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Differential gene expression associated with dietary methylmercury (MeHg) exposure in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*)

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Abstract

The objective of this study was to identify and evaluate conserved biomarkers that could be used in most species of teleost fish at most life-stages. We investigated the effects of sublethal methylmercury (MeHg) exposure on developing rainbow trout and zebrafish. Juvenile rainbow trout and young adult zebrafish were fed food with MeHg added at 0, 0.5, 5 and 50 ppm. Atomic absorption spectrometry was applied to measure whole body total Hg levels, and pathologic analysis was performed to identify MeHg-induced toxicity. Fish at six weeks were sampled from each group for microarray analysis using RNA from whole fish. MeHg-exposed trout and zebrafish did not show overt signs of toxicity or pathology, nor were significant differences seen in mortality, length, mass, or condition factor. The accumulation of MeHg in trout and zebrafish exhibited dose- and time-dependent patterns during six weeks, and zebrafish exhibited greater assimilation of total Hg than rainbow trout. The dysregulated genes in MeHg-treated fish have multiple functional annotations, such as iron ion homeostasis, glutathione transferase activity, regulation of muscle contraction, troponin I binding and calcium-dependent protein binding. Genes were selected as biomarker candidates based on their microarray data and their expression was evaluated by QPCR. Unfortunately, these genes are not good consistent biomarkers for both rainbow trout and zebrafish from QPCR evaluation using individual fish. Our conclusion is that

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Conflict of interest

Nan Jiang is employed by Roche NimbleGen. His contribution to this study is not related to his employment. All other authors declare that they have no conflict of interest.

biomarker analysis for aquatic toxicant assessment using fish needs to be based on tissue-, sex- and species-specific consideration.

Keywords

methylmercury; zebrafish; rainbow trout; biomarkers

Introduction

Mercury is released into the environment by anthropogenic and natural activities, and is transformed into methylmercury (MeHg) by microorganisms within anoxic aquatic sediments (Benoit et al. 1998). Through trophic transfer, MeHg undergoes biomagnification to achieve its highest concentration in predatory fish or carnivorous sea mammals (Evers et al. 2011). It has been reported that MeHg can accumulate in multiple tissues and organs of fish (Depew et al. 2012; Sandheinrich and Wiener, 2011) and cause multiple problems including visual deficits, teratogenesis, delayed mortality, and apoptosis of ovarian follicular cells (Drevnick et al. 2006; Samson et al. 2001; Weber et al. 2008). Impaired survival skills (foraging and predator evasion) in larval of Atlantic croaker (*Micropogonias undulatus*) were observed after maternal exposure to MeHg (Alvarez et al. 2006).

Juvenile stages represent the transition from embryonic to adult stages. Zebrafish and rainbow trout develop and mature at different rate. In zebrafish, the transition from the embryonic stage to the larval stage takes three days. After 30 days, zebrafish enter the juvenile stage, which lasts 60 days before zebrafish become adults. During the juvenile period, zebrafish undergo development of their fins, scales, teeth and adult pigment pattern (Van der Heyden et al. 2000; Parichy et al. 2003; Sire et al. 2004; Goldsmith et al. 2006). As a cold-water fish, the juvenile stage of rainbow trout lasts even longer so as to achieve maturation only after years (Sturgess et al. 1978; Whitworth et al. 1983). Previous studies of MeHg and TCDD exposure in fish mainly focused on embryonic and adult stages. However, investigations on MeHg and TCDD exposure in juvenile fish were rarely documented. In order to fill the knowledge gap around MeHg-induced toxicity in juvenile fish, the studies of MeHg exposure in juvenile fish are important and will provide outcomes that are likely different from those seen in embryos and adults. The rainbow trout is a powerful model system for studies of carcinogenesis, comparative immunology, stress physiology, and molecular genetics (Thorgaard et al. 2002). As a model organism supported by the National Institutes of Health, the zebrafish is a powerful tool for developmental and molecular toxicology research with substantial resources including a sequenced genome and established approaches for large-scale chemical and mutagenic screens (Carvan et al. 2008). Our hypothesis is that, using two species separated by 300 million years of evolution one can identify conserved toxicant-responsive biomarkers through parallel comparative toxicogenomic analyses.

In the laboratory, mercury exposure occurs via direct water-borne exposure, intracoelomic injection, or in the diet. We have chosen dietary exposure for our studies because it more accurately models exposure in wild populations of fish (Depew et al. 2012). Most published investigations have focused on the effects of MeHg in embryonic and adult fish, and there are few reports on the effects of dietary MeHg exposure in juvenile fish. The limited number of studies describing the effects of chronic dietary exposure in juvenile fish report that MeHg caused delayed growth in blackfish (*Orthodon microlepidotus*) (Houck et al., 2004), suppressed reproductive behavior in fathead minnow following maturation (Sandheinrich et al. 2006), and induced hyperplasia in the gill epithelium in rainbow trout (*Oncorhynchus mykiss*) (Wobeser, 1975).

The objective of this study was to identify and evaluate pan-teleost conserved biomarkers that could be used in field studies at most teleost fish life-stages. Consequently, we chose rainbow trout and zebrafish, which are separated by considerable evolutionary distance, and fed juveniles of both dietary MeHg with 0 ppm, 0.5 ppm, 5 ppm and 50 ppm for six weeks. Microarray based differential expression of gene orthologs of whole rainbow trout and zebrafish RNA samples were then compared to real-time quantitative reverse transcriptase polymerase chain reaction (QPCR) results. Analytical chemistry and pathologic analyses were performed to assess MeHg bioaccumulation and toxicity of MeHg. Chronic dietary MeHg exposure did not cause overt toxicity in juvenile rainbow trout and zebrafish at tissue residue levels above 10 ppm.

Materials and methods

The materials and methods are summarized below. More detail is provided as supplementary material.

Animals and exposure

Eyed rainbow trout eggs were obtained from the US Fish and Wildlife Service Ennis National Fish Hatchery (MT) and reared at 10 °C. Dietary exposures were initiated 45 days after hatching. Wild-type Ekkwill (EK) zebrafish (stock originally purchased from Ekk Will Waterlife Resources, Ruskin, FL) were reared at 28 °C. All fish from a single treatment were housed in a single 40 L polycarbonate tank to reduce hazardous waste. Treatment was initiated after a two week acclimation period. The fish were fed Biodiet starter (Bio-Oregon, 4% body weight per day) with MeHg (Sigma-Aldrich Co., St. Louis, MO) added at 0 ppm, 0.5 ppm, 5 ppm and 50 ppm, Ethanol was used as a vehicle in each treatment including 0 ppm. It was impossible to monitor individual fish for food consumption, however, mercury accumulation was very consistent between individuals suggesting equal food consumption. Fish were collected at 0, 1, 2, 4 and 6 weeks for Hg assessment and at six weeks for histopathological and gene expression analyses. The gonadal sex of fish was determined by direct observation. Body weight and standard length were measured. Individual fish were quick frozen in liquid nitrogen and stored at –80 °C.

Mercury assessment

Total Hg levels were measured in six homogenized, whole rainbow trout and zebrafish samples for each treatment and time point. Pilot studies of individual variance in Hg content determined that six samples provided sufficient coverage for accurate determination. Samples were analysed by atomic absorption spectrophotometry based on the methods of Nam and Basu (2011) and Nam et al. (2011). All concentrations are reported as ppm (µg/g) on a wet-weight basis. Tissue residue levels were statistically analyzed using two-way ANOVA with Student-Newman-Keuls (SNK) test for post-hoc multiple comparisons on log-transformed total Hg values (to achieve normality).

RNA isolation

Total RNA from individual fish was isolated using Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA samples (< 45 µg in a total volume of 100 µl RNase-free water) were digested with DNase I and further RNA purification was performed using the RNeasy MiniElute cleanup kit (Qiagen, MD). The concentration and A260/280 of cleaned RNA was determined by ND-1000 NanoDrop Spectrophotometer (Thermo Scientific, DE). RNA quality in trout was determined by agarose gel electrophoresis. Quality of zebrafish RNA was determined using the Experion Automated Electrophoresis System (Bio-Rad, Hercules, CA) with a RNA StdSens chip.

cDNA synthesis, labeling and Microarray hybridization

Rainbow trout—This common reference design study (Fig. 1) used the consortium for Genomics Research on All Salmonids Project (cGRASP; University of Victoria, Canada) 16K microarray platform to analyze gene expression in rainbow trout (von Schalburg et al. 2005). Total RNA from five individual fish from each treatment group were randomly selected for comparison to the common reference. Complementary DNA (cDNA) targets were labeled using the Array 900 Expression Array Detection kit (Genisphere, PA) according to the manufacturer's protocol. Following hybridization and washing, the slides were dried by centrifugation.

Zebrafish—NimbleGen Gene Expression 12X135K zebrafish microarrays (NimbleGen, Madison, WI) were used for gene expression analysis. RNA from three randomly selected male and three female zebrafish was used for microarray experiments. cDNA targets were labeled using the One-Color DNA Labeling Kit (NimbleGen). Following hybridization and washing, slides were spin-dried in a NimbleGen microarray drier.

Array Scanning, data extraction and normalization

Rainbow trout—The 16K cGRASP microarrays were scanned using the ScanArray Express (PerkinElmer) and two-channel intensities were quantified with ImaGene 6.0 (BioDiscovery). The R (version 2.12.2) statistical package LIMMA (version 2.18.3) in Bioconductor (version 2.4) was used for background correction and normalization.

Zebrafish—NimbleGen zebrafish microarrays were scanned at MOgene, LC (St. Louis, MO) using on a NimbleGen MS200 scanner. Expression data were normalized with the Robust Multichip Average (RMA) algorithm as described previously (Irizarry et al. 2003).

Microarray data analysis

Differential expression analysis was performed with Multiple Array Viewer (MeV, Dana-Farber Cancer Institute, MA) software (version 4.8.1). Three statistical methods included in MeV were reviewed and tested for analysis: Bayesian Estimation of Temporal Regulation (BETR), one-way ANOVA and Significance Analysis of Microarrays (SAM). False discovery rate (FDR) was controlled by Benjamini and Hochberg procedure (Benjamini et al. 1995). Of the three methods, BETR consistently predicted the lowest FDR and therefore was used for differential expression analysis of all data. Statistical significance was set at $FDR < 0.05$. Data from rainbow trout, male and female zebrafish were analyzed separately as three individual groups.

A BLAST-based Reciprocal Best Hit (RBH) method was performed to identify putative zebrafish-rainbow trout orthologs. Comparison of dysregulated genes between Rainbow trout and zebrafish were performed using the geneIDs of these RBH putative orthologs. Gene Ontology (GO) annotations (BP and MF) were based on comparison against all protein sequences of the Amigo database at amigo.geneontology.org (Carbon et al. 2009). The "GOHyperGAll" script from BioConductor was employed to conduct GO enrichment analysis. Rainbow trout gene symbols follow the guidelines established in Shaklee et al. (1990) for protein coding loci in fish. Zebrafish gene symbols follow the zebrafish nomenclature guidelines as established by the Zebrafish Nomenclature Committee and outlined at ZFIN.org. When referring to multiple species, the guidelines of Shaklee et al. (1990) are followed unless otherwise indicated.

All microarray data sets were submitted to the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE32431 (Sub-Series Nos. are GSE32429 for rainbow trout and GSE32430 for zebrafish).

Real-time QPCR

Real-time quantitative polymerase chain reaction (QPCR) was performed to evaluate the dose-dependent expression of target genes. For zebrafish, in order to boost statistical power, one more male and female fish were incorporated into each group except for the male 5 ppm group (because we did not find enough male fish samples in this group).

QPCR was conducted using AffinityScript™ Multi Temperature cDNA synthesis Kit (Agilent) with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Guanine nucleotide-binding protein (G protein), beta polypeptide 2-like 1 (*gnb2l1*) was used as a normalizer gene. We found that *gnb2l1* expression is stable under conditions of MeHg exposure in both zebrafish and rainbow trout. We tested other normalizer candidates (Tang et al. 2007; McCurley et al. 2008), including ribosomal protein L13a (*rpl13a*); actin, beta 1 (*actb1*); tubulin, alpha 1 (*tuba1*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) and elongation factor 1-alpha (*eef1a1a*), and expression of these transcripts was not stable between different MeHg treatment conditions and, therefore, could not be used as normalizers. Gene-specific primers (Supplementary Table 1) were designed using Primer 3 (<http://frodo.wi.mit.edu/primer3/>). Each amplification product from a primer pair was tested using melting curve analysis to verify that the primers amplified a single product and that there were no primer dimers. Amplification efficiencies of selected primer pairs ranged from 90–110%.

Each sample was run in duplicate. The relative starting quantity (RQ) of each transcript was determined using the comparative C_T method for relative quantification with assumption of 100% efficiencies (Livak et al. 2001). For each target gene, the individual sample with the lowest expression (highest C_T value) was set as a calibrator and its RQ to be equal to 1. Gene expression data were presented as mean (\pm standard error) RQ relative to the calibrator.

Statistical analysis of QPCR data was performed using SigmaStat 11.0 (Systat Software, Inc., CA). One-way analysis of variance (ANOVA) was used to detect effects of MeHg exposure on the expression of each target gene. If the differences in the median values among the treatment groups were greater than would be expected by chance ($p < 0.05$), the Tukey test was used for pair-wise multiple comparisons. If the data failed the Shapiro-Wilk normality test or Equal Variance Test, Kruskal-Wallis One Way Analysis of Variance on Ranks was used; and significant differences between treatments and control were evaluated using the Dunn's test. Significant differences were identified at $p < 0.05$.

Results

Total mercury in MeHg-treated fish

During six weeks of feeding, accumulation of whole body total Hg exhibited dose- and time-dependent increases (Fig. 2). There was a statistically significant interaction between exposure week and dose in both species (two-way ANOVA; $p = < 0.001$, $DF=9$). All pairwise multiple comparisons (SNK method) showed significant differences among doses within each week. At six weeks, 50 ppm dietary MeHg caused an accumulation of 30.6 ppm of total Hg in zebrafish and 10.7 ppm of total Hg in rainbow trout.

Growth of MeHg-treated fish

Neither significant mortalities nor influences of growth (body length and weight) were found in MeHg-treated rainbow trout or zebrafish (Supplementary Table 2) using multiple comparison analysis with one-way ANOVA ($p < 0.05$). At six weeks, body weight and length of trout in the 0 ppm group were 0.54 ± 0.03 g, 3.25 ± 0.06 cm, and in the 50 ppm group were 0.45 ± 0.07 g, 3.05 ± 0.12 cm; body mass and length of zebrafish (both male and female) in the

0 ppm group were 0.19 ± 0.02 g, 2.20 ± 0.06 cm, in the 50 ppm group were 0.17 ± 0.02 g, 2.08 ± 0.08 cm. Histopathological analysis revealed no apparent pathologies in MeHg-treated rainbow trout or zebrafish (data not shown).

Gene expression analysis

Rainbow trout—A total of 20 microarrays were used to identify genes dysregulated in MeHg-treated rainbow trout, and 162 probes were found to be significantly dysregulated ($FDR < 0.05$) following MeHg exposure for six weeks (Supplementary Table 3). The top enriched GO terms (Table 1) were mainly related to iron ion transport, iron ion homeostasis, metal ion transport, iron binding, and glutathione transferase activity.

Zebrafish—A total of 24 microarrays were used to identify genes dysregulated in MeHg-treated zebrafish (12 array for male and female, respectively). 1975 and 320 dysregulated genes were found for female and male fishes, respectively; ($FDR < 0.05$; Supplementary Table 4 and 5). There were only 31 dysregulated genes in common between MeHg-treated female and male zebrafish, indicating MeHg-induced dysregulation of gene expression exhibited sex-dependent differences. The top 15 significantly enriched GO terms of biological process and molecular function are shown in Table 2. In female zebrafish, the enriched GO terms are RNA splicing, regulation of striated muscle contraction, mRNA processing, troponin I binding and calcium-dependent protein binding. In male zebrafish, the dysregulated genes were mainly involved in the processes of cellular assembly and organization, such as protein metabolism, protein folding and axoneme assembly.

QPCR analysis of selected MeHg-induced genes in zebrafish and rainbow trout

Based on the microarray data, *coll1a1a* was the only gene that was significantly dysregulated among three groups (rainbow trout and male and female zebrafish). In order to increase biomarker candidates, additional 11 genes were selected for QPCR analysis because they were highly dysregulated in either rainbow trout or zebrafish. Totally, these 12 genes were considered as putatively conserved biomarker candidates. Multiple comparison analysis of QPCR data (Fig. 3) was performed to determine statistically significant differences between MeHg-treated groups and the control group. Ten genes were significantly dysregulated either in MeHg-treated zebrafish or rainbow trout after six weeks of dietary exposure. Up- or down-regulation of their expression by MeHg exposure was not completely consistent between zebrafish and rainbow trout. Based on the microarray data, *coll1a1a* was the only gene that was significantly dysregulated both in zebrafish and trout. However, its expression was not significant in male zebrafish by QPCR. MeHg (50 ppm) caused a five-fold induction in *coll1a1a* in female zebrafish, while it caused a two-fold decrease in trout that was not statistically significant. Based on the QPCR data (Fig. 3), *MGST3*, *SPARC* and *TNNT3* may be good biomarkers for MeHg-exposed trout; *coll1a1a*, *mylpfa*, *sparc*, *tmx2a* and *tnnt3a* can be applied to MeHg-exposed female zebrafish; *mylpfa* and *ptmaa* can be used as biomarkers for MeHg-treated male zebrafish. However, none of these 12 genes can be used as biomarkers among rainbow trout, female and male zebrafish.

Discussion and summary

Growth and mercury accumulation in fish

MeHg is absorbed by the digestive tract and is distributed throughout the body via the blood stream, primarily bound to red blood cells, to different organs (Giblin et al. 1975) including kidney, muscle, liver and spleen (Baatrup et al. 1987; Oliveira et al. 2008). MeHg can cross the blood-brain barrier and accumulate to a significant degree in the brain and induce neurotoxicity (Kerper et al. 1992; Berntssen et al. 2003; Gonzalez et al. 2005). Research in rainbow trout (Giblin et al. 1973) and zebrafish (Cambier et al. 2010, 2012) shows that

MeHg can accumulate in lens, brain, skeletal muscle, blood and spleen. Recent research in spotted dogfish (*Scyliorhinus canicula*) shows that the bioaccumulation of mercury followed the order muscle > heart > liver > gills > pancreas (Coelho et al. 2010).

In the current study, accumulation of total Hg exhibited time- and dose-dependent increases in dietary MeHg treated fish. The measurement was conducted using the whole body, instead of individual organs or tissues. After six weeks, total mercury accumulated to levels three times greater in zebrafish (30.6 ppm) than in rainbow trout (10.7 ppm), indicating that trout may absorb mercury slower and/or excrete mercury faster than zebrafish. In addition, it has been reported that temperature can influence MeHg uptake in the intestines of channel catfish (*Ictalurus punctatus*) with higher temperature enhancing the absorbance of MeHg (Leaner et al. 2002). Rainbow trout is a cold-water fish (10 °C) and zebrafish require much warmer water (28 °C). These environmental differences may partially explain the differences in MeHg assimilation. Previous research by Wobeser (1975) showed that the accumulation of 30 ppm MeHg in muscle was associated with hyperplasia in the gill epithelium of juvenile rainbow trout (dosing initiated at 29 g bw) with no significant mortality after 105 days of exposure. The rainbow trout used in our study were younger and smaller than in Wobeser's study, and the accumulation of 10.7 ppm in rainbow trout and 30.6 ppm in zebrafish did not cause mortality or any significant pathology in tissues or organs.

Gene expression analysis of microarray and QPCR data

Microarrays constitute a powerful tool with which to explore the molecular mechanisms involved in MeHg-induced toxic effects and to identify the genes involved in molecular pathways. We used individual fish to perform the microarray experiments and assess biologic variability in gene expression. The cGRASP 16K microarray contains 13,421 Atlantic salmon and 2,576 rainbow trout cDNA probes. The cGRASP cDNA microarray has been shown to be an effective genomic tool for identifying transcriptome responses to environmental stress (Lewis et al. 2010), and gene expression alterations due to domestication in rainbow trout (Tymchuk et al. 2009). The NimbleGen zebrafish genome-wide microarray contains almost the entire zebrafish transcriptome. Less than 10% of dysregulated zebrafish genes were in common between MeHg-treated females and males, indicating sex-specific responses to MeHg exposure in zebrafish.

Identifying biomarkers from microarray data is an important step towards understanding of molecular pathways of MeHg-induced toxicity in fish. The candidate biomarkers selected are associated with many of the enriched GO terms in rainbow trout (Table 1) and zebrafish (Table 2). Analysis of the Biological process (BP) and molecular function (MF) terms support much of what we know about the effects of MeHg toxicity. For example, glutathione transferase activity (MF) is highly related to detoxification and metabolism of xenobiotics and protection from oxidative stress caused by MeHg. MGST3 is involved glutathione-dependent detoxification and metabolism processes (UniProtKB O14880) and PRDX1 is known to function as an antioxidant enzyme (UniProtKB Q06830). MYL2, TNNT3, TNN2 and TPM1 function in the muscle contraction process, including ventricular myocardium (Margossian et al. 1987; Macera et al. 1992; Levine et al. 1998; Moran et al. 2008). It has been reported that MeHg influences heart rate and increases systolic and diastolic pressure in rat by depressing the ventricular myocardium (Cunha et al. 2001; de Assis et al. 2003). In the present study, GO terms of molecular function related to muscle contraction were significantly enriched, such as calcium-dependent ATPase activity, troponin I binding, and troponin C binding.

However, none of the 12 genes of interest proved to be robust biomarkers in both rainbow trout and zebrafish of both sexes. *COL1A1* was the only microarray-identified common

gene, but no significant changes were observed in MeHg-treated male zebrafish by QPCR. In previous studies, the strategies for identifying biomarkers from microarray data have been based on robust expression (Gunnarsson, et al. 2007), expression signature or patterns (Wang et al. 2008), specific location of tissue (Tilton et al. 2008), and linkage to the protein levels (Deng et al. 2006). In the current project, we focused on the identification of conserved gene expression biomarkers based on common dysregulated genes in whole rainbow trout and zebrafish. We chose whole fish because it is much more convenient in field assessments to collect whole animals for analysis, especially when fish are small. In field situations, individual tissues in small juvenile fish may be difficult to work with and, in some cases, may not provide sufficient material for gene expression analysis.

Our goal was to identify easily-assayed robust pan-teleost biomarkers and a number of factors may have influenced the outcome beyond the previously-mentioned influence of temperature on Hg accumulation. The difference in tissue Hg levels may have made direct comparisons more difficult. In addition, the experiment was initiated when the trout and zebrafish were juveniles and approximately the same size. However, rainbow trout and zebrafish exhibit dramatically different rates of growth and maturity. By the end of the experiment, the zebrafish were fully-developed young adults with differentiated sexes. The juvenile stage of rainbow trout can last years (Sturgess et al. 1978; van den Hurk et al. 1979; Whitworth et al. 1983), so the rainbow trout were still juveniles following our six-week feeding period. By the time gene expression was assessed, the two species were at dramatically different developmental stages which added substantial variability to the data.

Our focus on the whole animal may have confounded our analysis of gene expression based on tissue-specific expression of some of the QPCR target genes. For example, *tnni2a.1* was identified as a potential conserved biomarker and is expressed almost exclusively in heart (Moran et al. 2008). The biologically inherent differences between rainbow trout and zebrafish and the methodological issues of the experiment may have generated sufficient noise that made the identification of conserved MeHg-responsive genes between the two species improbable.

It may be possible to identify conserved teleost biomarkers for specific environmental stressors in whole fish, especially when exposures can be correlated with specific lesions. However, for mercury such analyses will require a focus on specific tissues in order to distinguish the signal from the background noise. In conclusion, we found that chronic dietary MeHg in juvenile rainbow trout and zebrafish resulted in dose- and time-dependent accumulation of Hg in whole fish, however, no pathological issues were observed and no conserved gene expression biomarkers were identified. Zebrafish exhibited greater assimilation of MeHg than rainbow trout. It is our conclusion that analysis of gene expression biomarkers in RNA samples collected from dietary MeHg exposed juvenile whole fish does not provide sufficient evidence of pan-teleost biomarkers, and that the development of pan-teleost biomarkers for mercury exposure will likely require analysis of tissue-specific biomarker expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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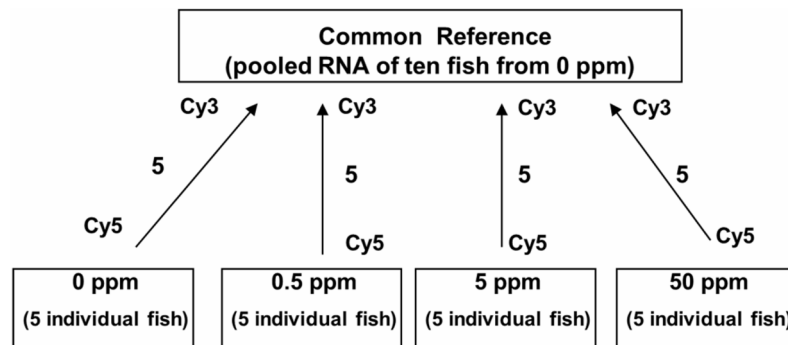


Fig. 1.

Common reference design of 16K cGRASP two-color microarray experiment analyzing rainbow trout samples collected at six weeks. The microarray experiment for rainbow trout samples included 20 arrays. The common reference was composed of pooled total RNA of ten fish from the 0 ppm group at six weeks. Five individual fish in each group at six weeks were randomly selected for comparison to the common reference. cDNA of the common reference was labeled with Cy3, and cDNA of individual samples was labeled with Cy5. The comparison between treatment group and common reference was calculated using the Cy5/Cy3 ratio.

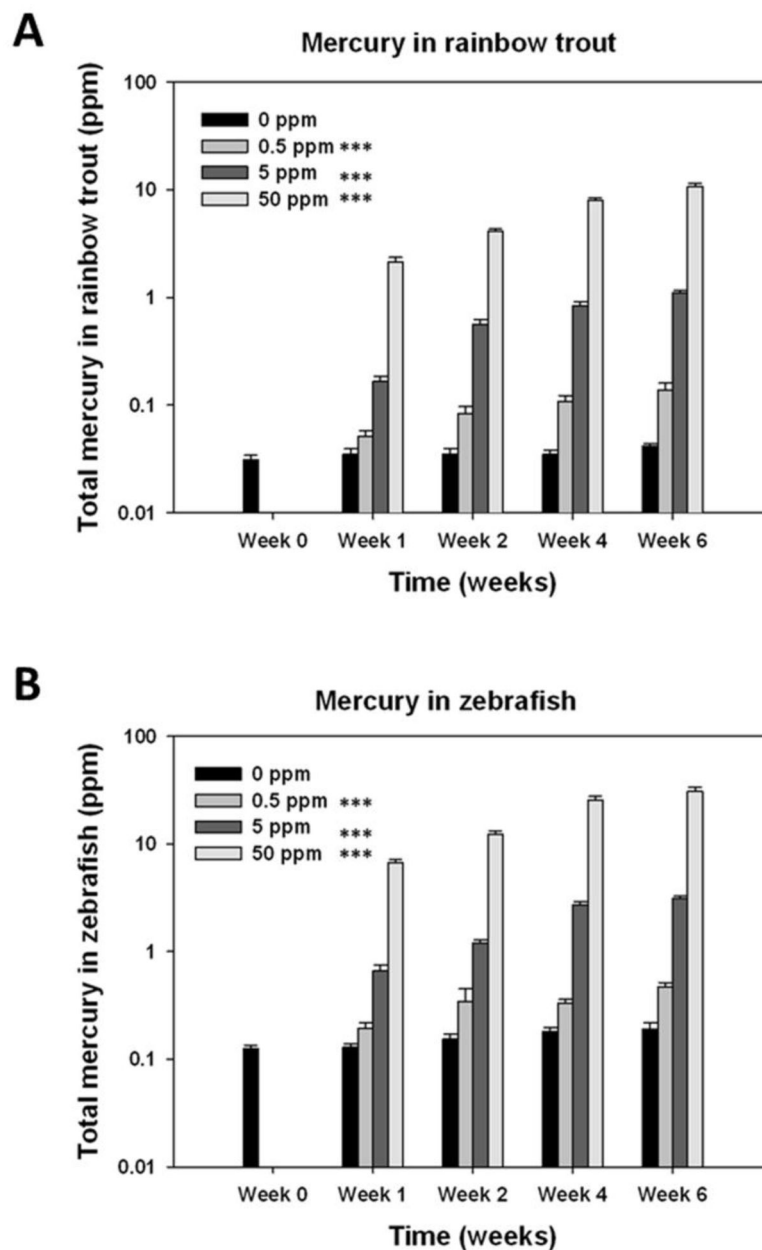
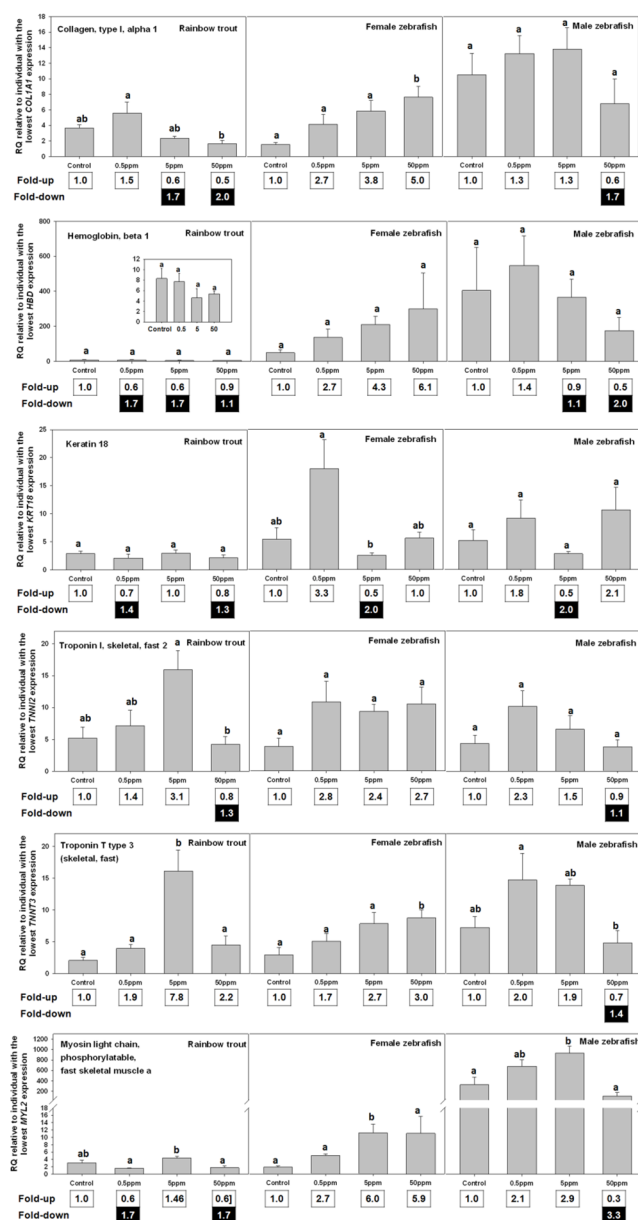


Fig. 2.

Accumulation of total Hg in MeHg-treated rainbow trout and zebrafish. Six fish were sampled at each time point for Hg measurements by atomic absorption spectrometry. All concentrations are reported as ppm ($\mu\text{g/g}$) on a wet weight basis. All pairwise multiple comparisons (Student-Newman-Keuls method) showed significant differences between doses within each week. There is a statistically significant interaction between WEEK and DOSE in both experiments (two-way ANOVA; *** $p < 0.001$, $DF=9$)



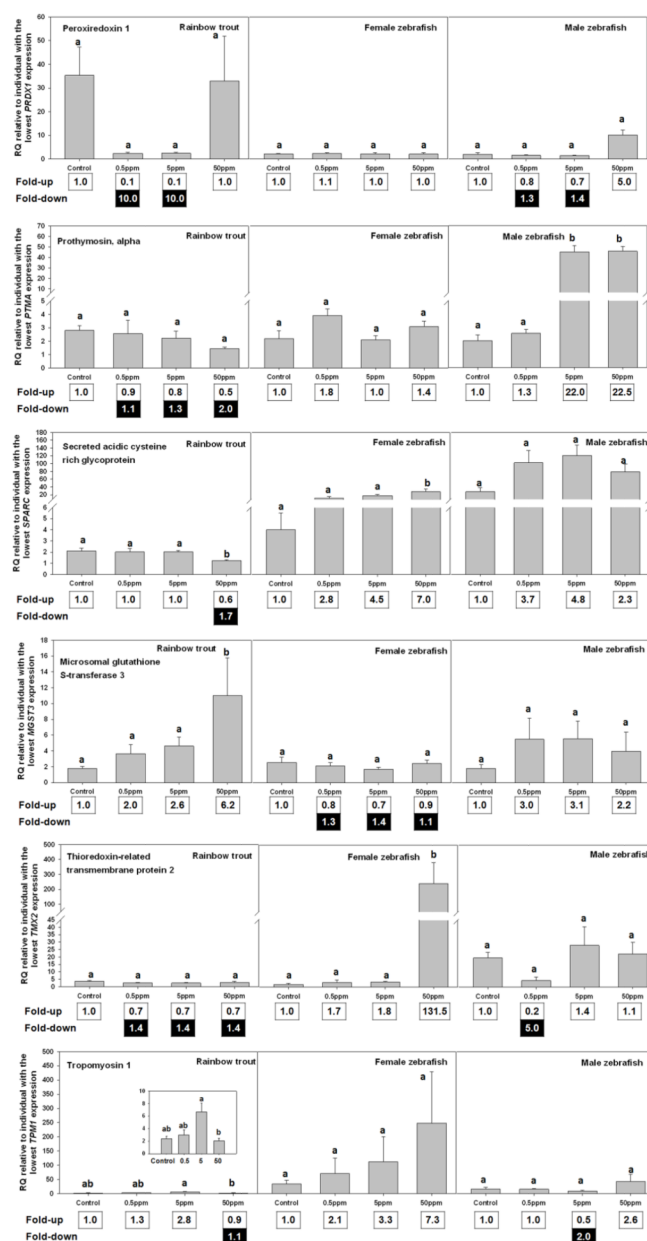


Fig. 3. QPCR analyses of 12 selected genes. Gene expression data are presented as mean (±standard error) RQ (relative quantity) normalized to guanine nucleotide binding protein (G protein), beta polypeptide 2-like 1 (gnb2l1) and calibrated to the individual with the lowest expression of target gene. Overall fold-changes were presented in the box under the bar. Fold change (Fold-up) was calculated as (average RQ of treatment group)/(average RQ of 0 ppm group). Down-regulation is indicated by a Fold-up value <1.0 and was converted to a Fold-down value (in black box) by taking the inverse of the Fold-up value. Lower case letters denotes statistically significant differences ($p < 0.05$) using multiple comparison by one-way ANOVA.

Table 1

Gene Ontology (GO) “Biological Process” and “Molecular Function” classifications of the 15 most significantly dysregulated genes in MeHg-treated rainbow trout ($p < 0.05$)

Biological process	Molecular function
iron ion transport	ferric iron binding
cellular iron ion homeostasis	iron ion binding
iron ion homeostasis	oxidoreductase activity
transition metal ion transport	cation binding
di-, tri-valent inorganic cation transport	ion binding
cellular di-, tri-valent inorganic cation homeostasis	metal ion binding
di-, tri-valent inorganic cation homeostasis	extracellular matrix structural constituent
cellular cation homeostasis	transition metal ion binding
cation homeostasis	glutathione transferase activity
cellular ion homeostasis	signal sequence binding
cellular chemical homeostasis	ER retention sequence binding
cellular homeostasis	binding
ion homeostasis	thrombospondin receptor activity
chemical homeostasis	heat shock protein binding
metal ion transport	transferase activity, transferring alkyl or aryl (other than methyl) groups

Table 2

Gene Ontology (GO) “Biological Process” and “Molecular Function” classifications of the 15 most significantly dysregulated genes in MeHg-treated female and male zebrafish ($p < 0.05$)

Biological process		Molecular function	
Female	Male	Female	Male
RNA splicing	cellular protein metabolic process	calcium-dependent ATPase activity	purine nucleotide binding
regulation of striated muscle contraction	protein metabolic process	troponin I binding	ribonucleotide binding
skeletal muscle contraction	tyrosyl-tRNA aminoacylation	tRNA-specific ribonuclease activity	purine ribonucleotide binding
mRNA metabolic process	axoneme assembly	troponin C binding	adenyl nucleotide binding
multicellular organismal movement	cilium axoneme assembly	nucleic acid binding	tyrosine-tRNA ligase activity
musculoskeletal movement	cilium movement	argininosuccinate lyase activity	purine nucleoside binding
mRNA processing	protein folding	melanin-concentrating hormone receptor activity	nucleoside binding
cellular biosynthetic process	tRNA aminoacylation for protein translation	calcium-dependent protein binding	nucleotide binding
nucleic acid metabolic process	amino acid activation	zinc ion binding	ATP binding
gene expression	tRNA aminoacylation	glucosamine-6- phosphate deaminase activity	adenyl ribonucleotide binding
ornithine metabolic process	microtubule bundle formation	ribonuclease P activity	oligosaccharyl transferase activity
induction of apoptosis via death domain receptors	cell projection assembly	alanine-tRNA ligase activity	protein serine/threonine kinase activity
arginine biosynthetic process via ornithine	post-translational protein modification	endoribonuclease activity	unfolded protein binding
nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	RNA interference	tropomyosin binding	tRNA binding
cellular nitrogen compound metabolic process	regulation of caspase activity	amidine-lyase activity	aminoacyl-tRNA ligase activity