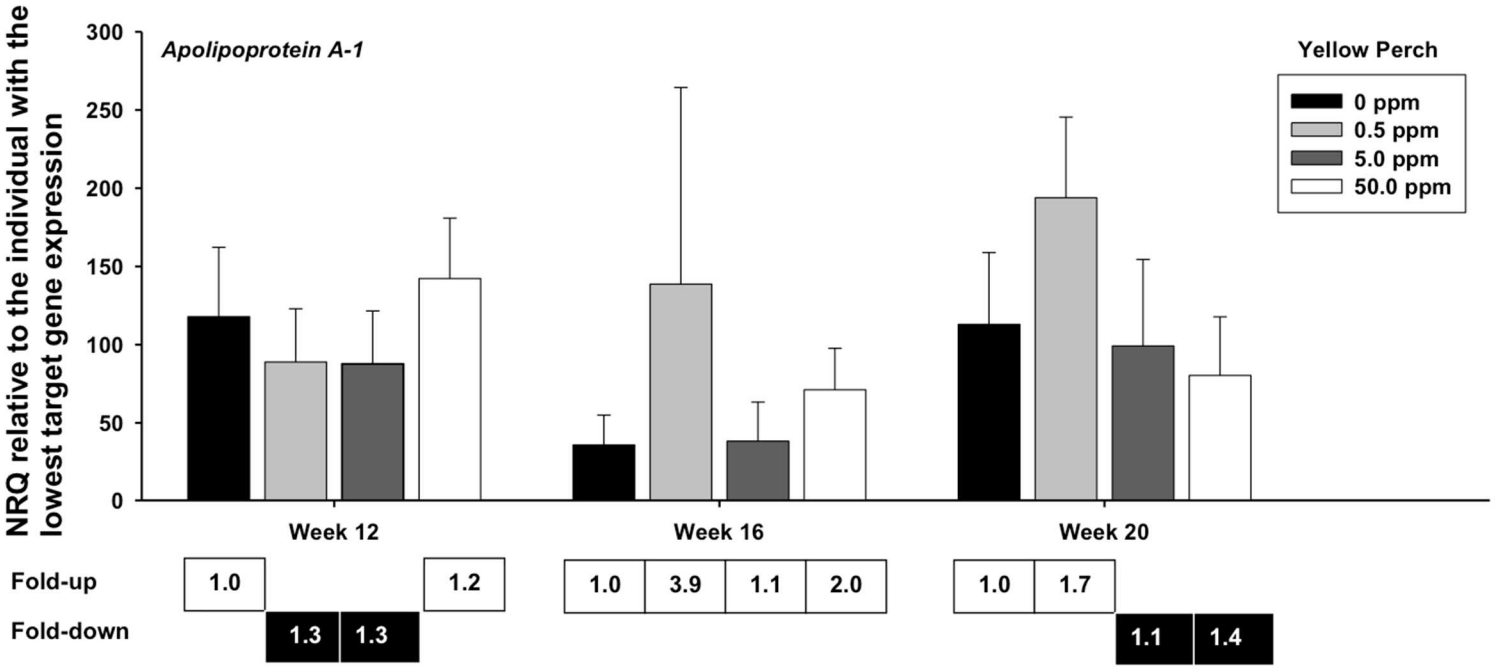


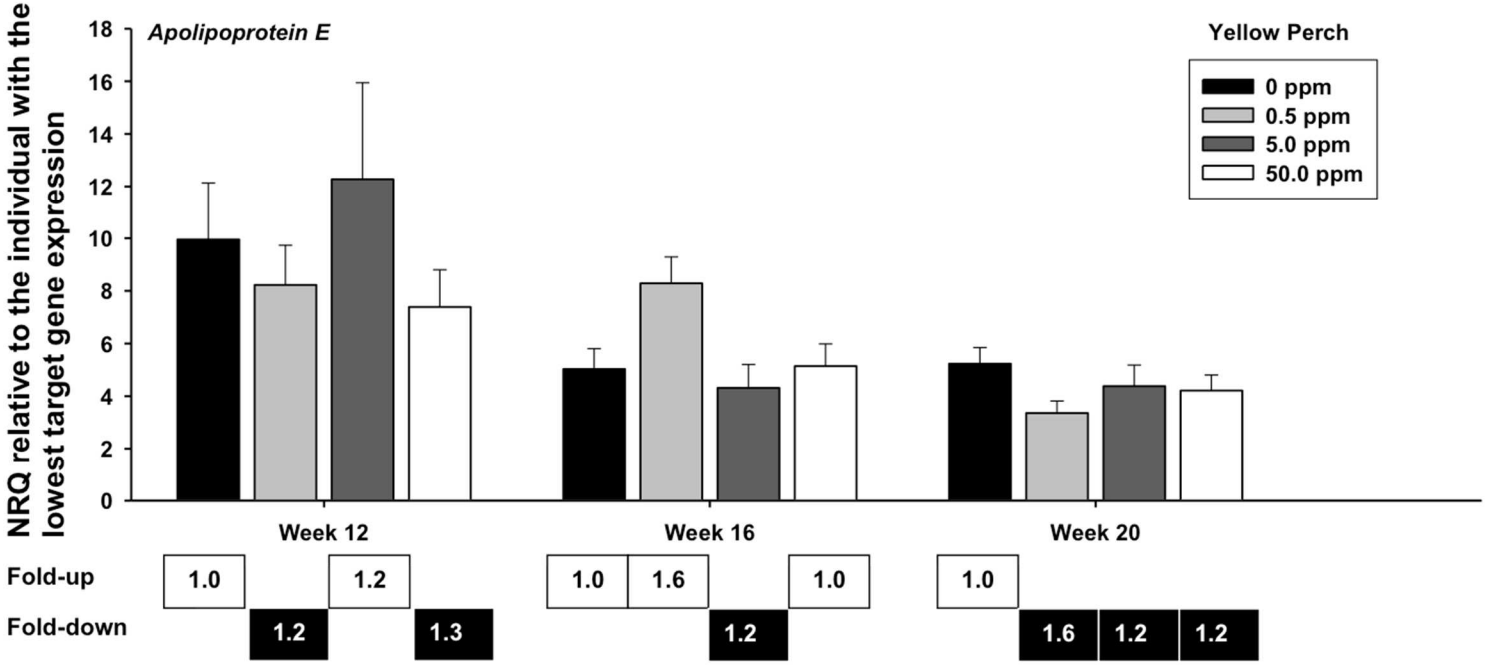
Figure 9. QPCR analysis of relative transcript abundance in yellow perch livers (error bars = SEM; *n* = 4-6 individuals per treatment group per sampling date). Genes included are: (A.) *vitellogenin Ab* and (B.) *vitellogenin C*. Data is shown as mean normalized relative quantity (NRQ) per treatment group; each individual sample was normalized to *ribosomal protein L13a* (*l13a*), *elongation factor 1a* (*ef1a*), and *elongation factor 2* (*ef2*). NRQ was calibrated to the individual with the lowest normalized quantity mean value. Overall fold change per exposure group is shown in the boxes below each exposure group. To calculate fold change, the average NRQ value of each exposure group at each sampling date was divided by the average NRQ of the control group of that same sampling date. As this calculated a fold-up value, the inverse of this value was taken to present the fold-down value for exposure groups where gene expression was down-regulated. Statistical analysis was calculated using a two-way ANOVA (factors = sampling date and exposure).

In the ovary, *apolipoprotein A-1* (*apoa1*) (Fig. 10A) and *vtgc* (Fig. 10E) were down-regulated across all MeHg exposures in the second sampling date, but MeHg did not significantly impact the expression of either gene (*apoa1*: p = 0.668; *vtgc*: p = 0.638). *Apolipoprotein E* (*apoe*) (Fig. 10B) and *vtgab* (Fig. 10F) expression were highest in the first sampling date, but MeHg did not alter the expression of these genes (*apoe*: p = 0.802, *vtgab*: p = 0.330). *Cytochrome P450, family 19, subfamily A, polypeptide 1a* (*cyp19a1a*) expression increased over time, and within each sampling dates, *cyp19a1a* was expressed at a higher level in yellow perch exposed to 50.0 ppm MeHg relative to control yellow perch (Fig. 10C); this trend was similar to the zebrafish results. The only significant difference occurred in the first sampling point, where *cyp19a1a* was up-regulated in yellow perch exposed to 50.0 ppm MeHg relative to yellow perch exposed to 0.5 ppm MeHg (p = 0.024**)**. Unlike in zebrafish, changes in the expression of *cyp19a1a* did not correlate with any other measured parameters. *Trypsin* (*try*), the closest orthologue to *protease, serine, 59, tandem duplicate 1* (*prss59.1*) in zebrafish was down-regulated in the highest MeHg concentration at all sampling dates (Fig. 10D). In the first sampling date, *try* was significantly down-regulated in the 0.5 ppm group relative to control fish, at the second sampling date, *try* was significantly down-regulated in the 5.0 ppm group relative to control fish, and at the third sampling time point, *try was* significantly down-regulated in the 50.0 ppm group relative to all other exposure groups (p = 0.002).

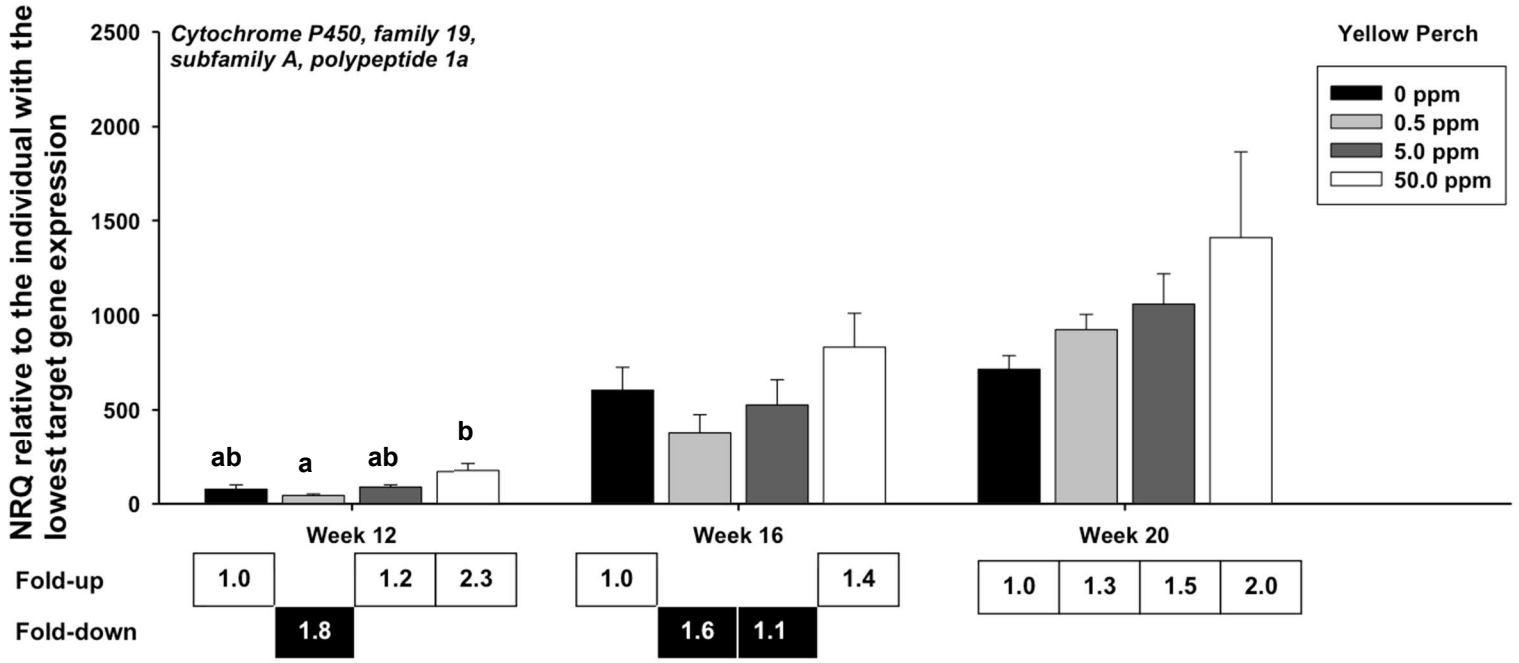
A.


Apolipoprotein A-1
Yellow Perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
NRQ relative to the individual with the lowest target gene expression
300
250
200
150
100
50
0
Week 12
Week 16
Week 20
Fold-up
Fold-down
1.0
1.2
1.3
1.3
1.0
3.9
1.1
2.0
1.0
1.7
1.1
1.4

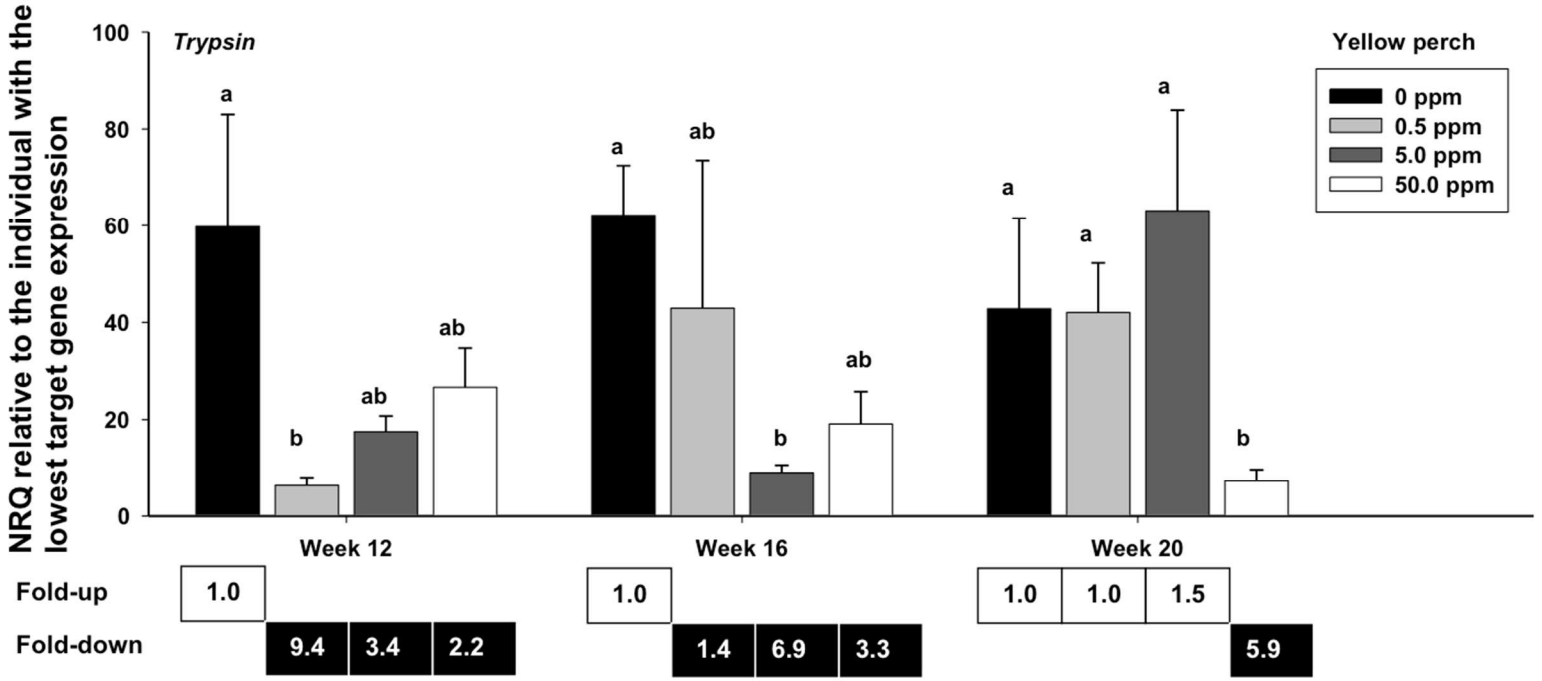
B.


Apolipoprotein E
Yellow Perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
NRQ relative to the individual with the lowest target gene expression
18
16
14
12
10
8
6
4
2
0
Week 12
Week 16
Week 20
Fold-up
Fold-down
1.0
1.2
1.2
1.3
1.0
1.6
1.0
1.2
1.0
1.6
1.2
1.2

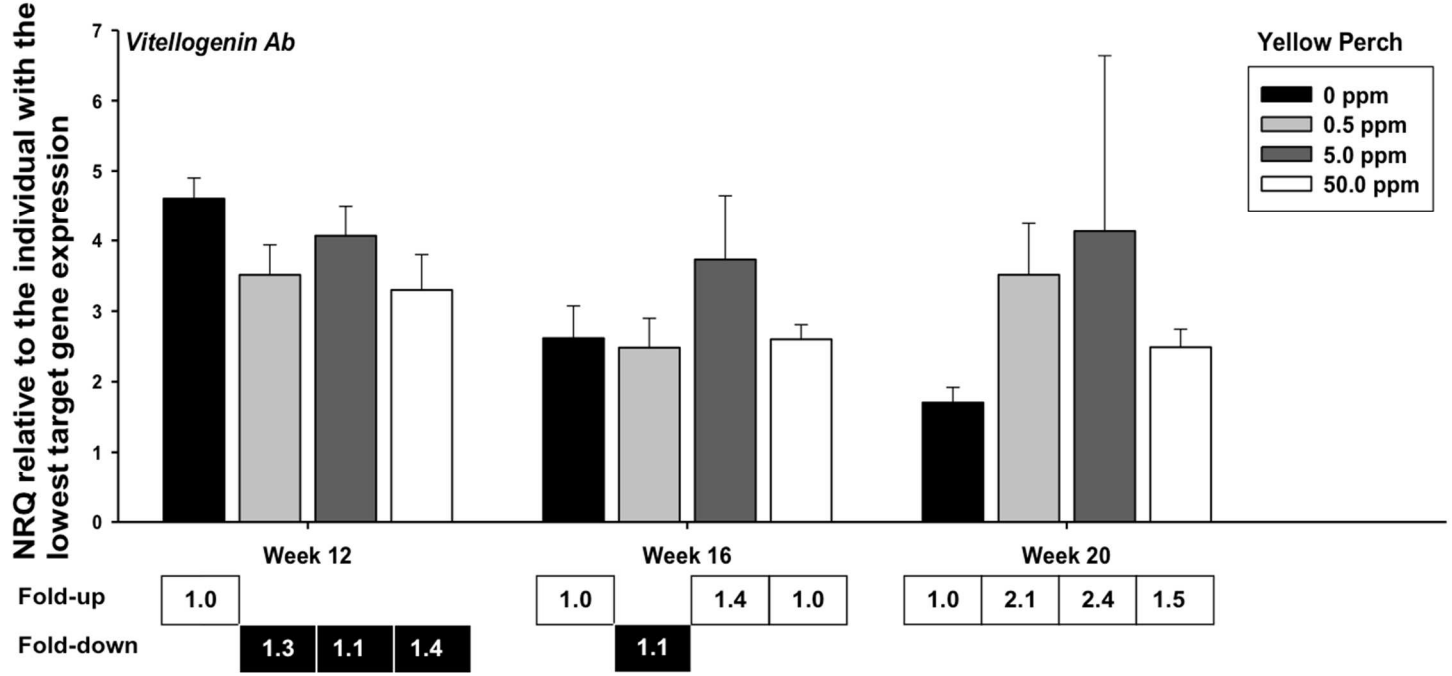
C.


Cytochrome P450, family 19, subfamily A, polypeptide 1a
Yellow Perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
NRQ relative to the individual with the lowest target gene expression
2500
2000
1500
1000
500
0
ab
a
ab
b
Week 12
Week 16
Week 20
Fold-up
Fold-down
1.0
1.2
2.3
1.8
1.0
1.4
1.6
1.1
1.0
1.3
1.5
2.0

D.


Trypsin
NRQ relative to the individual with the lowest target gene expression
100
80
60
40
20
0
Yellow perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
a
b
ab
ab
a
ab
b
ab
a
a
a
b
Week 12
Week 16
Week 20
Fold-up
1.0
1.0
1.0
1.0
1.5
Fold-down
9.4
3.4
2.2
1.4
6.9
3.3
5.9

E.


Vitellogenin Ab
Yellow Perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
NRQ relative to the individual with the lowest target gene expression
0
1
2
3
4
5
6
7
Week 12
Week 16
Week 20
Fold-up
Fold-down
1.0
1.3
1.1
1.4
1.0
1.1
1.4
1.0
1.0
2.1
2.4
1.5

F.

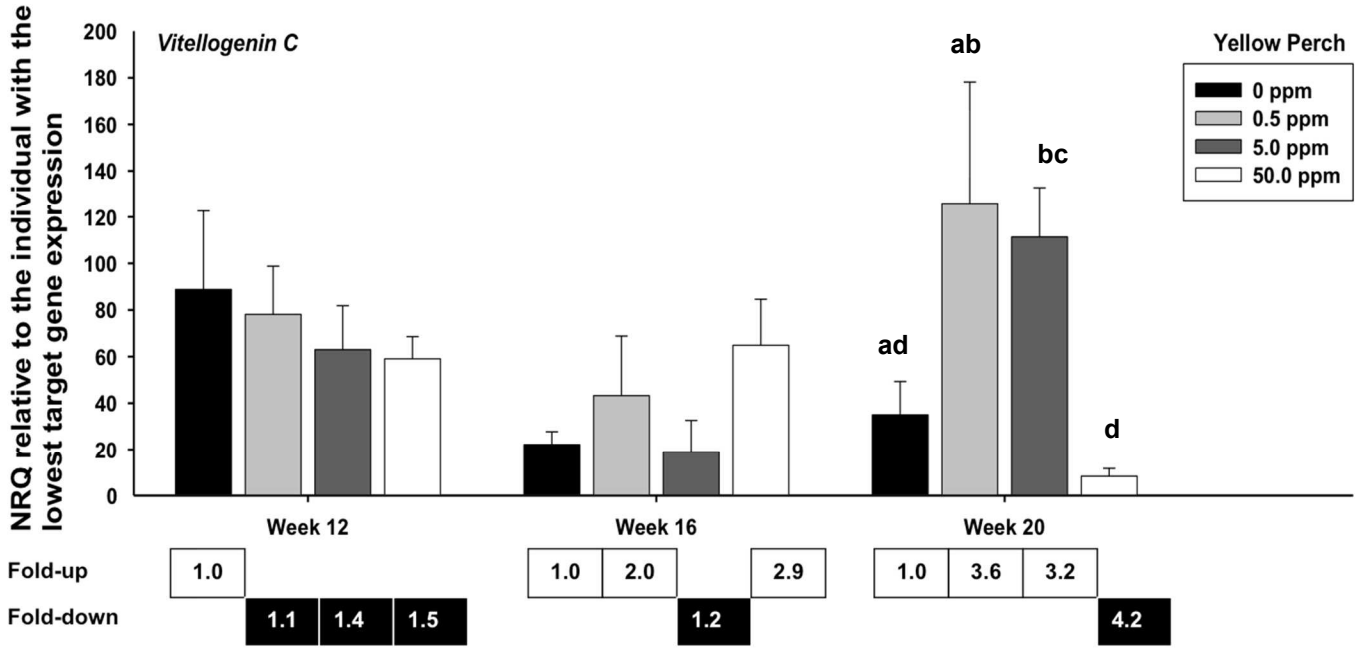


Figure 10. QPCR analysis of relative transcript abundance in yellow perch ovaries (error bars = SEM; *n* = 4-6 individuals per treatment group, outliers removed). Genes included are: (A.) *apolipoprotein A-1*, (B.) *apolipoprotein E*, (C.) *cytochrome P450, family 19, subfamily A, polypeptide 1a*, (D.) *trypsin*, (E.) *vitellogenin Ab*, (F.) *vitellogenin C*. Data is shown as mean normalized relative quantity (NRQ) per treatment group; each individual sample was normalized to *ribosomal protein L13a* (*l13a*), *elongation factor 1a* (*ef1a*), and *elongation factor 2* (*ef2*). NRQ was calibrated to the individual with the lowest normalized quantity mean value. Overall fold change per exposure group is shown in the boxes below each exposure group. To calculate fold change, the average NRQ value of each exposure group at each sampling date was divided by the average NRQ of the control group of that same sampling date. As this calculated a fold-up value, the inverse of this value was taken to present the fold-down value for exposure groups where gene expression was down-regulated. Lower case letters denote statistically significant changes in expression levels, as calculated using a two-way ANOVA (factors = sampling date and exposure; $p < 0.05$).

4. Discussion

While published studies suggest that MeHg impacts the ovary and reduces the reproductive capacity of female fish, evidence presented in this study demonstrates that the effects of environmentally relevant levels of MeHg exposure on female fish reproduction in a laboratory setting are minimal and vary by fish species.

The zebrafish component of this study was unique, relative to previous work elucidating the effects of MeHg on female fish reproduction (e.g. Hammerschmidt et al. 2002, Drevnick and Sandheinrich 2003, Drevnick et al. 2006), in that zebrafish from this study were exposed for their whole-life cycle. Rather than targeting a limited window in development, these fish received MeHg from both parental and dietary exposures. This had the advantage of mimicking a realistic scenario of chronic, long-term exposure. While whole-life exposure was expected to alter reproductive ability of zebrafish used in this study, the impact at both the molecular and physiological levels was minimal.

Utilization of yellow perch as a laboratory organism to study the effects of MeHg exposure was also distinct. Information on the female reproductive effects of MeHg on higher order teleost fish is currently limited (Depew et al., 2012). The yellow perch in this study were fed dietary MeHg for up to 20 weeks, incorporating the start of seasonal ovarian development.

Molecular-level results were variable in zebrafish and did not reveal considerable effects of MeHg on gene expression in the ovaries. RNA-seq is a relatively new tool in evaluating changes in gene expression, providing

sequencing depth for determining subtle, yet significant, changes in gene expression by providing actual transcript counts. RNA-seq analysis showed that many genes were only slightly dysregulated. Several of these genes are not well annotated in zebrafish and, therefore, are limited in their application to our analysis of impacts on reproduction and extrapolation to other fish species. Among the number of dysregulated and annotated genes found in this study, several were involved in processes one would expect to see in reproduction, including response to estradiol stimulus, lipid localization, and lipid transporter activity.

The two gene expression tools used in this study, RNA-seq and QPCR, yielded fairly different results. A lack of consensus between RNA-seq and QPCR with most genes may have been a direct result of using pooled RNA from multiple fish for RNA-seq, which normalizes individual variation, and RNA from individual fish for QPCR. This does not necessarily refute the validity of RNA-seq results, but rather highlights individual variation in how genes are expressed in zebrafish ovaries 24 hours following the previous spawning event. For example, *elastase 3l* (*ela3l*) and *protease, serine, 59, tandem duplicate 1* (*prss59.1*) were selected exclusively for their large fold-changes in RNA-seq results and clear dose response; QPCR results of these genes did not align with the RNA-seq results, but rather showed a large degree of variation. Translating these results to yellow perch was also problematic. In yellow perch, expression of *trypsin* (*try*), which most closely matches *prss59.1* in zebrafish, had significant dysregulation, but the results were not consistent among exposures or sampling dates.

In zebrafish, *cyp19a1a* expression was significantly up-regulated in response to MeHg exposure, making it the only gene that aligned with RNA-seq results. In yellow perch, *cyp19a1a* was up-regulated in the highest exposure group, relative to control, at all three sampling dates, but the results were not statistically significant.

The effect of MeHg on the expression of vitellogenin genes in zebrafish ovaries was not entirely clear. Vitellogenin, the main yolk precursor protein, is synthesized in the liver primarily in response to 17β-estradiol cues and transported to the oocytes (Kwon and Mugiya, 1994, Babin et al., 2007). In the ovary, the purpose of vitellogenin expression is not as well known. Wang et al. (2005) found *vitellogenin 2* (*vtg2*) expression in adipose tissues in the ovary, but not in any stage of oocytes, suggesting that vitellogenin in the ovaries is a supplemental source of vitellogenin for maturing vitellogenic oocytes.

While the results were not statistically significant, *vtg2* was down-regulated in the ovaries of exposed zebrafish. As estrogen regulate the synthesis of vitellogenin in the liver, perhaps this indicates that circulating estradiol has decreased as a result of MeHg exposure (although this was not measured). If this were the case, it would be consistent with findings by Klaper et al. (2006), who showed a significant reduction in vitellogenin expression in the liver of fathead minnows (*Pimephales promelas*) exposed to similar levels of dietary MeHg, and the findings of Drevnick and Sandheinrich (2003), who found reduced estradiol in fathead minnows exposed to MeHg. Surprisingly, the ovarian aromatase gene, *cyp19a1a*, which is involved in catalyzing the conversion of

androgens to estrogens (Devlin and Nagahama, 2002) was up-regulated in response to MeHg. Previous studies have shown that estradiol and brain aromatase (*cyp19a1b*) expression decrease concurrently (Ankley et al., 2003), although brain and ovary aromatase are not regulated in the same manner. The promoter region in *cyp19a1b* contains an estrogen response element (ERE), and *cyp19a1a* contains a steroiogenic factor-1 (SF-1) regulatory element (Callard et al., 2001, Dorts et al., 2009). Simultaneous suppressed brain aromatase and estradiol along with increased ovarian aromatase has been previously documented with aromatase inhibitors (Villeneuve et al., 2006, Villeneuve et al., 2009, Skolness et al., 2011). Villaneuve et al. (2009) found that increased *cyp19a1a* expression in fathead minnows exposed to a short-term treatment of fadrozole is correlated with a return of estradiol levels back to control levels. Induction of *cyp19a1a* in this study might suggest some sort of compensatory mechanism, accounting for suppressed estradiol levels.

In yellow perch ovaries, neither form of vitellogenin mRNA examined appeared to be impacted by MeHg. However, in yellow perch livers, vitellogenin Ab (*vtgab*), the orthologue of *vtg2*, was down-regulated at all three sampling dates (2.2, 1.2, 1.3, respectively) in the 50.0 ppm exposure group relative to control. The results were not statistically significant, but again, this trended alongside *cyp19a1a* up-regulation.

When analyzing QPCR data for *vitellogenin 3* (*vtg3*), it is worth noting several individuals expressed this gene more than 1000x higher than the individual with the least expression, contributing to the large variation in results.

This phenomenon has been previously reported (Susnik, 2010). Zebrafish ovaries undergo asynchronous development, resulting in oocytes at various stages (Selman et al., 1993), as was apparent with histological analysis. Large variations in gene expression may result from follicles in several developmental stages within each ovary. Yellow perch ovaries mature throughout the course of a spawning season, and follicles should all be in a similar stage, although ovary staging was not completed in yellow perch in this study. QPCR results were overall much less variable within each sampling date in yellow perch than in zebrafish.

The trends with *apolipoprotein Eb* (*apoeb*) and *apolipoprotein A-1a* (*apoa1a*) in zebrafish also support the idea that estradiol levels might be reduced in those zebrafish due to MeHg exposure. Apolipoproteins are involved in lipid transport and metabolism (Goetz et al., 2009) and there is a relatively high degree of conservation between fish and human apolipoproteins (Babin, 1987). Estradiol supplementation in postmenopausal women increases circulating apolipoprotein A-1 and decreases circulating apolipoprotein E (Yasui et al., 2002). If this relationship holds true for fish, apolipoprotein results are consistent with both *cyp19a1a* and *vtg2* results in zebrafish. Decreased levels of *apoa1a* and a slightly increased level of *apoeb* at the highest exposure concentrations suggests that estradiol levels are low in fish exposed to MeHg, but perhaps not low enough to create statistically significant differences. In yellow perch, neither apolipoprotein gene showed any trend in gene expression.

Yellow perch pituitaries were analyzed for *follicle stimulating hormone,*

beta polypeptide (*fshb*) and *luteinizing hormone, beta polypeptide* (*lhb*) expression. These hormones are associated with maturation of the ovaries and ovulation (Yaron et al., 2003, Campbell et al., 2006, Goetz et al., 2011), and expression of both of these genes was unchanged in fish exposed to MeHg.

Unfortunately, due to sampling limitations, it was not possible to gather the same physiological evidence of reproductive impairment in yellow perch as was obtained for zebrafish. In an attempt to correlate molecular results with reproductive output, fecundity, embryo mortality, and oocyte staging were analyzed only in zebrafish. In the highest treatment group, ovary development was shifted more towards mid- and late-vitellogenic follicles, fecundity was slightly increased, and embryo mortality at 24 hpf was increased; however the results were not significant. A shift towards mid- and late-vitellogenic follicles suggests that these fish are closer to spawning. Although these results were not statistically significant, this may indicate a biological significance, where fish are compromising egg quality, perhaps mediated through reduced vitellogenin in ovarian follicles.

In yellow perch, it was expected that the OSI and circulating estradiol would be decreased due to MeHg exposure, but MeHg exposure did not impact either the development of the ovary or estradiol levels at any time point. Molecular evidence in zebrafish implied that estradiol levels were suppressed in response to MeHg exposure, but this did not hold true for yellow perch. Although yellow perch also experienced an increase in *cyp19a1a* expression and decrease in *vtgab* expression in the ovary and liver, respectively, which can be suggestive

of decreased estradiol levels, the impact of MeHg was not as drastic, nor did the evidence manifest in any other measured genes or physiological parameters.

Overall, whole-life cycle dietary MeHg exposure appears to have a slight impact in zebrafish, and yellow perch are resilient to short-term exposure to dietary MeHg. The results from the zebrafish component of this study are consistent with previously published studies using the fathead minnow (e.g. Drevnick and Sandheinrich, 2003, Klaper et al., 2006), but the measured endpoints in this study did not provide dramatic evidence that MeHg is significantly affecting reproductive output. Based on an abundance of molecular and physiological evidence collected along the Hypothalamus-Pituitary-Liver-Gonadal (HPLG) axis in yellow perch, MeHg exposure does not appear to affect yellow perch ovaries or reproductive signaling at the doses or at the time points of ovarian development measured in this study.

The yellow perch exposure included a much higher dose than what was used in zebrafish (50.0 ppm), but surprisingly, these fish did not accumulate total mercury to the same extent as the zebrafish. Relative to the amount of MeHg in their diets, yellow perch accumulated much lower levels of mercury in muscle tissue than zebrafish did in ovary tissue (Tables 1 and 3). Because the ovary and muscle Hg concentrations are directly correlated in yellow perch (personal communication, Jessica Head, March 9, 2011; Supplementary Table 3), it can be assumed that yellow perch ovaries were much lower in mercury than zebrafish. This indicates that yellow perch are more successful at preventing MeHg from sequestering into tissue and implies that MeHg is either not as easily absorbed

through the intestine or is eliminated more efficiently from the body. This could account for the limited effects of MeHg on yellow perch reproduction.

The failure to find any significant effects in yellow perch is accordant with a field-based study, where female yellow perch in a MeHg hotspot in Canada did not experience significant changes in reproductive parameters (Batchelar et al., 2013), but this area needs further investigation. Furthermore, yellow perch are metal-tolerant fish species and proliferate in areas of heavier metal contamination (Couture and Rajotte, 2003, Pyle et al., 2005), although the mechanism allowing these fish to withstand these conditions is unclear.

One possible explanation for limited effects of MeHg in yellow perch reproduction is the chronic, long-term exposure yellow perch populations have experienced in the United States for several generations. If large quantities of MeHg entered the food web in the late 1800s (Pirrone et al., 1998, Wiener et al., 2012a), and yellow perch reach sexual maturity at two to four years of age (Schneider, 1984), the lineage of yellow perch that naturally inhabit North America may have been chronically exposed for more than 50 generations, allowing them time to adapt to MeHg or face local extinction. Fathead minnows, a common test species for MeHg toxicity, are also native to the Great Lakes, but this species spawns throughout the summer (Markus, 1934), unlike yellow perch, which only have one spawning event per year (Krieger et al., 1984). Individuals who do not successfully spawn at their one opportunity per year will not contribute their alleles to the overall population; this could create a strong selection pressure for fish that are less sensitive to MeHg.

The zebrafish used in this study, on the other hand, have been maintained in small populations in a laboratory setting for many generations, making them susceptible to genetic drift (Harper and Lawrence, 2010). Any resistance to MeHg that is normally present in wild zebrafish populations may have been lost in the laboratory over time.

Successful adaptation to a polluted environment has been documented before, both in individuals directly exposed to a toxicant and in a successive generation (Meyer and Di Giulio, 2003). Also, as recently shown in human populations, single nucleotide polymorphisms exist that elicit better elimination of MeHg from the body; such a mutation could exist in yellow perch populations that has been selected for over time to reduce population decline in response to MeHg exposure (Custodio et al., 2004, Goodrich et al., 2011).

Adaptation is not without the paradox of fitness trade-off, where the energetic costs of being successful in one environment inhibit some aspect of fitness when presented with other stressors (Hereford, 2009). Although purely speculation, if yellow perch successfully adapted to an influx of MeHg into the Great Lakes prior to the introduction of alewives or dreissenids, at least in terms of reproductive capacity, then their fitness trade-off may have contributed to their reduced natural population levels resulting from factors such as invasive species.

4.1 Recommendations

To further understand the physiological effects of MeHg on female reproduction in Great Lakes yellow perch, three further studies should be completed in yellow perch: a laboratory study of MeHg exposure over the entirety of ovarian development during a spawning season, a laboratory study pairing MeHg exposure with relevant stressors a fish would experience in a region of interest, and a detailed field study. This study only showed a snapshot of ovarian development, and even the last sampling date was months away from spawning. If MeHg is impacting reproduction, it may not become apparent until the ovary is fully developed and the fish is ready to spawn.

Additionally, MeHg is rarely a single stressor in the Great Lakes basin, with the introduction of alewives and dreissenids being substantial factors in the decline of yellow perch (Marsden and Robillard, 2004). Simulating the kinds of stress these introduced species would have (i.e. nutritional decline, less desirable habitat, etc.) would be a more realistic way to test the effects MeHg would have on female reproductive physiology in wild fish, now that a baseline of pure MeHg exposure has been established at environmentally relevant levels.

These proposed studies could be conducted along with field studies to anchor these results in realistic exposure scenarios, collecting fish at several points during ovarian development in a year and in areas of differing MeHg contamination. A lack of sequencing information is a current limitation to these kinds of studies in non-model organisms, but as sequencing becomes lower in cost and more easily accessible, this barrier may be overcome. These research

paradigms could then be completed in fishes exhibiting different reproductive life strategies in order to determine the relevance of cross-species comparisons.

4.2 Conclusions

Exposing both zebrafish and yellow perch to dietary MeHg at environmentally relevant levels allowed us to determine the efficacy of using zebrafish as a model for yellow perch. While zebrafish were considerably easier to use in the lab, the results of this study indicate that zebrafish are more sensitive to dietary MeHg at a molecular level, and therefore may not be an adequate model for assessing the effects of MeHg exposure in yellow perch or developing molecular biomarkers of MeHg exposure. Additionally, the effects in zebrafish were not drastic and suggested that a compensation mechanism was alleviating the burden of constant exposure to MeHg, possibly through the induction of *cyp19a1a*. This does not discredit the use of zebrafish as a surrogate for other wild fish species, but rather suggests that zebrafish results may be better correlated with fish species that share reproductive strategies.

Two features of this experimental design limit the conclusions of using zebrafish as a model for yellow perch: timing of dietary MeHg exposure and collection dates of the ovaries. Zebrafish experienced whole-life cycle dietary exposure, whereas yellow perch were limited in the duration of exposure, incorporating a considerable part of ovarian development, but not affecting the maturation processes early in development of the whole organism or through spawning. Moreover, the timing of ovary collection may have resulted in

differences in gene expression between fish species. Zebrafish ovaries were collected 24 hours after a spawning event, whereas yellow perch ovaries were removed at three separate time points in seasonal development of the ovary. The zebrafish transcriptome directly after spawning may not resemble yellow perch at any of the sampling dates, leading to differences in expression of genes directly tied to reproductive processes.

Overall, the effects of MeHg on female reproduction are complicated and not as clear as predicted. The molecular reproductive effects in zebrafish do not clearly manifest at a physiological level, nor do they translate to yellow perch. To consider the potential impact this might have on wild fish species, it is critical to appreciate that contaminated ecosystems are a slew of pollutants, invasive species, and other anthropogenically-driven changes, not one single contaminant. What may be a low-level impact in a laboratory setting might compound with other stressors and cause high-level impacts on reproduction. Furthermore, as climate change increases temperatures in local water bodies, the amount of bioavailable MeHg may increase, shifting what is considered “environmentally relevant.” A fish that has adapted to a range of MeHg exposure might suddenly be sensitive as water quality parameters shift. The results of this study highlight the complexity of sensitivity to MeHg among various fish species, and further examination may better elucidate any ubiquitous impacts of MeHg on recruitment in wild populations.

5. References

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Appendix: Supplementary Tables

Supplementary Table 1. Total Hg in diets of both generations of zebrafish (*n* = 3) and yellow perch (*n* = 7-12) (± SEM). For the sake of simplicity, diets were categorized based on intended target Hg concentrations.

Fish Generation/Species	Nominal Hg (ppm)	Measured Hg (ppm)
G_0 Zebrafish	0 ppm	0.12 ± 0.00
	0.5 ppm	0.61 ± 0.12
	5.0 ppm	4.48 ± 0.31
	50.0 ppm	47.35 ± 0.62
G_1 Zebrafish	0 ppm	0.05 ± 0.01
	1 ppm	1.11 ± 0.02
	3 ppm	3.62 ± 0.07
	10 ppm	11.16 ± 0.37
Yellow Perch	0 ppm	0.04 ± 0.00
	0.5 ppm	0.46 ± 0.06
	5.0 ppm	3.86 ± 0.31
	50.0 ppm	47.11 ± 4.54

Supplementary Table 2. Accession numbers, primer sequences, primer efficiencies, and primer concentrations for all primers used in zebrafish and yellow perch.

	Primer	Accession Number	Forward Primer (Primer Sequence 5′–3′)	Reverse Primer (Primer Sequence 5′–3′)	Efficiency (%)	Concentration
Zebrafish	*actb1*	NM_131031.1	TGTGGATCAGCAAGCAGGAG	GGTTGGTCGTTCGTTTGAATC	90.44	2.5 µM
	rpl13a	NM_212784.1	TTGTCCCAGCTGCTCTCAAG	GGTGATGGCCTGGTACTTCC	97.18	1 µM
	snrpd1	NM_173252.2	GAGTCACTCAGCATCCGAGG	CGCGCACAAAAGTACAGTGG	90.1	1 µM
	apoa1a	NM_131128.1	TAAGCTGACCGAGCGTCTTG	TCTGTGCGAATGTGGTCCTC	107.7	1 µM
	apoeb	NM_131098.1	TGACATGACCGACGCTAAGG	TGTAGGTTGCTACGGTGTTGC	98.58	1 µM
	cyp19a1a	NM_131154.2	TCATCGAGGGCTACAACGTG	CACCATGGCAATGTGCTTCC	100.9	1 µM
	ela3l	NM_001024408.1	AGCCTCTCATGTCTCGTGTG	TAGTTGCGGCCAGAACTGATG	104	1 µM
	prss59.2	NM_199605.2	TCTGACCGCGACTGTAACAAC	CAGGATGATTCTTCTCAGCACAGC	91.87	1 µM
	vtg2	NM_001044913.1	CTGCAAGCTTTGAGACATTCGC	GTGACTGACGGTTTTGAAGGG	97.18	1 µM
	vtg3	XM_688789.7	TGAGGCTCGGTTCATACAGC	TTTGTCACGCCGATAAAGCC	103.7	1 µM
	Primer	Accession Number	Forward Primer (Primer Sequence 5′–3′)	Reverse Primer (Primer Sequence 5′–3′)	Efficiency (%)	Concentration
Yellow Perch Ovary	*l13a*		CAAAATGGTCCTCTGAATCGGC	TCCCCACCAATGACAAGGG	91.26	1 µM
	ef1a		CGACAAGATGAGCTGGTTCAAG	ACAGTTCCGATACCGCCAATC	93.32	1 µM
	ef2		GATGAGGCTGCCATGGGTATC	CCTTCTTTCCAGGGACATAGTTTG	90.84	1 µM
	apoa1		GGTGCGATCTTCGTCCTCAG	GCAGATGGCTACTCCCTACG	97.61	5 µM
	apoe	FJ800037	AAGACACCGAGGAGATTCGC	TCACTCAGCTTCTGGGATGC	99.93	1 µM
	cyp19a1a	DQ984126	AGCTTCAGAACGGGGACTG	AAAACTCTGTCCGGTGCATGTG	99.8	1 µM
	try		GCTACCCTGATCGTCTGAGG	TAACCCCAGGACACCACACC	94.64	2.5 µM
	vtgab	FJ804421	GGCTCTCCCTATTGCTCTGC	ACTCAGCTGCATGAGCCTTG	100.26	1 µM
	vtgc	FJ804422	CTGCCATGGTCTCCGGTATC	ACACATTGGATGGGGAGCTG	107.41	2.5 µM

Yellow Perch Pituitary	Primer	Accession Number	Forward Primer (Primer Sequence 5′–3′)	Reverse Primer (Primer Sequence 5′–3′)	Efficiency (%)	Concentration
	actin	AY332493	TGCCATGTACGTTGCCAT	AGCTGTGGTGGTGAATGA		5 pM
	fshb		GTTGTCATGGCAGCAGTGCT	CCTTCAATGTGTTTCACCTCGTA		5 pM
	lhb		TGCCAGCTCATCAACCAGAC	CTCTCGAAGGTGCAGTCGGA		5 pM
Yellow Perch Liver	Primer	Accession Number	Forward Primer (Primer Sequence 5′–3′)	Reverse Primer (Primer Sequence 5′–3′)	Efficiency (%)	Concentration
	l13a		CAAAATGGTCCTCTGAATCGGC	TCCCCACCAATGACAAGGG	90.77	1 µM
	ef1a		CGACAAGATGAGCTGGTTCAAG	ACAGTTCCGATACCGCCAATC	93.06	1 µM
	ef2		GATGAGGCTGCCATGGGTATC	CCTTCTTTCCAGGGACATAGTTTG	91.58	1 µM
	vtgab	FJ804421	GGCTCTCCCTATTGCTCTGC	ACTCAGCTGCATGAGCCTTG	90.56	1 µM
	vtgc	FJ804422	CTGCCATGGTCTCCGGTATC	ACACATTGGATGGGGAGCTG	91	2.5 µM

Supplementary Table 3. Total mercury in muscle and ovary in yellow perch from an unpublished study (personal communication, Jessica Head, March 9, 2011) using the same exposure conditions (± SEM; *n* = 6).

Exposure	Muscle			Ovary		
	Sampling 1	Sampling 2	Sampling 3	Sampling 1	Sampling 2	Sampling 3
0 ppm	0.07 ± 0.02	0.07 ± 0.01	0.08 ± 0.01	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
0.5 ppm	0.08 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.03 ± 0.01
5.0 ppm	0.37 ± 1.64	0.47 ± 0.06	0.67 ± 0.10	0.23 ± 0.07	0.29 ± 0.10	0.28 ± 0.06
50.0 ppm	11.87 ± 1.64	8.15 ± 1.52	7.77 ± 0.76	7.04 ± 0.74	8.15 ± 1.41	3.36 ± 0.57

Supplementary Table 4. Sample quality of zebrafish ovary RNA was confirmed by the Bio-Rad Experion. Only samples with an RNA Quality Indicator (RQI) value of 8.0 or greater were used for downstream analysis

Sampling Date	MeHg Exposure	Sample #	RQI Classification	RQI Alert
R1	1 ppm	#6	9.5	Green
R1	1 ppm	#7	9.5	Green
R1	1 ppm	#9	9.5	Green
R1	1 ppm	#11	9.9	Green
R1	1 ppm	#8	9.7	Green
R1	1 ppm	#4	9.2	Green
R1	3 ppm	#10	2.3	Red
R1	3 ppm	#11	9.8	Green
R1	3 ppm	#5	9.8	Green
R1	3 ppm	#7	9.8	Green
R1	3 ppm	#6	9.8	Green
R1	3 ppm	#4	N/A	RNA conc. low
R1	10 ppm	#6	8.3	Green
R1	10 ppm	#8	8.4	Green
R1	10 ppm	#7	9.9	Green
R1	10 ppm	#5	9.6	Green
R1	10 ppm	#4	9.8	Green
R1	0 ppm	#10	9.5	Green
R1	0 ppm	#11	10	Green
R1	0 ppm	#12	8.9	Green
R1	0 ppm	#7	9.5	Green
R1	0 ppm	#9	9	Green
R1	0 ppm	#8	9.3	Green
R2	1 ppm	#7	9.6	Green
R2	1 ppm	#9	7.8	Green
R2	1 ppm	#10	9.8	Green
R2	1 ppm	#4	9.1	Green
R2	1 ppm	#8	8.9	Green
R2	1 ppm	#6	7.8	Green
R2	3 ppm	#10	9.8	Green
R2	3 ppm	#9	9.4	Green
R2	3 ppm	#6	9.7	Green
R2	3 ppm	#8	8.8	Green
R2	3 ppm	#5	9.8	Green
R2	3 ppm	#7	9.7	Green
R2	10 ppm	#8	9.8	Green
R2	10 ppm	#6	9.8	Green

Sampling Date	MeHg Exposure	Sample #	RQI Classification	RQI Alert
R2	10 ppm	#5	9.9	Green
R2	10 ppm	#7	9.9	Green
R2	10 ppm	#4	9.9	Green
R2	0 ppm	#11	3.5	Red
R2	0 ppm	#12	9.5	Green
R2	0 ppm	#4	9	Green
R2	0 ppm	#6	10	Green
R2	0 ppm	#5	9.8	Green
R2	0 ppm	#9	9.6	Green
R3	1 ppm	#10	9.6	Green
R3	1 ppm	#11	9.9	Green
R3	1 ppm	#8	9.8	Green
R3	1 ppm	#9	9.8	Green
R3	1 ppm	#4	9.4	Green
R3	1 ppm	#12	8.8	Green
R3	1 ppm	#6	8.2	Green
R3	3 ppm	#7	9.8	Green
R3	3 ppm	#12	9.1	Green
R3	3 ppm	#4	9.7	Green
R3	3 ppm	#6	9.8	Green
R3	3 ppm	#8	8.9	Green
R3	3 ppm	#9	9.6	Green
R3	10 ppm	#6	9.6	Green
R3	10 ppm	#7	9.6	Green
R3	10 ppm	#5	10	Green
R3	10 ppm	#4	9.1	Green
R3	0 ppm	#4	10	Green
R3	0 ppm	#10	9.7	Green
R3	0 ppm	#7	9.9	Green
R3	0 ppm	#6	9.4	Green
R3	0 ppm	#8	9.5	Green
R3	0 ppm	#9	7.8	Green

Supplementary Table 5. RNA sample quality of yellow perch ovaries was confirmed by the Agilent Bioanalyzer. Only samples with an RNA Integrity Number (RIN) of 7.0 or greater were used for downstream analysis.

Sampling Date	Tank #	Fish #	MeHg Exposure	RIN
Week 12	1	1	5.0 ppm	8.3
Week 12	1	2	5.0 ppm	9.7
Week 12	1	3	5.0 ppm	7
Week 12	1	4	5 ppm	8.4
Week 12	2	2	0.5 ppm	9.5
Week 12	2	3	0.5 ppm	9.1
Week 12	2	4	0.5 ppm	9.2
Week 12	3	1	0 ppm	7.5
Week 12	3	2	0 ppm	10
Week 12	3	3	0 ppm	9.4
Week 12	3	4	0 ppm	9.4
Week 12	4	1	0.5 ppm	9.8
Week 12	4	2	0.5 ppm	8.4
Week 12	4	3	0.5 ppm	9.5
Week 12	4	4	0.5 ppm	7.5
Week 12	5	1	0 ppm	8.9
Week 12	5	3	0 ppm	9.6
Week 12	6	2	5.0 ppm	9.4
Week 12	6	3	5.0 ppm	9
Week 12	6	4	5.0 ppm	9
Week 12	7	1	50 ppm	8.9
Week 12	7	2	50 ppm	9.6
Week 12	7	3	50 ppm	8.7
Week 12	7	4	50 ppm	9.4
Week 12	8	2	0.5 ppm	9.5
Week 12	8	4	0.5 ppm	8.9
Week 12	9	1	5.0 ppm	9.3
Week 12	9	2	5.0 ppm	9.7
Week 12	9	3	5.0 ppm	9.5
Week 12	9	4	5 ppm	9.4
Week 12	10	1	50 ppm	8.5
Week 12	10	2	50 ppm	9.4
Week 12	10	3	50 ppm	9.7
Week 12	10	4	50 ppm	9.6
Week 12	11	1	50 ppm	9.4
Week 12	11	3	50 ppm	9.3
Week 12	11	4	50 ppm	9.5

Sampling Date	Tank #	Fish #	MeHg Exposure	RIN
Week 12	12	2	0 ppm	10
Week 12	12	3	0 ppm	9.7
Week 12	12	4	0 ppm	9.1
Week 16	1	2	5.0 ppm	9.7
Week 16	1	3	5.0 ppm	9.2
Week 16	1	4	5.0 ppm	8.2
Week 16	2	1	0.5 ppm	7.3
Week 16	2	2	0.5 ppm	9.6
Week 16	2	3	0.5 ppm	9.8
Week 16	2	4	0.5 ppm	8.6
Week 16	3	2	0 ppm	8.5
Week 16	3	3	0 ppm	9.7
Week 16	3	4	0 ppm	8.3
Week 16	4	1	0.5 ppm	8.5
Week 16	4	2	0.5 ppm	8.9
Week 16	4	3	0.5 ppm	9.1
Week 16	4	4	0.5 ppm	6.5
Week 16	5	1	0 ppm	9.2
Week 16	5	2	0 ppm	8.8
Week 16	5	3	0 ppm	9.5
Week 16	5	4	0 ppm	7.8
Week 16	6	1	5.0 ppm	9.5
Week 16	6	2	5.0 ppm	8.1
Week 16	6	3	5.0 ppm	9.2
Week 16	6	4	5.0 ppm	9.3
Week 16	7	1	50 ppm	8.6
Week 16	7	2	50 ppm	8.9
Week 16	7	3	50 ppm	6.7
Week 16	7	4	50 ppm	9.4
Week 16	8	1	0.5 ppm	9
Week 16	8	2	0.5 ppm	9.3
Week 16	8	3	0.5 ppm	9.6
Week 16	8	4	0.5 ppm	9.4
Week 16	9	1	5.0 ppm	9.3
Week 16	9	4	5.0 ppm	8.4
Week 16	10	2	50 ppm	9.5
Week 16	10	3	50 ppm	7.7
Week 16	10	4	50 ppm	9.2

Sampling Date	Tank #	Fish #	MeHg Exposure	RIN
Week 16	11	1	50 ppm	9.4
Week 16	11	2	50 ppm	8.6
Week 16	11	3	50 ppm	9.8
Week 16	11	4	50 ppm	9.1
Week 16	12	1	0 ppm	8.5
Week 16	12	2	0 ppm	7.9
Week 16	12	3	0 ppm	8.8
Week 20	1	1	5.0 ppm	9
Week 20	1	2	5.0 ppm	9.5
Week 20	1	3	5.0 ppm	9.3
Week 20	1	4	5.0 ppm	3.4
Week 20	2	2	0.5 ppm	9
Week 20	2	3	0.5 ppm	8.9
Week 20	3	2	0 ppm	8.3
Week 20	3	3	0 ppm	5.9
Week 20	3	4	0 ppm	9.3
Week 20	4	2	0.5 ppm	8.7
Week 20	4	4	0.5 ppm	9.4
Week 20	5	1	0 ppm	7.2
Week 20	5	2	0 ppm	8.5
Week 20	5	3	0 ppm	9
Week 20	6	1	5.0 ppm	8.7
Week 20	6	3	5.0 ppm	9
Week 20	6	4	5.0 ppm	9.3
Week 20	7	1	50 ppm	8.6
Week 20	7	2	50 ppm	8.3
Week 20	7	3	50 ppm	7.7
Week 20	8	1	0.5 ppm	9.4
Week 20	8	2	0.5 ppm	9.6
Week 20	8	3	0.5 ppm	9.4
Week 20	8	4	0.5 ppm	9.2
Week 20	9	1	5.0 ppm	9.5

Sampling Date	Tank #	Fish #	MeHg Exposure	RIN
Week 20	9	2	5.0 ppm	9.6
Week 20	9	3	5.0 ppm	9.1
Week 20	9	4	5.0 ppm	7.8
Week 20	10	1	50 ppm	8.9
Week 20	10	2	50 ppm	5.8
Week 20	10	3	50 ppm	9
Week 20	10	4	50 ppm	9.1
Week 20	11	1	50 ppm	3
Week 20	11	2	50 ppm	8.9
Week 20	11	3	50 ppm	8
Week 20	12	1	0 ppm	9.4
Week 20	12	2	0 ppm	9.1
Week 20	12	3	0 ppm	9
Week 20	12	4	0 ppm	9.3

Supplementary Table 6. RNA sample quality of yellow perch livers was confirmed by the Agilent Bioanalyzer. All samples were used for downstream analysis.

Sampling Date	Tank #	Fish #	MeHg Exposure	RIN
Week 12	3	2	0 ppm	7.8
Week 12	5	1	0 ppm	7.1
Week 12	7	1	50 ppm	7.1
Week 12	11	1	50 ppm	7.1
Week 12	5	2	0 ppm	7.7
Week 12	3	1	0 ppm	7.4
Week 12	11	4	50 ppm	7.9
Week 12	12	2	0 ppm	7.7
Week 12	12	1	0 ppm	7.7
Week 12	10	1	50 ppm	7.1
Week 12	7	4	50 ppm	6.6
Week 12	11	2	50 ppm	7
Week 16	5	1	0 ppm	8.9
Week 16	3	3	0 ppm	8.7
Week 16	11	4	50 ppm	8.5
Week 16	10	2	50 ppm	9
Week 16	11	1	50 ppm	8.7
Week 16	12	2	0 ppm	8.8
Week 16	12	1	0 ppm	8.3
Week 16	7	3	50 ppm	7.6
Week 20	12	2	0 ppm	8.1
Week 20	7	1	50 ppm	8.9
Week 20	10	1	50 ppm	9.1
Week 20	3	2	0 ppm	8.9
Week 20	5	2	0 ppm	9
Week 20	10	3	50 ppm	8.6
Week 20	12	3	0 ppm	9.1
Week 20	11	3	50 ppm	9.4

Supplementary Table 7. Significant differentially expressed genes in zebrafish, using RNA-seq values. Gene locus and ZFIN ID are shown for each gene. Samples 1 and 2 indicate which samples are being compared, based on MeHg exposure concentration. Values 1 and 2 are the FPKM values. Fold change is calculated as the quotient of Value 1 and Value 2.

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
1:39291548-39318676	-	ppm0	ppm1	0	20.9087	inf
14:19522724-19525451	-	ppm0	ppm1	11.8415	35.825	1.59711
16:27371530-27374477	-	ppm0	ppm1	18.0363	86.6374	2.26408
19:11893550-11896472	-	ppm0	ppm1	153.805	89.2017	-0.785961
2:51633349-51641977	-	ppm0	ppm1	10.5152	32.9261	1.64675
20:44290995-44292151	-	ppm0	ppm1	541.266	776.363	0.520393
22:25001744-25087130	-	ppm0	ppm1	0	3.32834	inf
24:40430029-40430526	-	ppm0	ppm1	170.345	88.0255	-0.952467
4:1704981-1705789	-	ppm0	ppm1	12.2054	51.0844	2.06536
5:11286549-11351885	-	ppm0	ppm1	10.3498	20.7384	1.0027
5:68530348-68554021	-	ppm0	ppm1	3.86788	0.973721	-1.98996
5:3731752-3732168	-	ppm0	ppm1	0	2.71574	inf
7:44131026-44132707	-	ppm0	ppm1	30.6864	85.4334	1.4772
9:20479270-20483073	-	ppm0	ppm1	8.6383	4.35037	-0.98961
9:58093972-58094925	-	ppm0	ppm1	6.74136	1.6976	-1.98954
Zv9_NA401:226-4681	-	ppm0	ppm1	23.1289	44.4143	0.941329
11:28955518-28956523	-	ppm0	ppm10	0	4.51	inf
14:48455765-48485251	-	ppm0	ppm10	2.74955	8.17993	1.57289
15:14793584-14793907	-	ppm0	ppm10	0	6.63777	inf
17:21713606-21714316	-	ppm0	ppm10	0	14.2646	inf
18:2814161-2934651	-	ppm0	ppm10	0.412568	2.42732	2.55666
18:299862-301548	-	ppm0	ppm10	38.3427	17.634	-1.12059
19:11893550-11896472	-	ppm0	ppm10	153.805	70.6398	-1.12255

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
20:16512083-16512584	-	ppm0	ppm10	0	2.51395	inf
21:26367280-26368453	-	ppm0	ppm10	1.11972	5.37637	2.26349
24:34453725-34454034	-	ppm0	ppm10	4.5231	0	NA
7:50536855-50728473	-	ppm0	ppm10	10.5283	21.5417	1.03286
8:25489225-25496439	-	ppm0	ppm10	0.461815	1.57765	1.77239
9:5289139-5330274	-	ppm0	ppm10	2.87262	9.13837	1.66957
Zv9_NA802:109-785	-	ppm0	ppm10	1445.56	803.063	-0.84804
12:48469981-48487060	-	ppm0	ppm3	4.13051	9.76375	1.24111
14:19522724-19525451	-	ppm0	ppm3	11.8415	40.148	1.76148
16:27371530-27374477	-	ppm0	ppm3	18.0363	258.536	3.84139
19:11893550-11896472	-	ppm0	ppm3	153.805	55.0821	-1.48145
20:44290995-44292151	-	ppm0	ppm3	541.266	836.111	0.627356
24:40430029-40430526	-	ppm0	ppm3	170.345	112.034	-0.604524
3:31744139-31756430	-	ppm0	ppm3	9.82366	15.4602	0.654227
7:44131026-44132707	-	ppm0	ppm3	30.6864	151.75	2.30603
7:44127596-44130896	-	ppm0	ppm3	0.346459	1.98122	2.51563
1:59728504-59756308	-	ppm0	ppm1	1266.89	2061.03	0.702072
1:59728504-59756308	-	ppm0	ppm10	1266.89	1998.71	0.657772
8:17129285-17132876	*gas5*	ppm0	ppm1	89.8508	34.2817	-1.39009
8:17129285-17132876	*gas5*	ppm0	ppm10	89.8508	21.2739	-2.07845
7:39550709-39575433	*abcc12*	ppm0	ppm1	2.74437	5.26552	0.940102
7:39550709-39575433	*abcc12*	ppm0	ppm10	2.74437	5.00867	0.867956
11:30851283-30871811	*ace2*	ppm0	ppm10	1.02454	5.11193	2.3189
12:18167910-18183279	*acta2*	ppm0	ppm10	20.1615	33.615	0.737503
2:42877223-42966373	*adcy8*	ppm0	ppm3	11.6854	3.71931	-1.6516
8:11594770-11626258	*ampd1*	ppm0	ppm1	8.05897	17.0322	1.0796

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
2:15433337-15447555	*amy2a*	ppm0	ppm1	2.10681	8.99166	2.09353
2:15433337-15447555	*amy2a*	ppm0	ppm3	2.10681	7.66492	1.86321
5:27222729-27227874	*anxa1a*	ppm0	ppm1	22.4273	36.4685	0.701396
25:35059902-35074189	*anxa2a*	ppm0	ppm1	41.747	94.1073	1.17263
25:35059902-35074189	*anxa2a*	ppm0	ppm3	41.747	83.7587	1.00457
5:39578957-39581978	*apoa1a*	ppm0	ppm10	0.612874	15.2037	4.63269
15:3556-5136	*apoa1b*	ppm0	ppm1	14.6065	3.09003	-2.24092
16:26200036-26202754	*apoeb*	ppm0	ppm1	59.7976	93.317	0.642052
16:26200036-26202754	*apoeb*	ppm0	ppm10	59.7976	119.97	1.00451
16:26200036-26202754	*apoeb*	ppm0	ppm3	59.7976	122.204	1.03113
25:20099784-20156179	*atp2b1b*	ppm0	ppm1	10.9113	4.37243	-1.31931
25:20099784-20156179	*atp2b1b*	ppm0	ppm10	10.9113	3.28822	-1.73044
25:20099784-20156179	*atp2b1b*	ppm0	ppm3	10.9113	6.01036	-0.860293
15:14409896-14420932	*ca4b*	ppm0	ppm3	1.43052	7.15254	2.32191
Zv9_scaffold3471:41640-46789	*ccbl2*	ppm0	ppm10	1.4688	0	NA
11:6296247-6300316	*ccl25b*	ppm0	ppm3	14.3531	5.6565	-1.34338
16:29024965-29053582	*cdh17*	ppm0	ppm10	0.359523	2.14482	2.5767
25:4211960-4228059	*ckap5*	ppm0	ppm10	1.0275	5.00586	2.28448
17:5308390-5325556	*clic5a*	ppm0	ppm3	1.23347	6.76309	2.45496
4:2089744-2111243	*cmah*	ppm0	ppm1	14.3515	22.7217	0.662871
4:2089744-2111243	*cmah*	ppm0	ppm10	14.3515	24.5682	0.77559
25:18935475-18957985	*cpa5|cpa1*	ppm0	ppm1	4.77205	15.1502	1.66665
25:18935475-18957985	*cpa5|cpa1*	ppm0	ppm10	4.77205	27.8791	2.5465
24:4835108-4845460	*cpb1*	ppm0	ppm1	1.47376	6.25323	2.0851
24:4835108-4845460	*cpb1*	ppm0	ppm10	1.47376	12.4248	3.07565
24:4835108-4845460	*cpb1*	ppm0	ppm3	1.47376	5.53513	1.90911

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
7:36522715-36533247	*ctrb1|zgc:136461*	ppm0	ppm1	3.48663	18.2317	2.38655
7:36522715-36533247	*ctrb1|zgc:136461*	ppm0	ppm10	3.48663	27.1144	2.95915
7:36522715-36533247	*ctrb1|zgc:136461*	ppm0	ppm3	3.48663	17.3674	2.31648
17:32828688-32841875	*ctsba*	ppm0	ppm1	172.493	257.115	0.575878
17:32828688-32841875	*ctsba*	ppm0	ppm3	172.493	272.656	0.660545
20:40753101-40761118	*cx32.2*	ppm0	ppm3	3.78634	12.8026	1.75756
18:38057823-38073637	*cyp19a1a*	ppm0	ppm1	11.2574	21.4957	0.93318
18:38057823-38073637	*cyp19a1a*	ppm0	ppm10	11.2574	20.9393	0.895339
12:32090597-32112707	*dclre1a*	ppm0	ppm1	11.3112	19.4584	0.78264
12:32090597-32112707	*dclre1a*	ppm0	ppm10	11.3112	19.5283	0.78781
12:32090597-32112707	*dclre1a*	ppm0	ppm3	11.3112	18.684	0.724046
12:49633437-49635564	*ddit4*	ppm0	ppm3	9.64948	18.249	0.919293
12:37715591-37723218	*dnai2a*	ppm0	ppm10	0.446278	4.24198	3.24872
12:37715591-37723218	*dnai2a*	ppm0	ppm3	0.446278	4.45043	3.31793
16:11307945-11319501	*ecm1b*	ppm0	ppm10	50.952	73.0836	0.520408
8:21891895-21910503	*ela2l*	ppm0	ppm10	2.80976	17.9388	2.67456
5:27756144-27763404	*ela3l*	ppm0	ppm1	2.19131	11.8901	2.4399
5:27756144-27763404	*ela3l*	ppm0	ppm10	2.19131	24.8294	3.50218
5:27756144-27763404	*ela3l*	ppm0	ppm3	2.19131	12.114	2.46681
12:29065814-29140231	*etv4*	ppm0	ppm10	9.52266	16.1093	0.758455
8:910207-920867	*fabp1b.1|fabp1b.2,fabp1b.1*	ppm0	ppm10	1.65634	18.6274	3.49136
21:28787114-28791414	*fabp6*	ppm0	ppm1	0	12.4079	inf
21:28787114-28791414	*fabp6*	ppm0	ppm10	0	18.997	inf
9:15638531-15700871	*fn1a*	ppm0	ppm3	4.76134	8.11877	0.769893
16:47879729-47908744	*gapdhs*	ppm0	ppm3	15.8993	31.8861	1.00396
7:72890102-72896535	*gba3*	ppm0	ppm1	33.2518	16.8874	-0.977486

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
7:72890102-72896535	*gba3*	ppm0	ppm10	33.2518	17.0454	-0.964052
5:25426867-25474322	*gbgt1l4|si:ch211-287c22.1*	ppm0	ppm1	346.454	147.878	-1.22826
5:25426867-25474322	*gbgt1l4|si:ch211-287c22.1*	ppm0	ppm3	346.454	228.97	-0.597507
2:19460033-19463977	*glula*	ppm0	ppm10	19.7374	30.6128	0.633205
20:47081824-47196730	*gpatch2*	ppm0	ppm1	10.6033	6.94863	-0.609719
14:29565306-29583093	*hmmr*	ppm0	ppm1	67.9956	41.9071	-0.698246
20:54322500-54336769	*hsp90aa1.2*	ppm0	ppm3	2.09886	8.98766	2.09834
21:19211801-19212563	*hspb11*	ppm0	ppm1	2.75011	11.0601	2.0078
21:19211801-19212563	*hspb11*	ppm0	ppm3	2.75011	12.5856	2.19422
5:38701555-38717941	*il13ra2*	ppm0	ppm1	70.2195	132.991	0.92139
1:25414013-25419172	*ints12*	ppm0	ppm1	8.40851	3.68051	-1.19195
9:26361937-26379928	*itm2bb*	ppm0	ppm10	34.7899	51.7793	0.573707
19:17847487-17853240	*klf2b*	ppm0	ppm1	1.12127	3.094	1.46434
19:17847487-17853240	*klf2b*	ppm0	ppm3	1.12127	4.93561	2.13809
23:10454076-10457982	*krt18*	ppm0	ppm3	35.8454	88.2443	1.29971
6:39239361-39243891	*krt4*	ppm0	ppm3	6.58425	15.4155	1.22729
23:10355853-10360516	*krt8*	ppm0	ppm1	60.7906	92.9704	0.612923
23:10355853-10360516	*krt8*	ppm0	ppm3	60.7906	126.126	1.05294
13:36441274-36451926	*lgmn*	ppm0	ppm10	82.1025	112.329	0.452229
18:37609476-37637846	*mapk6|si:ch211-235f12.2*	ppm0	ppm10	5.37789	10.5038	0.965796
21:13837531-13877870	*mmp11b*	ppm0	ppm10	5.82615	10.5149	0.851816
15:29731016-29735742	*mrps23*	ppm0	ppm1	78.251	111.702	0.513475
5:38450812-38454978	*nccrp1*	ppm0	ppm1	49.6324	73.0308	0.557222
16:33815911-33821400	*ndrg1b*	ppm0	ppm1	10.7748	23.4745	1.12343
5:53469466-53472796	*otpb*	ppm0	ppm1	6.47455	2.17496	-1.57379
5:53469466-53472796	*otpb*	ppm0	ppm3	6.47455	1.67174	-1.95343

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
5:53694116-53708973	*papd4*	ppm0	ppm1	29.3761	13.9034	-1.07921
5:53911219-53925762	*papd4*	ppm0	ppm1	11.1309	22.9838	1.04604
5:53694116-53708973	*papd4*	ppm0	ppm10	29.3761	11.1587	-1.39648
5:53911219-53925762	*papd4*	ppm0	ppm10	11.1309	21.9182	0.977552
5:53694116-53708973	*papd4*	ppm0	ppm3	29.3761	16.0311	-0.873773
1:9030572-9040849	*pdia2*	ppm0	ppm1	29.74	43.0105	0.532282
7:7457930-7473128	*pnp5a*	ppm0	ppm10	3.51654	7.12645	1.01903
5:59563352-59576469	*prpf4*	ppm0	ppm10	4.02894	9.42654	1.22633
7:25543156-25557970	*ptgr1*	ppm0	ppm1	41.5242	60.1628	0.534919
9:57999657-58048041	*rab3gap1*	ppm0	ppm1	4.49693	1.16472	-1.94896
15:5697321-5708818	*rbp2a*	ppm0	ppm10	2.68056	27.5024	3.35895
16:44302075-44305173	*rpp25l*	ppm0	ppm1	57.7374	32.5258	-0.827923
16:44302075-44305173	*rpp25l*	ppm0	ppm3	57.7374	39.5034	-0.547527
23:31665071-31675103	*rps12*	ppm0	ppm3	422.081	601.402	0.510808
7:59990937-59995522	*rps20*	ppm0	ppm3	438.321	615.549	0.489883
12:48443853-48466573	*ryr2a*	ppm0	ppm3	4.38952	10.3562	1.23836
16:24862111-24863680	*s100t*	ppm0	ppm10	5.18629	16.9433	1.70794
7:58352182-58369985	*scamp2l*	ppm0	ppm10	17.2977	10.6758	-0.696245
9:17806411-17850876	*scel*	ppm0	ppm1	4.52001	14.5589	1.6875
9:17806411-17850876	*scel*	ppm0	ppm10	4.52001	11.409	1.33578
8:32283493-32291376	*sepp1a*	ppm0	ppm10	58.6027	81.1009	0.468753
9:8422554-8427723	*si:ch1073-75o15.4*	ppm0	ppm1	286.3	437.623	0.612161
9:8422554-8427723	*si:ch1073-75o15.4*	ppm0	ppm10	286.3	415.652	0.537851
5:26238786-26381394	*si:ch211-106a19.1*	ppm0	ppm1	9.15546	5.52999	-0.727354
25:37405980-37406927	*si:ch211-113a14.28,zgc:173587,zgc:171759,zgc:112234*	ppm0	ppm10	0	1.61627	inf

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
9:13161977-13164389	*si:ch211-167j6.3*	ppm0	ppm1	57.6553	101.994	0.822962
19:32141803-32208780	*si:ch211-194e15.5*	ppm0	ppm1	0.665553	2.5912	1.961
4:7210119-7210735	*si:ch211-240l19.8*	ppm0	ppm10	6.03229	40.8501	2.75956
4:7210119-7210735	*si:ch211-240l19.8*	ppm0	ppm3	6.03229	28.793	2.25494
19:6353698-6392621	*si:ch211-264f5.6*	ppm0	ppm3	1.6521	4.61184	1.48104
1:50031468-50064622	*si:ch211-281g13.5*|*zgc:163136*|*si:ch211-281g13.4*	ppm0	ppm1	6.58636	11.5982	0.816352
3:55950129-55957631	*si:ch211-5k11.8,si:xx-by187g17.1,hbaa1*	ppm0	ppm3	14.9071	35.0363	1.23284
16:28257798-28259131	*si:ch73-103b2.3*	ppm0	ppm1	4.81348	22.7847	2.24292
16:28257798-28259131	*si:ch73-103b2.3*	ppm0	ppm10	4.81348	44.7352	3.21626
16:28257798-28259131	*si:ch73-103b2.3*	ppm0	ppm3	4.81348	23.5963	2.29341
24:40705325-40731067	*si:ch73-171a6.3*	ppm0	ppm1	8.10065	4.54041	-0.835216
24:40705325-40731067	*si:ch73-171a6.3*	ppm0	ppm3	8.10065	4.24518	-0.932211
19:25238195-25243855	*si:dkey-154b15.1*|-	ppm0	ppm1	38.1888	15.8167	-1.2717
19:25238195-25243855	*si:dkey-154b15.1*|-	ppm0	ppm10	38.1888	16.4271	-1.21707
19:25238195-25243855	*si:dkey-154b15.1*|-	ppm0	ppm3	38.1888	14.3291	-1.4142
9:23690315-23706171	*si:dkey-189g17.2*	ppm0	ppm1	29.6769	48.6342	0.712632
18:7047760-7050665	*si:dkey-238c7.13*	ppm0	ppm10	20.8513	80.4202	1.94742
21:19729177-19760478	*si:dkey-246j23.5,c7b*	ppm0	ppm3	17.838	28.7455	0.688383
5:24189933-24208758	*si:dkey-27p18.3*	ppm0	ppm1	3.59578	9.19594	1.35469
22:25480410-25481227	*si:dkey-4c23.8*	ppm0	ppm3	237.672	391.921	0.721591
2:35202988-35218374	*si:dkey-4i23.7*	ppm0	ppm10	8.55807	1.69671	-2.33455
12:19494030-19545787	*si:dkey-7e14.7*	ppm0	ppm3	32.3846	16.7482	-0.951301
8:31348617-31443914	*si:dkey-91m11.5*	ppm0	ppm10	3.56177	6.30919	0.824861
16:24475466-24492851	*si:dkey-92i15.4*	ppm0	ppm1	2.71361	6.94542	1.35585

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
21:6117510-6120175	*si:dkey-93m18.3*	ppm0	ppm1	7.45548	22.8842	1.61798
18:7032703-7035514	*si:dkeyp-1h4.9*	ppm0	ppm10	22.046	36.1598	0.713875
22:25613373-25614100	*si:dkeyp-20e4.8*	ppm0	ppm3	430.873	769.853	0.837318
25:27621528-27624918	*si:dkeyp-73b11.8*	ppm0	ppm10	0	4.95218	inf
16:26983256-27019601	*si:dkeyp-84f3.3*	ppm0	ppm1	1.66253	0	NA
11:21827227-21847773	*slc6a14*	ppm0	ppm1	1.00983	4.84998	2.26386
11:21827227-21847773	*slc6a14*	ppm0	ppm3	1.00983	6.99309	2.79181
16:20931669-20956155	*slc6a19b*	ppm0	ppm1	4.56813	1.18433	-1.94754
16:20931669-20956155	*slc6a19b*	ppm0	ppm3	4.56813	1.29153	-1.82252
7:72195885-72211300	*spata6l*	ppm0	ppm10	31.8672	12.4705	-1.35355
16:29963110-29977411	*steap4*	ppm0	ppm3	3.55352	1.50566	-1.23885
6:48993275-49002798	*sycp1*	ppm0	ppm10	11.652	5.50716	-1.08119
5:32256931-32259642	*tcnl*	ppm0	ppm10	0	2.22325	inf
2:16581395-16592930	*tfa*	ppm0	ppm10	25.6849	44.4063	0.789845
24:26813695-26827806	*tfr1b*	ppm0	ppm10	6.41646	11.2609	0.811478
24:26813695-26827806	*tfr1b*	ppm0	ppm3	6.41646	13.4827	1.07126
16:33834934-33902920	*tg*	ppm0	ppm1	1.78148	4.13852	1.21604
Zv9_scaffold3522:24530-41501	*tgm5l*	ppm0	ppm1	1.30692	5.47301	2.06616
Zv9_scaffold3522:24530-41501	*tgm5l*	ppm0	ppm3	1.30692	9.75318	2.8997
5:27297049-27313337	*tmc1*	ppm0	ppm1	9.20626	3.37784	-1.44652
9:56198177-56199762	*tmsb4x*	ppm0	ppm10	287.995	401.501	0.479362
19:27505096-27509684	*tnfa*	ppm0	ppm3	0	3.1387	inf
16:27320044-27344563	*try*	ppm0	ppm10	6.98123	46.2643	2.72834
16:11935768-11944511	*tubb5*	ppm0	ppm1	15.7242	9.08237	-0.791848
16:11935768-11944511	*tubb5*	ppm0	ppm10	15.7242	8.27536	-0.926094

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
16:11935768-11944511	*tubb5*	ppm0	ppm3	15.7242	9.7462	-0.690077
16:24543993-24565999	*tuft1a*	ppm0	ppm1	0.682506	2.1632	1.66425
16:24543993-24565999	*tuft1a*	ppm0	ppm3	0.682506	2.51256	1.88024
17:6121993-6148052	*txlnbb*	ppm0	ppm10	77.8255	44.3282	-0.812017
3:61284748-61316486	*ubald2*	ppm0	ppm1	12.9191	8.40459	-0.620253
5:72956617-72980648	*ugt2a4,ugt2a1*	ppm0	ppm1	8.41138	15.7481	0.904764
5:72956617-72980648	*ugt2a4,ugt2a1*	ppm0	ppm10	8.41138	15.4683	0.878895
5:72956617-72980648	*ugt2a4,ugt2a1*	ppm0	ppm3	8.41138	15.6411	0.894925
3:25648468-25747094	*usp43b*	ppm0	ppm1	7.1258	19.8076	1.47493
9:45977992-46020818	*vil1*	ppm0	ppm10	0.355301	2.47282	2.79905
7:10089246-10103115	*vimp*	ppm0	ppm1	28.4507	18.9589	-0.58559
22:25342159-25353294	*vtg2*	ppm0	ppm1	105.432	56.8942	-0.889959
22:25342159-25353294	*vtg2*	ppm0	ppm3	105.432	57.809	-0.866946
11:7505708-7526742	*vtg3*	ppm0	ppm3	19.9022	10.391	-0.937594
18:44007257-44031137	*wt1b*	ppm0	ppm3	2.83982	7.22772	1.34774
19:44161937-44176092	*zgc:103438|si:dkey-228a15.1*	ppm0	ppm1	4.71702	15.4779	1.71426
19:44161937-44176092	*zgc:103438|si:dkey-228a15.1*	ppm0	ppm3	4.71702	13.5277	1.51997
7:36516808-36521137	*zgc:112160*	ppm0	ppm10	0.634284	5.90263	3.21816
17:50535158-50579487	*zgc:113886*	ppm0	ppm10	5.59129	483.671	6.4347
15:34230671-34240322	*zgc:113969,zgc:66024|si:ch73-95l15.3*	ppm0	ppm1	331.58	582.749	0.813518
15:34230671-34240322	*zgc:113969,zgc:66024|si:ch73-95l15.3*	ppm0	ppm10	331.58	528.777	0.673302
9:8449880-8453901	*zgc:153499*	ppm0	ppm1	1213.69	1845.73	0.604791
11:11589957-11592854	*zgc:153629*	ppm0	ppm10	22.8125	35.3994	0.633903
11:11589957-11592854	*zgc:153629*	ppm0	ppm3	22.8125	37.9191	0.733102
8:18969451-19019038	*zgc:153738*	ppm0	ppm10	34.5692	19.7493	-0.807686

Locus	ZFIN ID	Sample 1	Sample 2	Value 1	Value 2	Log_2(fold_change)
6:39267594-39275123	*zgc:158846*	ppm0	ppm10	0.451187	3.57877	2.98766
13:33894410-33896169	*zgc:163030*	ppm0	ppm1	163.312	111.4	-0.551884
11:16403709-16406187	*zgc:173544*	ppm0	ppm1	98.2194	182.843	0.896527
5:35558112-35577766	*zgc:63972*	ppm0	ppm1	12.4865	4.59214	-1.44313
17:53940051-53946421	*zgc:65772*	ppm0	ppm10	28.6701	16.2059	-0.823027
16:28253005-28254288	*zgc:66382*	ppm0	ppm1	5.79185	32.4519	2.48621
16:28253005-28254288	*zgc:66382*	ppm0	ppm10	5.79185	49.0602	3.08246
16:28253005-28254288	*zgc:66382*	ppm0	ppm3	5.79185	34.3345	2.56756
18:7029381-7031332	*zgc:77650*	ppm0	ppm10	189.199	278.339	0.556936
18:7029381-7031332	*zgc:77650*	ppm0	ppm3	189.199	273.173	0.529907
22:7477197-7482391	*zgc:92041*	ppm0	ppm1	5.86378	20.9654	1.83811
22:7477197-7482391	*zgc:92041*	ppm0	ppm10	5.86378	37.5223	2.67785
22:7477197-7482391	*zgc:92041*	ppm0	ppm3	5.86378	24.6277	2.07038
16:27366215-27369356	*zgc:92590*	ppm0	ppm10	1.17977	7.80121	2.7252