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Running Head: Effects of dietary MeHg on female yellow perch and zebrafish

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

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ABSTRACT

The purpose of this study was to evaluate the effects of environmentally relevant dietary MeHg exposures on adult female yellow perch (*Perca flavescens*) and female zebrafish (*Danio rerio*) ovarian development and reproduction. Yellow perch were used in the study for their socioeconomic and ecological importance within the Great Lakes basin, and the use of zebrafish allowed for a detailed analysis of the molecular effects of MeHg following a whole life-cycle exposure. Chronic whole life dietary exposure of F_1 zebrafish to MeHg mimics realistic wildlife exposure scenarios, and the twenty-week adult yellow perch exposure (where whole life-cycle exposures are difficult) captures early seasonal ovarian development. For both species, target dietary accumulation values were achieved prior to analyses. In zebrafish, several genes involved in reproductive processes were shown to be dysregulated by RNA-sequencing and quantitative real-time polymerase chain reaction (QPCR), but no significant phenotypic changes were observed regarding ovarian staging, fecundity, or embryo mortality. Yellow perch were exposed to dietary MeHg for 12, 16, or 20 weeks. In this species, a set of eight genes were assessed by QPCR in the pituitary, liver, and ovary, and no exposure-related changes were observed. The lack of genomic resources in yellow perch hinders the characterization of subtle molecular impacts. The ovarian somatic index, circulating estradiol and testosterone, and ovarian staging were not significantly altered by MeHg exposure in yellow perch. These results suggest that environmentally relevant MeHg exposures do not drastically reduce the reproductively important endpoints in these fish, but to capture realistic exposure scenarios, whole life-cycle yellow perch exposures are needed.

Key Words: zebrafish, yellow perch, methylmercury, reproductive toxicity, transcriptomics, aquatic toxicology

1. Introduction

Methylmercury (MeHg) is a pervasive contaminant in aquatic ecosystems, particularly within the Great Lakes region, where historic industrial practices have impacted the waterways [1]. MeHg bioaccumulates and biomagnifies through trophic levels, reaching potentially harmful concentrations [1,2]. Although MeHg has been identified as an endocrine disrupting chemical in both male and female fish, knowledge is currently limited on population-level reproductive effects of chronic sub-lethal exposure of fish to MeHg [3–5]. Dietary exposures of MeHg at higher-end environmentally relevant concentrations have been shown to reduce the ovarian somatic index (OSI; *i.e.*, the percentage of total body weight contributed by the gonads) in female fathead minnows (*Pimephales promelas*), which may indicate hindrance of egg development [6,7]. Moreover, MeHg exposure has also been shown to decrease vitellogenin gene expression in the liver concurrent with reduced levels of circulating E2 [3,4,8]

Currently, little is understood about the effects of chronic Hg pollution on female reproduction in native omnivorous fish species such as the yellow perch. Yellow perch (*Perca flavescens*) are a socially and economically important native fish species to the Great Lakes region and serve as an important intermediate in the trophic transfer of MeHg; total Hg burdens of yellow perch found in the Great Lakes are correlated with piscivorous fish species inhabiting the same waters [9]. In the Great Lakes region, fish are most likely to experience low-level chronic exposure throughout their lifespan [1]. To address the effects of low-level chronic MeHg exposure throughout the lifespan, the zebrafish (*Danio rerio*) was adopted as a model.

Mora-Zamorano *et al.* [10,11] have reported commonalities in altered neurobehavior in larval zebrafish and yellow perch exposed to MeHg. Moreover, common biomarkers of MeHg-induced neurotoxicity in zebrafish and yellow perch larvae have been identified [12]. These biomarkers include a circadian rhythm gene (*per3*) and a gene involved in the astrocytic glutamate uptake pathway (*slcla2a*) [12]. As the zebrafish in Mora-Zamorano *et al.* [11,12] were the offspring of the fish used in this study, the impacts of maternal dietary MeHg provided the basis to investigate deeper into the effects of MeHg on reproductively-important endpoints.

The evidence provided in Mora-Zamorano *et al.* [10–12] supports the possibility of analogous MeHg-mediated reproductive effects between zebrafish and yellow perch. Zebrafish have the advantage of a short generation time and become sexually mature at three to six months of age [13]. Consequently, a whole-life cycle exposure, from parental exposure through adulthood, is feasible in zebrafish thereby mimicking a realistic environmental exposure scenario. Zebrafish also have a sequenced genome, allowing for high-throughput transcriptomic analyses such as RNA-sequencing (RNA-seq) to discover candidate biomarkers of MeHg exposure. In contrast, female yellow perch do not reach sexual maturity until three to four years of age [14], and the genome is not completely characterized [15]. Collectively, these factors considerably limit experimental design capabilities (*e.g.*, whole-life exposure paradigm, RNA-seq, bioinformatics, *etc.*) with yellow perch.

The objectives of this study were to: (1) evaluate the effects of dietary MeHg exposure on female reproduction in yellow perch and zebrafish; (2) phenotypically-anchor possible gene dysregulation in several organs (*i.e.*, pituitary, liver, and ovary) of

109 the HPLG axis in zebrafish and yellow perch [7,16] and; (3) explore the efficacy of the
110 zebrafish as a model for the yellow perch. Zebrafish were subjected to whole-life cycle
111 dietary MeHg exposure, thereby simulating realistic exposure conditions in the Great
112 Lakes. Yellow perch were exposed to dietary MeHg for 12, 16, or 20 weeks,
113 encompassing a period of seasonal ovarian development. A diverse experimental
114 approach (*e.g.*, ovarian staging, RNA-seq, and hormone analyses) was employed to
115 thoroughly investigate the potentially overt reproductive impairment resulting from
116 dietary MeHg exposure in fish. Taken together, these data were used to evaluate the
117 effects of MeHg on female fish reproduction and attempt to establish relevant
118 biomarkers relating exposure and subsequent reproductive effects.
119
120

Materials and Methods

All animal procedures were approved by the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee. Materials and methods are summarized here; more information is provided as Supplemental Material. Experimental details of the quantitative real-time polymerase chain reaction (QPCR) studies focused on zebrafish ovary and yellow perch ovary and liver are summarized according to Minimum Information for Publication of QPCR Experiments (MIQE) guidelines in Supplemental Tables 1 and 2. Unless otherwise noted, all statistical analyses were performed using SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA). A p-value <0.05 was considered statistically significant in all analyses unless otherwise noted.

2.1 Zebrafish

MeHg exposure

Adult EK zebrafish were reared using standard husbandry conditions (flow-through dechlorinated municipal water at 26-28°C with a 14 h light: 10 h dark cycle) at the National Institute for Environmental Health Sciences Children's Core Center at the University of Wisconsin-Milwaukee. Upon commencement of MeHg exposures, zebrafish were fed under static conditions to prevent Hg waste from entering the wastewater. Tanks were siphoned each afternoon and filters were placed in the outflows of the tanks to capture any remaining Hg-contaminated waste.

To simulate a whole life-cycle exposure scenario, adult females (F_0) were exposed to dietary MeHg (nominal concentrations of 0.0, 0.5, 5.0, and 50.0 ppm; Supplemental Table 3), providing a realistic route of maternal transfer of MeHg to embryos (F_1) [11]. No further analyses were conducted with the F_0 zebrafish, therefore

any further mention of zebrafish will be regarding the F_1 zebrafish. F_1 zebrafish larvae were given micro-encapsulated food (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 (nominal concentrations of 0.0, 1.0, 3.0, or 10.0 ppm; Supplemental Table 3). When normalized to caloric density, these nominal concentrations reflect environmentally realistic exposures occurring in aquatic ecosystems with MeHg-contaminated food webs [7]. The Hg-contaminated diet was supplemented with feedings of *Artemia* nauplii (platinum grade Argentemia, Argent Laboratories, Redmond, WA), which occurred as a separate daily feeding event to ensure proper development of fish, as flake food alone does not provide a sufficient diet. This feeding regime was maintained daily for approximately seven months. Males and females were separated upon development of visible sexual characteristics such as brighter coloration in males and protruding stomachs in females.

Egg production and embryo mortality

F_1 females were spawned with F_1 males from a tank of the same exposure group; to establish cyclicity, spawning occurred weekly prior to a final spawning. The number of eggs produced from all females in each tank was counted at the final spawning point, and the number of eggs per female was estimated by dividing the total number of eggs in each tank by the number of females in that tank. Embryo mortality at 24 hours post-fertilization (hpf) was calculated as a percent of dead embryos relative to the number of eggs laid. Egg production and mortality were both analyzed using a one-way analysis of variance (ANOVA).

Tissue collection

Approximately 24 hours post-spawning, nine of twelve F_1 females in each tank were euthanized, using an overdose of neutral-buffered MS-222 (Western Chemical, Inc., Ferndale, WA) followed by transection of the cervical spinal cord, and the ovaries were collected. Of the nine ovaries, three were used for total Hg analysis and six were used for RNA isolation. Ovaries were dissected from F_1 fish immediately after euthanasia and inserted individually into 1.7 ml microcentrifuge tubes (MidSci, St. Louis, MO). Ovaries used for RNA isolation were placed in tubes containing 200 µL RNA*later* (Qiagen, Hilden, Germany). All samples were flash frozen in liquid nitrogen and placed on dry ice until long-term storage at -80°C. The three remaining zebrafish were euthanized six days post-spawning and collected for histological analysis.

Mercury analysis

Total Hg content was determined by atomic absorption spectrophotometry using a DMA-80 (Milestone, Inc., Shelton, CT) based on the methods of Nam and Basu [24]. All concentrations are reported as ppm (mg/kg) wet-weight (w.w.). Total Hg burdens were measured in 200 embryos per F_0 spawning group (n = 3 clutches per exposure) approximately every two weeks. Log-transformed total Hg values, relative to parental exposure, were analyzed using a one-way ANOVA and Holm-Sidak post-hoc test. Total Hg burden was also measured in nine ovaries from each exposure group of F_1 zebrafish, and log-transformed ovarian Hg values were analyzed using a non-parametric Kruskal-Wallis one-way ANOVA on ranks and Dunn's post-hoc test.

Histology analysis

Zebrafish were euthanized as described above and an incision was made along the ventral surface. Whole fish were preserved individually in cassettes submerged in

10% neutral buffered formalin. 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 using a NanoZoomer Digital Pathology system (Hamamatsu Photonics K.K., Hamamatsu City, Shizuoka, Japan) at the Medical College of Wisconsin in the Histology Core (Milwaukee, WI) [17]. Two sections from five different females were analyzed (blinded) from both 0.0 ppm and 10.0 ppm MeHg exposure groups. These two exposure groups were chosen to assess any overt changes in ovarian staging; had differences been discovered, the other exposure groups would have been analyzed. Area and perimeter of the ovary were measured for each section, and follicles were classified as pre-vitellogenic, mid-vitellogenic, and late-vitellogenic [26]. To normalize variation in ovary size, individual follicle types were calculated as a proportion of total follicles in an ovary. Statistical analyses were conducted via unpaired t-test.

RNA isolation

RNA was isolated from individual ovaries (n = up to 6 samples per tank on each sampling date, accounting for slight mortality in the highest exposure group) using the Direct-zol™ RNA MiniPrep kit (Zymo Research, Irving, CA) per manufacturer's instructions. See Supplemental Methods for details regarding RNA quality control (data are presented in Supplemental Table 4). To reduce biological variation, equal amounts of RNA were pooled from zebrafish ovaries collected from each tank of 0.0, 1.0, and 3.0, and 10.0 ppm exposure groups (n = 3, replicates at level of tank). From each tank, 2.0 µg of pooled RNA 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).

RNA-seq bioinformatics

Briefly, adapters and low quality bases were removed from the initial 2x101bp Illumina TruSeq and trimmed using Cutadapt [18]. FastQC was used to ensure that cleaned reads were of higher quality than initial raw reads supplied by the sequencer. The cleaned reads for each sample were independently aligned to the reference zebrafish genome (Zv9, UCSC) using TopHat (v. 2.0.11) [19,20]. 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. Alignment data was confirmed against the Zv9 reference transcriptome using RNAseQC [21], and an ovary-specific transcriptome was assembled using Cufflinks [19], with the Zv9 transcriptome as a reference to correct fragment biases [22]. Each sample transcriptome was then merged into a single ovary transcriptome using Cuffmerge. Differential expression analysis was conducted with Cuffdiff using pooled dispersion, geometric normalization, and the merged ovary transcriptome. TopHat alignments were grouped using MeHg exposure levels. RNAseq data have been deposited in the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/). The data is accessible through GEO Series accession number GSE73615.

Prior to gene set enrichment analysis (GSEA), gene sets of interest were selected based on relevance to reproductive function. Zebrafish gene symbols were converted to human gene symbols using the zebrafish-human ortholog file from ZFIN. GSEA was done using the gage function from gage R package [23], and the non-

parametric Kolmogorov-Smirnov test was used on unpaired samples to calculate the statistics and p value for each gene set. A default cut-off q-value (0.05) was used to determine the gene sets that were significantly enriched in exposed fish compared to control fish.

Identification of QPCR biomarkers

QPCR was used to identify putative biomarkers that could be analyzed in yellow perch. Target genes were selected based on the zebrafish RNA-seq results, relevance to fish reproduction, and availability of annotation in yellow perch. Normalizer genes were selected based on previous literature [24] and stability of expression upon MeHg-exposure compared to control according to the RNA-seq data. QPCR was performed using a StepOne Plus™ system (Applied Biosystems, Foster City, CA). 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. A normalized quantity mean (NQM) was determined by the StepOnePlus™ software (version 2.2.2), accounting for primer efficiencies and normalizer genes [25]. A normalized relative quantity (NRQ) was calculated using the individual sample with the lowest expression level, based on the smallest NQM value, as the calibrator (NRQ=1) [26]. 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: $Cq' = log_2(NRQ)$ [27]. One-way ANOVA and Holm-Sidak post-hoc test were used to determine the 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 test followed by a Dunn's multiple comparison test was used. Fold-change of expression was calculated via the average NRQ value of each exposure group divided by the average NRQ of the control group. This calculated a fold-up value; when applicable, the inverse of this value was taken to present the fold-down value.

2.2 Yellow perch

MeHg exposure of yellow perch

Adult females were exposed to dietary MeHg (0.0, 0.5, 5.0, or 50.0 ppm, 0.35% body weight per day; Sigma-Aldrich Co., St. Louis, MO, USA) via contaminated Biodiet starter (Bio-Oregon), using ethanol as the vehicle. All fish (three tanks of 12 fish per exposure group) were fed under static conditions and tanks were siphoned daily to remove any Hg-contaminated waste. To simulate conditions in which gonadal maturation and vitellogenesis would occur in the fall in Cambridge, MD, USA (38°34'N, 76°05'W; the location whereby this line of fish originated) the temperature was maintained at 20°C for 12 weeks and then gradually decreased until reaching 11°C in the final three weeks [28]. An AstroDial Suntracker™ (Paragon® Electrical Products, Albuquerque, NM) was used to mimic the natural changing of the photoperiod. The initiation point of this experiment occurred eight months prior to the natural spawning season of yellow perch, allowing for progression of ovarian development within those 12 weeks.

Measurements and tissue collection

Four females per tank were euthanized (via overdose of neutral-buffered MS-222) at 12, 16, and 20 weeks to capture different time points in ovarian development. Whole fish weight, ovary weight, and fish length were measured. Ovarian Somatic Index (OSI) was calculated using the following equation: $OSI = \frac{WO}{WT} \times 100$ (WO = ovary weight and WT= total fish weight). OSI was analyzed via two-way ANOVA (factors = exposure and sampling date) and Holm-Sidak post-hoc test. Ovaries, livers, and pituitaries were collected to assess the impact of MeHg exposure on gene expression and approximately 1.0 g of muscle tissue was collected for total Hg analysis. All tissues were flash frozen upon dissection and stored at -80°C. Approximately 0.5 ml of blood was also collected from the caudal vein of each fish for estradiol analysis.

Mercury analysis

Total Hg content in yellow perch muscle and food was determined as described above. Log-transformed total Hg values (ppm w.w.) for exposures within each sampling date were analyzed using a one-way ANOVA and Holm-Sidak post-hoc test.

Histology analysis

Approximately 2.0 g of ovary tissue was placed in cassettes, submerged in Altmann's Fixative for a week, rinsed three times in cold tap water, and stored in 70% ethanol for an additional week. The tissues were then embedded in paraffin and stained with hematoxylin and eosin, and the slides were scanned as described above. As with zebrafish, only the control and highest exposure (50.0 ppm group) were analyzed for ovarian development (see Supplemental Methods for more information). Differences among group means were assessed (separately for previtellogenic and vitellogenic data

sets) using two-way ANOVA (factors= exposure and time) and Fishers honest significant difference post-hoc test (Minitab) [29].

Estradiol and Testosterone analysis

Circulating estradiol and testosterone was measured using radioimmunoassay using procedures of the U.S. EPA (EPA/600/R-01/067). Estradiol and testosterone levels were analyzed with two-way ANOVA (factors = exposure and sampling date) and Holm-Sidak post-hoc test.

RNA isolation and QPCR for ovary and liver

RNA was isolated from six individual ovaries per exposure group per sampling date using the Direct-zol™ RNA MiniPrep kit (Zymo Research) per the manufacturer's instructions. 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 exposure (control and 50.0 ppm exposure groups) were used per sampling date. If a significant difference had been observed from this experiment, liver samples from each of the other exposure groups would have been analyzed. Ovarian and liver RNA samples were treated with RQ1 RNase-Free DNase (per the manufacturer's instructions; Promega, Madison, WI) prior to QPCR. See Supplemental Methods for details regarding RNA quality control (data for ovary and liver are presented in Supplemental Tables 5 and 6, respectively).

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. cDNA was synthesized as described for zebrafish, with ovary reactions diluted to a final concentration of 8.0 ng/µL and liver reactions diluted to 1.0 ng/µL. 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. Relative quantification of gene expression was measured relative to the three normalizer genes, which showed stable expression across individuals. Data analysis was completed using the StepOnePlus™ software, as described above. To determine any effects of MeHg exposure on the expression of each target gene, a two-way ANOVA (factors = exposure and sampling date) and Holm-Sidak post-hoc test was used. Fold change was calculated as described above.

RNA isolation and QPCR for pituitary

RNA was extracted from yellow perch pituitaries according to Goetz *et al.* [30]. RNA concentration was measured using a NanoDrop ND1000 spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE). cDNA was synthesized from approximately 500 ng RNA using the ImProm-II RT system (Promega, Madison, WI) according to manufacturer's instructions. Genes for QPCR were selected based on their importance to fish reproduction. Both *follicle stimulating hormone, beta polypeptide* (*fshb*) and *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 sequences [30]. To ensure a single band of the correct size, PCR products from the amplification of each primer set were run on an agarose gel. QPCR was used for the analysis of *fshb* or *lhb* transcript levels in twelve fish per exposure level per sampling date. Raw data were processed with Real-Time PCR Miner

348 [31]. The relative messenger RNA concentration (R_0) was calculated for each gene per
349 individual sample using *beta actin* (*actin*) as the normalizer gene [10]. Results were
350 analyzed via two-way ANOVA (factors = exposure and sampling date) and Holm-Sidak
351 post-hoc test.

352 **3. Results**

353 **3.1 Zebrafish**

354 *Mercury accumulation and phenotypic endpoints in F_1 zebrafish*

355 Dosimetric results revealed that the tissue concentrations yielded from all
356 pairwise comparisons of Hg exposure levels were significantly different among
357 exposure groups ($p < 0.001$) by week 9; these embryos were reared to adulthood
358 (Supplemental Fig. 1). Whole-life cycle dietary exposure to MeHg in these fish yielded a
359 concentration-dependent accumulation of Hg in the ovary, accumulating 0, 0.8, 2.1, and
360 6.5 ppm Hg, respectively, based on exposures (Supplemental Table 7).

361 Neither the number of offspring nor embryo mortality in F_1 zebrafish was
362 significantly affected by whole-life cycle exposure to MeHg at the concentrations tested
363 ($p > 0.05$; Supplemental Table 7). Ovarian follicle development was also minimally
364 impacted by MeHg exposure (Fig. 1A-C); however, controls did have a greater
365 proportion of pre-vitellogenic follicles, whereas the 10 ppm-exposure group had a
366 greater proportion of mid- and late-vitellogenic follicles. Only the proportion of mid-
367 vitellogenic follicles was statistically different between groups ($p > 0.05$).

368 *RNA-seq*

369 RNA-seq revealed a total of 117 independent genes that were significantly
370 dysregulated in zebrafish ovary exposed to MeHg (false discovery rate [FDR] < 0.05;

fold-change ≥ 2; Fig. 2). A list of all dysregulated genes is presented in Supplemental Table 8. Overall, MeHg exposure induced up-regulation of ovarian gene expression, but there was limited overlap in the number of significant genes dysregulated among the three exposure groups. Gene Set Enrichment Analysis (GSEA) identified gene sets related to reproduction that were dysregulated; up-regulated gene sets included those involved with the metabolism of lipids and lipoproteins, response to estradiol, and estrogen receptor targets, while down-regulated gene sets included response to gonadotrophins and metabolism of lipids and lipoproteins. Information regarding which exposure group impacted each gene set are included in Supplemental Table 9.

QPCR analysis

Seven genes were selected from the RNA-seq data for QPCR analysis based on their availability in yellow perch sequence datasets: *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 expressed at lower levels relative to controls in a concentration-dependent manner (Fig. 3A). Zebrafish exposed to 10.0 ppm MeHg expressed *apoeb* at greater levels than those exposed to 1.0 ppm MeHg (p = 0.034; Fig. 3B). Only *cyp19a1a* was expressed at significantly greater levels in all zebrafish exposed to MeHg relative to controls (p < 0.001; Fig. 3C). Expression of both *ela3l* (Fig. 3D) and *prss59.1* (Fig. 3E) was extremely variable among individual zebrafish, but MeHg exposure did not impact the expression of either gene (*ela3l*: p = 0.792 and *prss59.1*: p = 0.683). In

MeHg-exposed zebrafish, *vtg2* expression was also reduced relative to control, although a high degree of variation existed ($p = 0.085$; Fig. 3F).

3.2 Yellow Perch

Mercury accumulation

Total Hg measurements of muscle tissue confirmed that MeHg accumulation was dependent upon dietary exposure at 12, 16, and 20 weeks ($p < 0.001$; Supplemental Table 10). At all time points of exposure, the fish accumulated approximately 0.05, 0.2, 1.5, and 9 μg/g total Hg in the 0, 0.5, 5.0, and 50.0 ppm exposure groups, respectively.

OSI, ovarian staging, estradiol, and testosterone analysis

The OSI increased throughout the time course of the experiment as the ovary developed (Fig. 4A), The OSI was significantly greater in the 50.0 ppm exposure group relative to the 5.0 ppm group at 20 weeks ($p = 0.004$), but the OSI was not impacted at either 12 or 16 weeks (Fig. 4A). MeHg exposure had no effect on estradiol, compared to control, within any sampling date ($p > 0.05$)); E2 did peak at the second sampling date, likely indicating the beginning of vitellogenesis (Fig. 4B) [28]. Moreover, MeHg exposure did not impact circulating concentrations of plasma testosterone, compared to control, regardless of sampling date (Fig. 4C; $p>0.05$)

No differences in follicle development were observed in 50.0 ppm MeHg-exposed female yellow perch, compared to control, at any sampling date (Supplemental Figure 2, Supplemental Table 11). In both 50.0 ppm MeHg and control exposure groups, the majority of follicles were pre-vitellogenic at 12 weeks, with some early vitellogenic follicles present, but there were no significant differences between exposure groups ($p >$

0.05, Supplemental Table 11). At 16 weeks, both exposure groups exhibited a significant increase ($p > 0.05$) (within follicle categories) in the percentage of vitellogenic follicles present relative to 12 weeks post exposure. By 20 weeks, the ovaries of the 50 ppm MeHg-exposed yellow perch showed a significant increase in the number of vitellogenic follicles compared to 16 weeks ($p < 0.05$, Supplemental Table 11).

QPCR analysis

MeHg exposure did not significantly alter the expression of any target genes, compared to control, in yellow perch pituitaries (Fig. 5) or livers (Fig. 6) *($p>0.05$)*. Interestingly, the expression of *vtgab* was lowered in the exposed yellow perch, but the results were not significant. At 12 weeks, *cyp19a1a* was up-regulated in yellow-perch ovary exposed to 50.0 ppm MeHg relative to yellow perch exposed to 0.5 ppm MeHg ($p = 0.024$, Fig. 7C). The expression of *trypsin* (*try*), the closest orthologue to *protease, serine, 59, tandem duplicate 1* (*prss59.1*) in zebrafish, was expressed at lower levels at each sampling date upon exposure to MeHg compared to control (Fig. 7D). At 20 weeks, dietary exposure to 0.05 ppm and 5.0 ppm MeHg significantly induced vitellogenin c (*vtgc*) expression, while exposure to 50.0 ppm MeHg significantly reduced expression of *vtgc* (compared to control; *$p<0.05$*, Fig. 7F). No other significant differences in ovarian gene expression were observed *($p>0.05$)*.

4. Discussion

The current study demonstrates that the effects of environmentally relevant concentrations of MeHg on female fish reproduction, in a laboratory setting, are minimal. The employment of a whole-life cycle exposure scheme using zebrafish is

unique relative to previous work elucidating the effects of MeHg on female fish reproduction [6,7,32]. Rather than targeting a limited window in development, zebrafish received MeHg from both maternal and dietary exposures, thus mimicking a realistic scenario of chronic, long-term exposure. Utilization of yellow perch, a non-model fish species, as a laboratory organism to study the effects of MeHg exposure was beneficial for elucidating the potential impacts of MeHg exposure on wild fish populations [4]. Although the results could not be directly compared primarily due to differences in exposures, general similarities might infer commonalities in impacted pathways. Overall, it did appear that zebrafish were more reproductively sensitive to the effects of MeHg than yellow perch based on molecular endpoints.

Molecular results were variable in zebrafish and did not reveal considerable effects of MeHg on gene expression in the ovaries. Several of the dysregulated genes from our study 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 here, several were involved in processes one would expect to see in reproduction, including response to estradiol stimulus, lipid localization, and lipid transporter activity. A lack of consensus between RNA-seq and QPCR results may have been a direct result of using pooled RNA from multiple fish for RNA-seq, which, in turn, normalized individual variation, in comparison to our use of RNA from individual fish for QPCR. For example, several individual zebrafish expressed *vtg3* more than 1000x greater than the individual with the lowest expression values, contributing to the large variation in results. Zebrafish ovaries also undergo asynchronous development, resulting in follicles at various stages [33],

possibly contributing to large variations in gene expression. During the design of QPCR primers, care was taken to avoid targets with known splice variants. However, the possibility exists that these sequences contain unknown variants which could influence accurate quantitation of expression levels [34]. Conversely, yellow perch ovaries mature throughout the course of a spawning season; therefore all follicles are in a similar developmental stage. QPCR results were, overall, much less variable within each sampling date in yellow perch than in zebrafish. In yellow perch, it was expected that because *cyp19a1a* was upregulated in the ovary and estradiol was slightly elevated at the final sampling point, that the increased estradiol would result in an increase in vitellogenin expression in the liver, however, expression of *vtgc* was not impacted and *vtga* expression decreased. Additionally, the factors driving the variability in expression of vitellogenin in the ovary are unclear and warrant further analysis.

In yellow perch, MeHg exposure did not affect OSI or circulating estradiol at any time point; this finding was in accordance with the results of Friedmann et al. [35] who found no significant difference in gonadal development in walleye (*Sander vitreus*), a closely related species to yellow perch. Additionally, MeHg exposure did not impact the developmental progression of the ovary across these sampling dates. Surprisingly, yellow perch that were not exposed to MeHg showed a regression in development at the third sampling date; although, this may have resulted from sampling bias toward the selection of larger fish earlier in the collection process rather than the experimental design.

In our previous work where we examined the effects of MeHg on neuroendocrine biochemicals on these same fish, we found that MeHg exposure significantly affect

brain dopamine receptor binding and affinity, monoamine oxidase, brain estrogen receptor, brain and liver androgen receptor [36]. However, modeling these neurotransmitter effects on vitellogenin production using a physiological based model calibrated for yellow perch (*e.g.* [37]), suggested that MeHg should have no effects on vitellogenin production [38], mostly because percids do not rely on dopamine inhibition to control final oocyte maturation [39–42]. While MeHg may affect neuroendocrine receptors in yellow perch and other species [36], the effects do not translate into impaired reproduction in percids. MeHg could; however, have reproductive effects in other fish taxa (*e.g.* cyprinids) that do rely on dopamine to control reproduction [43,44].

Our hypothesis regarding the overarching effect of MeHg on zebrafish reproduction is that it causes suppression of circulating estradiol levels. Supporting evidence includes the downregulation of *vtg2* in the ovaries of MeHg-exposed zebrafish, possibly indirectly suggesting a concurrent reduction in E2, which is known to regulate vitellogenin synthesis in the liver [45]. A pattern of decreased E2 resulting from MeHg exposure was demonstrated with fathead minnows in Drevnick and Sandheinrich using comparable exposure levels of MeHg [6]. Additionally, induction of *cyp19a1a* suggests a compensatory mechanism, in response to the suppressed estradiol levels. Simultaneous suppression of brain aromatase and estradiol along with increased ovarian aromatase has been previously documented with aromatase inhibitors, where increased *cyp19a1a* expression correlates with a return of estradiol levels back to normal levels [46].

Although molecular evidence in zebrafish implied that estradiol levels were suppressed in response to MeHg exposure, this was not the case for yellow perch.

509 Yellow perch also experienced an increase in *cyp19a1a* expression and decrease in
510 *vtgab* expression in the ovary and liver, respectively. These results may suggest
511 decreased estradiol levels; however, serum estradiol and testosterone concentrations,
512 as well as all other endpoints of female reproduction were unchanged in MeHg-exposed
513 yellow perch compared to control. Together, these results coupled with previous studies
514 [36,37] suggest the possible role of a compensatory mechanism, which may involve the
515 neurotransmitter dopamine, warranting further investigation. Future research into the
516 proteome or metabolome could provide a better understanding of underlying
517 mechanisms.

518 Molecular endpoints of this study indicated the potential for reproductive
519 impairment in zebrafish, but phenotypic endpoints evaluated in this study did not
520 support that MeHg significantly impacts reproduction in female fish, as was expected
521 based on previous studies with fathead minnows [3,32]. It is possible that zebrafish are
522 less sensitive to the reproductive effects of dietary MeHg than fathead minnows, and/or
523 that exposure duration is a critical component of reproductive-level effects of MeHg.
524 However, in his dissertation, Drevnick [47] found a similar lack of phenotypic impact of
525 dietary MeHg on zebrafish reproduction and postulated that this resulted from
526 supplementary brine shrimp included in the diet. *Artemia* given to these fish may have
527 been a confounding factor allowing the zebrafish to maintain reproductive function [48]
528 although the mechanism is unknown. Snarski and Olson [49] also found that toxicity
529 thresholds were greater in fathead minnows exposed to mercuric chloride with *Artemia*
530 diets over those solely fed a trout diet, attributing the cause to the *Artemia* providing
531 sufficient nutrition to overcome early toxic effects of mercury in sensitive life stages.

532 This study provides a future avenue to explore a similar experimental paradigm under
533 varying dietary conditions.

534 Based on the current investigation of the HPLG axis in yellow perch, MeHg
535 exposure does not significantly affect hypothalamus-pituitary-ovary-liver signaling in
536 yellow perch at the exposures or time points of ovarian development measured in this
537 study. The failure to find any significant effects in yellow perch is in accord with field-
538 based studies that suggest that female yellow perch are a metal-tolerant fish species
539 and proliferate in areas where greater metal exposure may occur [50–52]. Obviously,
540 several other factors are at play in a field-based study, including the severity of
541 exposure and other stressors, therefore, this lab-based study may provide insight into a
542 field-based scenario and highlights the need for future studies in the reproductive
543 effects of MeHg.

544 *Conclusion*

545 This study indicates that whole life-cycle exposed zebrafish are more sensitive to
546 dietary MeHg at a molecular level than yellow perch exposed during egg maturation;
547 however, the effects in zebrafish were subtle and suggest that a compensatory
548 mechanism was alleviating the burden of constant exposure to MeHg, possibly via
549 induction of *cyp19a1a*. Limitations in yellow perch exposure conditions and tissue
550 collection may have inhibited any strong parallel effects that would otherwise be seen in
551 identical exposure scenarios. Moreover, it is possible that dietary MeHg exposure may
552 not impact reproduction in yellow perch until the ovary is fully developed and the fish is
553 ready to spawn. Overall, this study suggests that factors more complex than MeHg
554 exposure alone are driving population declines in wild yellow perch. 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.

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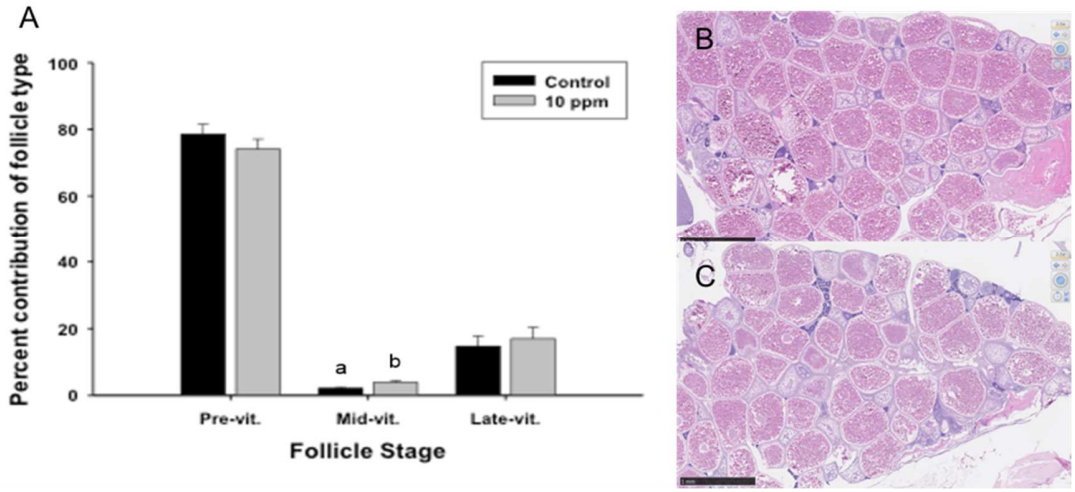
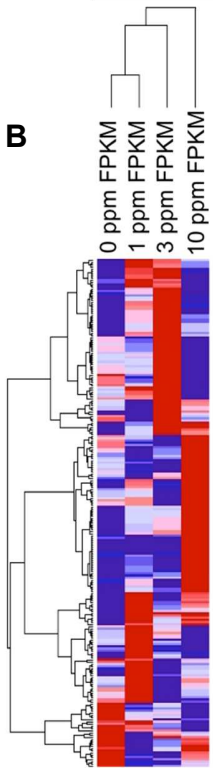
FIGURE 1.


A
Percent contribution of follicle type
100
80
60
40
20
0
Control
10 ppm
a
b
Pre-vit.
Mid-vit.
Late-vit.
Follicle Stage
B
C

A

Exposure	Total dysregulated genes	Up-regulated	Down-regulated
1.0 ppm	53	33	20
3.0 ppm	45	37	8
10.0 ppm	56	36	20



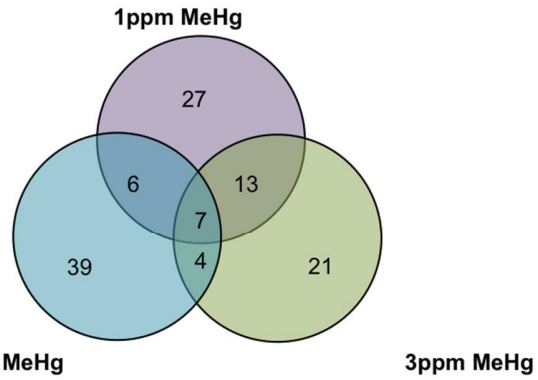


FIGURE 2.

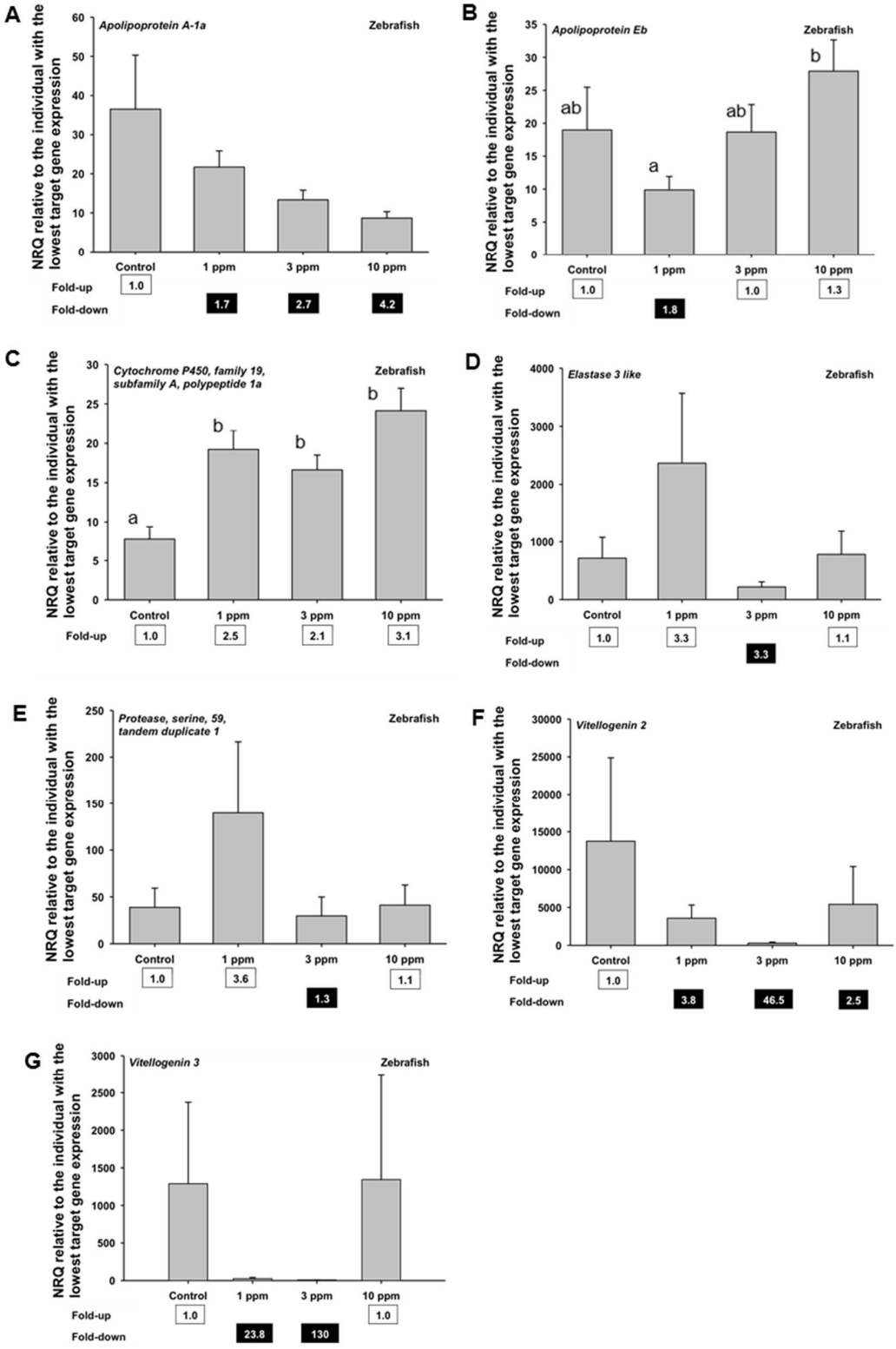

A
Apolipoprotein A-1a
Zebrafish
NRQ relative to the individual with the lowest target gene expression
Control
1 ppm
3 ppm
10 ppm
Fold-up
1.0
Fold-down
1.7
2.7
4.2
B
Apolipoprotein Eb
Zebrafish
ab
a
ab
b
Fold-up
1.0
1.0
1.3
Fold-down
1.8
C
Cytochrome P450, family 19, subfamily A, polypeptide 1a
Zebrafish
a
b
b
b
Fold-up
1.0
2.5
2.1
3.1
D
Elastase 3 like
Zebrafish
Fold-up
1.0
3.3
1.1
Fold-down
3.3
E
Protease, serine, 59, tandem duplicate 1
Zebrafish
Fold-up
1.0
3.6
1.1
Fold-down
1.3
F
Vitellogenin 2
Zebrafish
Fold-up
1.0
Fold-down
3.8
46.5
2.5
G
Vitellogenin 3
Zebrafish
Fold-up
1.0
1.0
Fold-down
23.8
130

FIGURE 3.

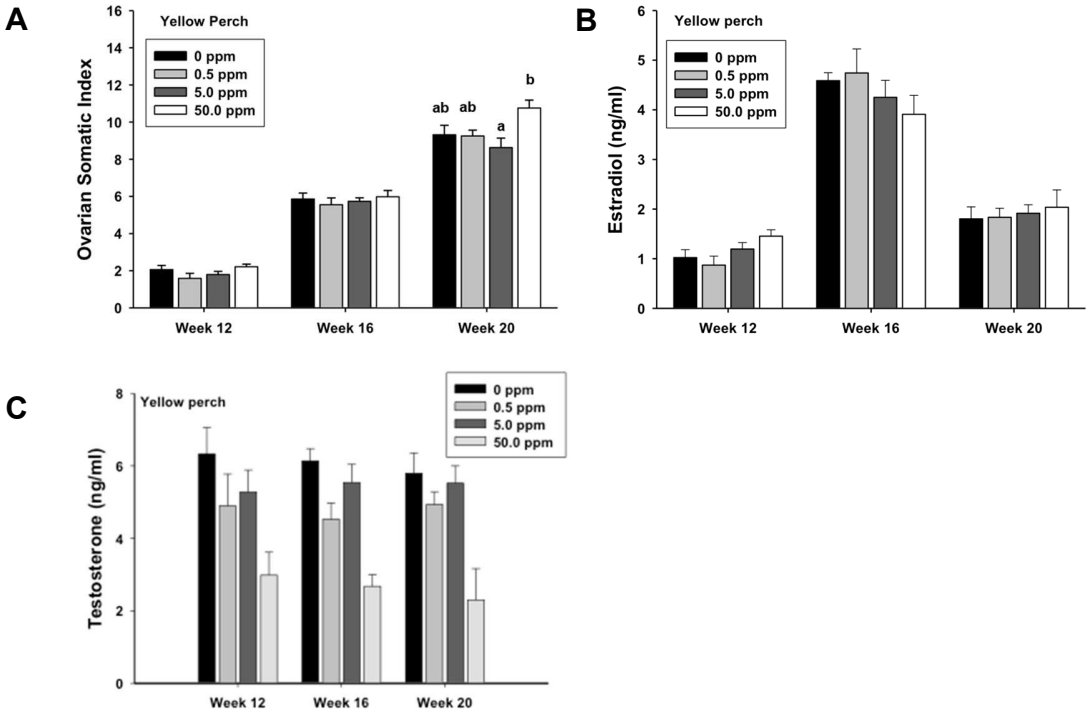

A
Yellow Perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
Ovarian Somatic Index
ab
ab
a
b
Week 12
Week 16
Week 20
B
Yellow perch
Estradiol (ng/ml)
C
Yellow perch
Testosterone (ng/ml)

FIGURE 4.

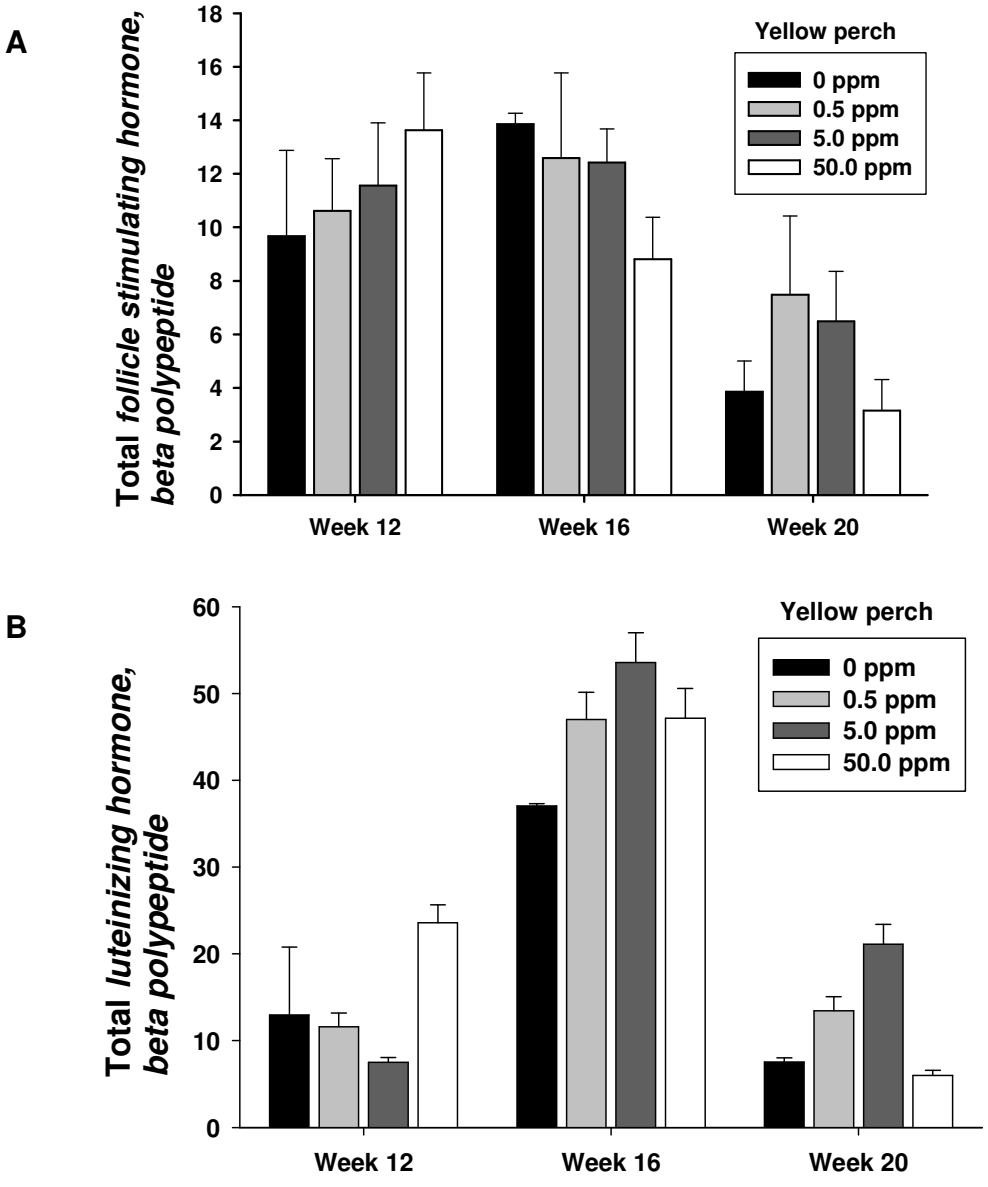
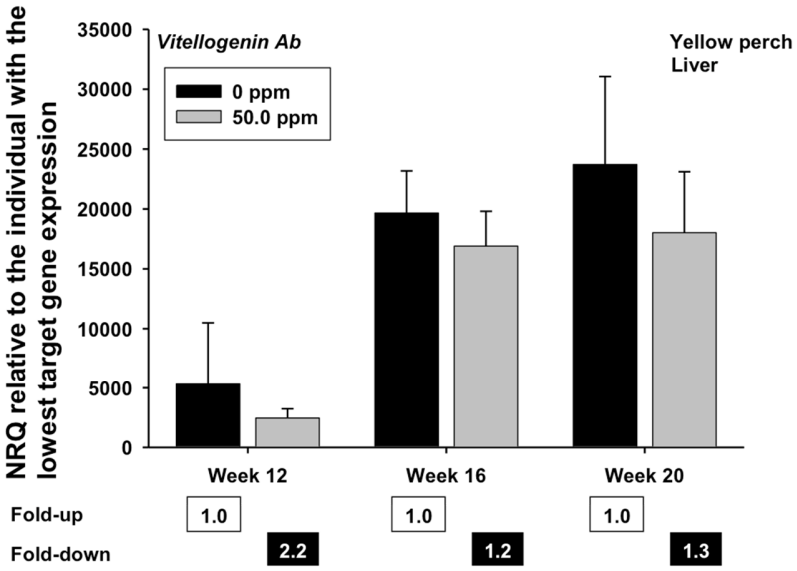

A
Total follicle stimulating hormone, beta polypeptide
Yellow perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
Week 12
Week 16
Week 20
B
Total luteinizing hormone, beta polypeptide
Yellow perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
Week 12
Week 16
Week 20

FIGURE 5.

A.


Vitellogenin Ab
Yellow perch
Liver
0 ppm
50.0 ppm
NRQ relative to the individual with the lowest target gene expression
35000
30000
25000
20000
15000
10000
5000
0
Week 12
Week 16
Week 20
Fold-up
1.0
1.0
1.0
Fold-down
2.2
1.2
1.3

B.

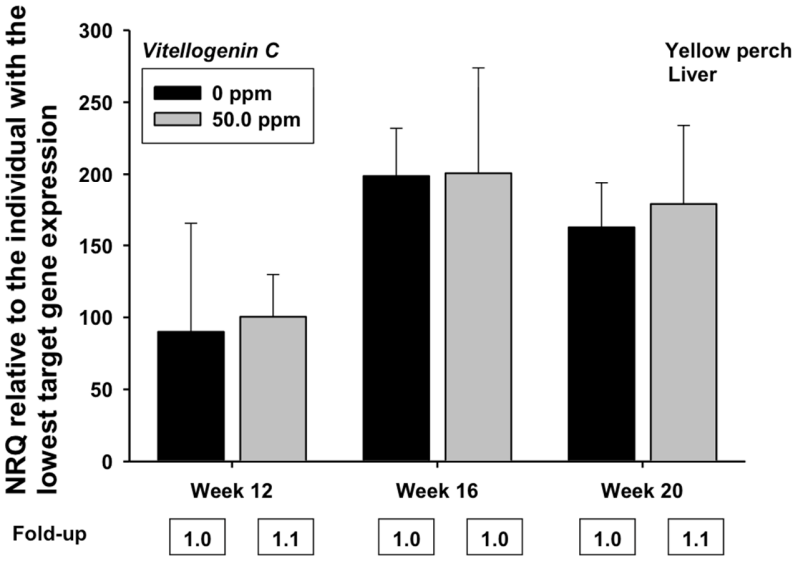

Vitellogenin C
Yellow perch
Liver
0 ppm
50.0 ppm
NRQ relative to the individual with the lowest target gene expression
300
250
200
150
100
50
0
Week 12
Week 16
Week 20
Fold-up
1.0
1.1
1.0
1.0
1.0
1.1

FIGURE 6.

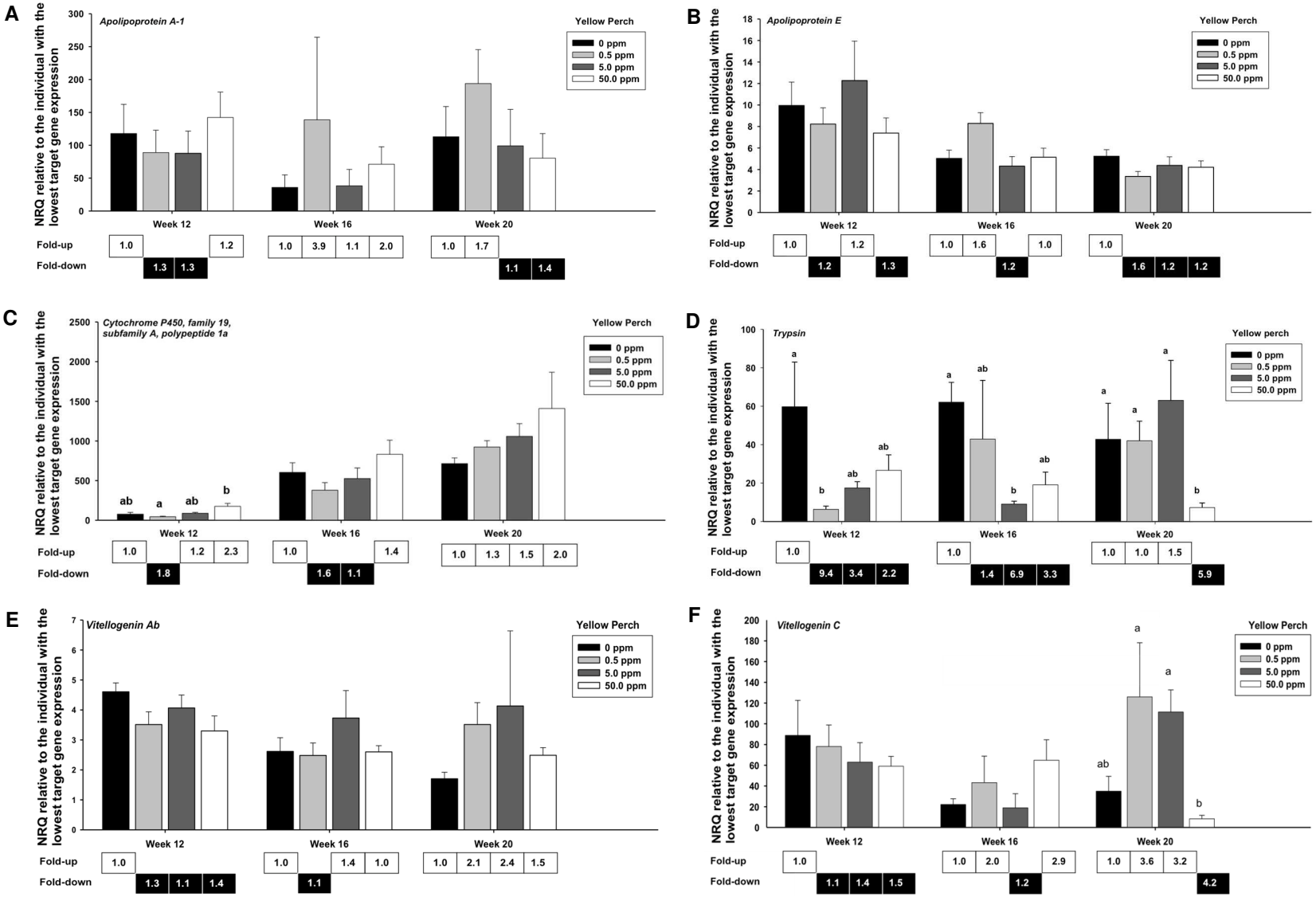

A
Apolipoprotein A-1
Yellow Perch
0 ppm
0.5 ppm
5.0 ppm
50.0 ppm
NRQ relative to the individual with the lowest target gene expression
Week 12
Week 16
Week 20
Fold-up
Fold-down
1.0 1.2
1.3 1.3
1.0 3.9 1.1 2.0
1.0 1.7
1.1 1.4
B
Apolipoprotein E
1.0 1.2
1.2 1.3
1.0 1.6 1.0
1.2
1.0
1.6 1.2 1.2
C
Cytochrome P450, family 19, subfamily A, polypeptide 1a
ab a ab b
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
Yellow perch
a b ab ab
1.0
9.4 3.4 2.2
a ab b ab
1.0
1.4 6.9 3.3
a a a b
1.0 1.0 1.5
5.9
E
Vitellogenin Ab
1.0
1.3 1.1 1.4
1.0 1.4 1.0
1.1
1.0 2.1 2.4 1.5
F
Vitellogenin C
ab a a b
1.0
1.1 1.4 1.5
1.0 2.0 2.9
1.2
1.0 3.6 3.2
4.2

FIGURE 7.