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Combined Transcriptomic and Proteomic Approach to Identify Toxicity Pathways in Early Life Stages of Japanese Medaka (*Oryzias latipes*) Exposed to 1,2,5,6-Tetrabromocyclooctane (TBCO)

Jianxian Sun,[†] Song Tang,[‡] Hui Peng,[*,†] David M.V. Saunders,[†] Jon A. Doering,[†] Markus Hecker,[†,‡] Paul D. Jones,[†,‡] John P. Giesy,[†,§,∥,⊥,#] and Steve Wiseman[*,†,▽]

[†]Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B3, Canada

[‡]School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5C8, Canada

[§]Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada

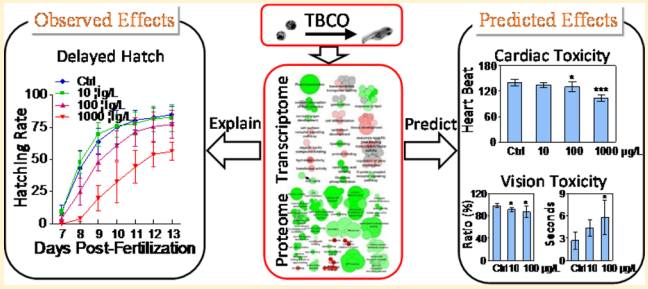
[∥]Zoology Department, Center for Integrative Toxicology, Michigan State University, East Lansing, Michigan 48824, United States

[⊥]School of Biological Sciences, University of Hong Kong, Hong Kong Special Administrative Region 999077, People's Republic of China

[#]State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, People's Republic of China

Supporting Information

ABSTRACT: Currently, the novel brominated flame retardant 1,2,5,6-tetrabromocyclooctane (TBCO) is considered a potential replacement for hexabromocyclododecane (HBCD). Therefore, use of TBCO could increase in the near future. To assess potential toxicological risks to aquatic organisms, embryos of Japanese medaka (*Oryzias latipes*) were exposed to 10, 100, or 1000 μg/L TBCO from 2 h postfertilization until 1 day post-hatch. TBCO accumulated in embryos in the order of 0.43−1.3 × 10^4-fold, and the rate constant of accumulation was 1.7−1.8 per day. The number of days to hatch and the hatching success of embryos exposed to the medium and the greatest concentrations of TBCO were impaired. Responses of the transcriptome (RNA-seq) and proteome were characterized in embryos exposed to 100 μg/L TBCO because this was the least concentration of TBCO that caused an effect on hatching. Consistent with effects on hatching, proteins whose abundances were reduced by exposure to TBCO were enriched in embryo development and hatching pathways. Also, on the basis of the responses of transcriptome and proteome, it was predicted that TBCO might impair vision and contraction of cardiac muscle, respectively, and these effects were confirmed by targeted bioassays. This study provided a comprehensive understanding of effects of TBCO on medaka at early life stages and illustrated the power of "omics" to explain and predict phenotypic responses to chemicals.



INTRODUCTION

Brominated flame retardants (BFRs), including polybrominated diphenyl ethers (PBDEs) and hexabromocyclododecanes (HBCDs), have been detected in various environmental matrices.[1−3] In response to concerns about their persistence, potential for bioaccumulation, and toxic potencies (PBT), production and usage of the technical mixtures, PentaBDE and OctaBDE, have been banned or phased out, and HBCD and DecaBDE were phased out in European markets in 2015 and will soon be phased out of use in North America.[4] Novel brominated flame retardants (NBFRs) are being developed to replace currently used BFRs that have been, or will be, phased out of use or banned. The NBFR 1,2,5,6-tetrabromocyclooctane (TBCO) (Figure S1), which is marketed as Saytex BCL-48, is a potential alternative to HBCD. Amounts of TBCO in the environment are expected to increase as its usage in textiles, paints, and plastics increases.[5] TBCO has been detected in dust (2 ng/g, dry mass (dm)) from homes in northern California,[6] in samples of sediments (1 ng/g dm), and in European dabs (*Limanda limanda*, 12 ng/g, wet mass (wm)) collected in 2013 from the German Bight in the North Sea.[7] On the basis of screening-level assessments, TBCO might be considered a hazardous substance because it has the potential to persist and bioaccumulate in the environment.[8] Thus, information on toxicity of TBCO to aquatic organisms, including fish, is critical for the assessment of its potential ecological and health risks.

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Little is known about adverse effects or underlying mechanism(s) of responses of organisms to exposure to TBCO. Results of in vitro experiments, including assays of hormone receptor activation with the yeast estrogen (YES) and androgen (YAS) screens and synthesis of sex steroids in H295R cells, demonstrated potential for TBCO to modulate endocrine function through interactions with estrogen and androgen receptors and via alterations in synthesis of 17-β-estradiol and testosterone.[9,10] Exposure to TBCO via diet for 21 days impaired reproductive capacity of Japanese medaka (*Oryzias latipes*), as indicated by lesser fecundity and altered expression of key regulatory genes along the hypothalamus−pituitary−gonad−liver (HPGL) axis.[11] Therefore, a more comprehensive assessment of effects of TBCO on aquatic organisms is warranted.

Demands for toxicity testing of an increasing number of chemicals while using fewer animals in these tests has spurred efforts to develop alternative testing strategies that are cost-effective and high-throughput and provide insight into a chemical mode of action. In this regard, use of early life stages (ELS) of fishes, especially embryos, which in most cases are considered the most sensitive life stage to chemical stressors, has received much attention.[12−14] Also, use of ELS for toxicity testing addresses concerns about animal welfare because, according to legislation within the European Union and North America, ELS of fish are not considered live animals until they begin exogenous feeding.[15]

The goal of the current study was to utilize next-generation transcriptomic and proteomic approaches to characterize molecular responses in ELS of Japanese medaka, a model species of teleost that has been used extensively in toxicity testing. Embryos were exposed to three incremental concentrations of TBCO, and effects on survival, time to hatch, hatching success, and incidences of malformations were determined. Responses of the transcriptome and proteome were investigated in embryos exposed to the medium concentration of TBCO, which was the least concentration to cause effects on hatching. Putative toxicity pathways were identified that might explain effects of TBCO on hatching of embryos. Other putative toxicity pathways that were identified predicted that exposure to TBCO impaired cardiac function and visual performance in embryos, and these effects were confirmed in separate exposures by use of bioassays to quantify these phenotypes. In addition to providing greater understanding of potential toxicities of TBCO, this study illustrated the sensitivity and power of combining "omics technologies" and bioassays with ELS of fishes for elucidating molecular mechanisms of toxicity and predicting toxicities of chemicals.

MATERIALS AND METHODS

Chemicals and Reagents. 1,2,5,6-Tetrabromocyclooctane (TBCO) and 6-fluoro-2,2′,4,4′-tetrabromodiphenyl ether (F-BDE-47) were obtained from Specs (Delft, Netherlands) and AccuStandard (New Haven, CT), respectively. All solvents were of analytical grade and obtained from Fisher Scientific (Ottawa, ON, Canada).

Exposure of Embryos to TBCO. Embryos of medaka were obtained from a breeding culture maintained at the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan (Saskatchewan, Canada). Medaka were maintained according to protocols approved by the University of Saskatchewan Committee on Animal Care and Supply and Animal Research Ethics Board (UCACS-AREB; no. 200090108).

Embryos were collected from breeding tanks, and those that had not yet passed the 32 cell stage of development (approximately 2 h postfertilization) were selected. Embryos were randomly assigned to 100 mm glass Petri dishes containing 30 mL of acetone in medium (0.1% v/v) as the solvent control or 10, 100, or 1000 μg/L nominal concentrations of TBCO. Exposure concentrations were based on a previous study in which fecundities were significantly decreased in adult medaka exposed to TBCO via the diet for 21 days.[11] These concentrations are comparable to those of TBCO detected in environmental matrices.[7] Approximately 100 embryos were assigned to each Petri dish, and exposures were replicated seven times, each with a separate batch of eggs (n = 7). Stock solutions of TBCO were prepared in acetone, and working solutions were prepared each day by spiking the stock solution into embryo rearing media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$). Concentrations of acetone did not exceed 0.1% (v/v) in any of the treatment or control groups. Exposures were conducted at 27 ± 0.5 °C and 16:8 h light−dark cycle, and exposures lasted until the first day post-hatch (8−13 days). Exposure media were changed twice daily. Any embryos that were dead were removed immediately, and mortalities and number of embryos that had hatched was recorded every 12 h. All embryos collected on the first day post-hatch were snap-frozen in liquid N_2 and stored at −80 °C for further study. For the purpose of this study, stages of development were based on stages of development of zebrafish (*Danio rerio*) outlined elsewhere.[12,16] Medaka were considered to be at the embryo stage of development until they hatched from eggs, at which time they were considered to be at the eleutheroembryo stage of development. However, the term "embryo" is used to describe both of these stages. Once medaka began exogenous feeding, they were considered to be at the larval stage of development.

Quantification of TBCO. Concentrations of TBCO in exposure media and in eggs were determined by modification of a method that was published previously.[1,11] A total of three eggs that had not hatched were collected randomly from each Petri dish on days 1, 3, 5, 7, and 9, and eggs from every two dishes were pooled as a sample so that the sample size was 3 (n = 3). Approximately 5 mL of media were collected from each of the seven Petri dishes of each exposure group prior to changing media on days 2, 4, and 6. TBCO was extracted and analyzed by use of an Agilent (Santa Clara, CA) 7890A gas chromatograph (GC) system coupled to an Agilent 5975C mass spectrometer (MS). Details of sample preparation and analysis of TBCO are given in the Supporting Information.

RNA-seq. Responses of the transcriptome to TBCO were determined in embryos at 1 day post-hatch. A total of five embryos from the same Petri dish were pooled as one sample, and libraries were constructed from three samples of embryos exposed to the solvent control and three samples of embryos exposed to the medium concentration of TBCO by use of the Tru-Seq RNA Sample Prep Kit (Illumina, San Diego, CA) according to the protocol provided by the manufacturer, as described previously.[17] Sequencing reads of each replicate were mapped independently against the reference genome of Japanese medaka[18] (Ensembl, v1.78) by use of TopHat2 (v0.6)[19] on Galaxy public server (http://galaxy-qld.genome.edu.au).[20] Levels of expression were calculated, and statistical significances of changes in abundances of transcripts were

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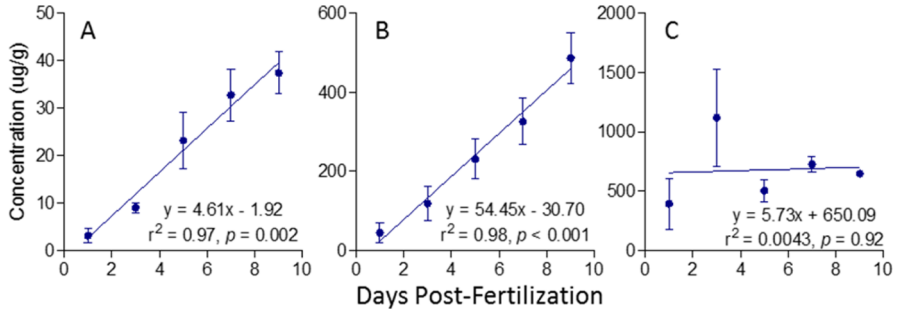

Figure 1. Uptake and bioaccumulation of TBCO in eggs of Japanese medaka exposed to (A) 10, (B) 100, or (C) 1000 μg/L TBCO. TBCO was analyzed by use of an Agilent 7890A gas chromatograph system coupled to an Agilent 5975C mass spectrometer operating in electron-impact-ionization mode. A total of three eggs were collected randomly from each Petri dish on days 1, 3, 5, 7, and 9, and eggs from pairs of dishes were pooled as a single sample so that the sample size was 3 (n = 3). Data are expressed as the mean ± standard deviation.

determined by use of Cuffdiff (v0.0.7). Details on RNA extraction, sequencing, and RNA-seq are provided in the Supporting Information.

Proteomics. To assess responses of the proteome to TBCO, we sampled 50 embryos collected at 1 day post-hatch from the same Petri dish, pooled as one sample, and four samples of embryos exposed to the solvent control (n = 4), and four samples of embryos exposed to the medium concentration of TBCO (n = 4) were selected randomly. Samples were analyzed by use of a LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific) equipped with a nanoLC-electrospray ion source (Proxeon, Mississauga, ON). Details of sample digestion, liquid chromatography–tandem mass spectrometry (LC–MS/MS) parameters and database search are provided in the Supporting Information. Label-free proteomics based on spectral counts was conducted, and proteins were defined as being differentially expressed if the p value was less than 0.05 and the fold-change in abundance at least ±1.5-fold. This cutoff was applied to exclude those proteins with very small changes in abundance, as described elsewhere.[21]

Gene Ontology Enrichment Analysis. Biological processes affected by TBCO were identified using transcripts and proteins whose abundances were affected in embryos exposed to 100 μg/L TBCO. Uniprot IDs of medaka proteins were converted to Zebrafish IDs using BioMart. Functions and pathway enrichment based on significant changes in abundance of transcripts or proteins were investigated using ClueGO (v2.1.6)[22] run through Cytoscape (v3.2.1).[23] Because of the paucity of annotation information, transcripts and proteins of medaka were matched to zebrafish orthologs on the basis of data sets, including the Kyoto Encyclopedia of Genes and Genomes (KEGG) and the Gene Ontology (GO) Consortium Biological Processes. The statistical test was a right-sided hypergeometrical test with a Bonferroni (step-down) p value correction and a κ score of 0.4.

Assessment of Visual Performance and Cardiac Function. New batches of embryos were used to assess if there were adverse effects of TBCO on visual performance and cardiac function, as predicted from transcriptomic and proteomic data, respectively. Exposures were performed using the same conditions described above except the duration of the exposure was different, and they were performed in quadruplicate (n = 4). Embryos were exposed to solvent control or TBCO (10, 100, or 1000 μg/L) from 2 h postfertilization until 14 days post-hatch to assess visual performance. A background-color-preference assay developed to assess visual performance of *Xenopus laevis*[14] was used to assess visual performance of larvae. For the assay, 20 larvae were selected randomly from each Petri dish. Larvae at 14 days post-hatch were selected for the assay because larvae collected prior to this day of development were not active enough. Visual performance of larvae exposed to the greatest concentration of TBCO could not be determined because larvae did not survive exposure until 14 days post-hatch.

To assess cardiac function, embryos were exposed to solvent control or TBCO (10, 100, or 1000 μg/L) from 2 h postfertilization until 5 days postfertilization because this is the critical period for development of this organ in medaka.[24] A total of three embryos from each of four Petri dishes per exposure group were randomly selected for the assay, and cardiac function was determined by quantifying numbers of heart beats per minute in each embryo. Details of these bioassays are provided in the Supporting Information.

Data Analyses. Statistical analyses were performed either by use of R software (v3.1.2, http://www.R-project.org; R Foundation for Statistical Computing, Vienna, Austria) or IBM SPSS Statistics v19.0 (IBM Corporation, Armonk, NY). Linear regression was performed to evaluate uptake of TBCO by embryos as a function of duration of exposure. Sample size for analysis of transcriptomics data was n = 3 for the control and n = 3 for the medium concentration of TBCO. The sample size for analysis of proteomics data was n = 4 for the control and n = 4 for the medium concentration of TBCO. Distance-based redundancy analysis (dbRDA) was performed using the "vegan", "ggplot2", and "ellipse" packages through R to evaluate statistical difference of responses to TBCO. A permutation test similar to a one-way analysis of variance (ANOVA) that was available in the "vegan" package was performed to determine which axes explained the greatest percentage of sample variation and if sample clustering was statistically different between control and TBCO groups. Effects of TBCO on heart rate and visual performance were assessed by use of ANOVA followed by a Dunnett's posthoc test. Prior to analysis, data were assessed for homogeneity of variance by use of Levene's test and for normality by use of the Shapiro–Wilk test, and data were log-transformed if necessary. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Uptake of TBCO. Concentrations of TBCO in exposure media at nominal concentrations of 10, 100, and 1000 μg/L were determined to be 2.7 ± 0.4, 29.6 ± 7.5, and 151.3 ± 81.9 μg/L, respectively (Table S1), while concentrations in the

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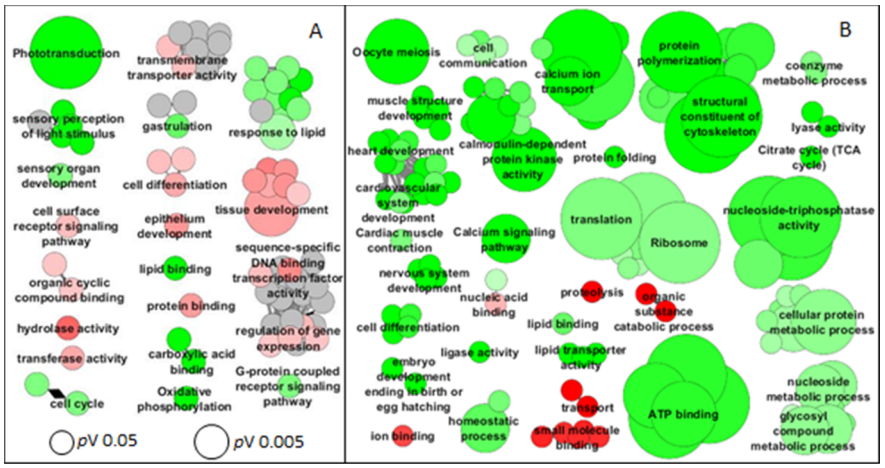

Figure 2. Cytoscape visualization of the ClueGo clustering results of significantly enriched gene ontology categories of (A) transcripts and (B) proteins that were of different abundances in embryos of Japanese medaka exposed to 100 μg/L TBCO compared to the control. Embryos were collected on the first day post-hatch (8 days and 8−10 days postfertilization for transcriptomes and proteomes, respectively). The significance of GO terms (p value, which is indicated as pV, was corrected with the Benjamini−Hochberg procedure) is expressed by the size of the node. Significantly decreased and increased GO terms are shown in green and red, respectively. The intensity of color is proportional to the extent of the decrease or increase. Gray indicates regulated pathways with both increased and decreased transcripts and proteins. A total of three samples of RNA (n = 3) and four samples of proteins (n = 4) from separate pools of embryos exposed to a control solution of acetone (0.1% v/v) and to 100 μg/L TBCO were analyzed.

solvent control were less than the limit of detection (LOD). The relatively small concentrations of TBCO measured in exposure media likely was because of uptake of the chemical by embryos, which has been reported previously.[25] Concentrations of TBCO in embryos exposed to the least and medium concentrations increased in a time- and an exposure-dependent manner (Figure 1). Concentrations in embryos exposed to the least concentration increased from 3.2 ± 1.5 μg/g wm on day 1 of exposure to 37.4 ± 4.4 μg/g wm on day 9 (p = 0.002), and concentrations in embryos exposed to the medium concentration increased from 45.4 ± 24.6 μg/g wm on day 1 to 486.5 ± 63.8 μg/g wm on day 9 (p < 0.001). However, concentrations of TBCO in embryos exposed to the greatest concentration increased only 2-fold from 394.7 ± 212.2 μg/g wm on day 1 of exposure to 647.7 ± 24.1 μg/g wm on day 9 of exposure, and the trend was not significant (p = 0.92). Because environmental chemicals can be sequestrated in the chorion,[26] the amount of TBCO measured in eggs might not be the actual concentration to which embryos were exposed. Therefore, concentrations of TBCO in embryos that hatched on day 9 of the exposure were quantified and were 35.0 ± 9.0, 423.7 ± 37.9, and 661.5 ± 380.8 μg/g wm in embryos exposed to the lowest, medium, and greatest concentrations of TBCO, respectively. Concentrations of TBCO in embryos that had hatched were similar to those in unhatched eggs collected on the same day, suggesting that TBCO accumulated in embryos rather than being sequestrated by the chorion. Less accumulation in fish exposed to great concentrations of chemical has been reported previously and was suggested to be due to saturation of binding biomolecules (e.g., proteins).[25,27,28] Therefore, studies to determine if a similar mechanism prevents accumulation of TBCO when concentrations in exposure media are great are warranted. The rate constant of accumulation of TBCO by embryos exposed to the least and medium concentrations were 1.7 and 1.8 per day, respectively, calculated based on a kinetic rate equation.[29] At day 9, concentrations of TBCO in embryos exposed to the least and medium concentrations were 1.4×10^4- and 1.6×10^4-fold greater, respectively, than concentrations in the exposure medium. These values are approximately 100-fold greater than those of HBCD (2×10^2-fold) in eggs of medaka after waterborne exposure to 5−50 μg/L of HBCD for 8 days.[30] Greater bioaccumulation of TBCO than HBCD might be due in part to the fewer bromine atoms incorporated into the molecule, the smaller size, and the smaller K_{OW} of TBCO. Greater bioaccumulation of TBCO highlights the potential risk of the compound as a replacement for HBCD.

Global Changes in Transcript and Protein Abundances. Among the 20 425 genes in the reference genome of Japanese medaka, abundances of 240 transcripts (141 up-regulated and 99 down-regulated) were significantly altered in embryos exposed to the medium concentration of TBCO (Figure S2A). Global proteome profiling showed that 1256 unique proteins at an FDR of 0.1% were identified in embryos. Among these, abundances of 189 proteins (68 up-regulated and 121 down-regulated) were significantly altered in embryos exposed to TBCO (Figure S2B). On the basis of distance-based redundancy analysis (dbRDA), expression profiles of the transcriptome (p = 0.04, Figure S3A) and proteome (p = 0.001, Figure S3B) of embryos exposed to TBCO were determined to be significantly different from embryos exposed to the solvent control. Poor segregation of transcripts of embryos exposed to TBCO from the control was partly due to the relatively small changes in abundances of transcripts (0.05−4.8-fold), which, in general, was less than changes in abundances of proteins (0.02−43.5-fold), indicating that the proteome of embryos might be more sensitive to longer-term exposure to TBCO. However, the greater sample size used for proteomics (four replicates with 50 embryos per replicate) than transcriptomics (three replicates with five embryos per replicate) also might contribute to better segregation of proteome data.

Although a significant correlation (r = 0.26, p < 0.001) between abundances of proteins (indicated by spectral counts) and transcripts (indicated by gene reads) was observed in embryos exposed to the solvent control (Figure S3C), the

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correlation coefficient was small and abundances of some proteins deviated from the log−linear regression. Several factors might explain these results. For example, different rates of turnover of mRNAs and proteins could lead to differences in abundances of transcripts and proteins when both are measured in samples taken at the same time point.[31] Alternatively, variation of transcriptome and proteome of embryos might be complicated by maternal transfer of biomolecules. Supporting this hypothesis, we identified several vitellogenin proteins (VTGs) on the basis of spectral counts as the most abundant proteins in embryos (spectral counts: 1755 for VTG1 and 872 for VTG6), while abundances of transcripts of these proteins were small. Although other processes might contribute to this observation, the great variation from the log−linear regression suggested that these VTG proteins might be maternally transferred to embryos, which is consistent with the function of VTG proteins as nutrients for developing embryos.[32]

Enrichment analysis of transcriptome data revealed that several processes, including development of sensory organs, lipid binding, and oxidative phosphorylation were significantly down-regulated, while several other processes, such as transmembrane transporter activity, were significantly up-regulated (Figure 2A). In contrast to analyses based on the transcriptome, enrichment analysis with proteome data revealed that most processes were down-regulated, including expression of ribosome proteins, translation, and protein folding, which suggested that exposure to TBCO might inhibit biosynthesis of proteins. Down-regulated proteins were also significantly enriched in processes such as cardiac-muscle contraction, oocyte meiosis, and metabolism (Figure 2B). Consistent with the weak correlation between responses of the proteome and transcriptome, enrichments of pathways as determined by analysis of the transcriptome and proteome were weakly correlated. This phenomenon has been documented previously[33] and might be due to post-transcriptional effects, different half-lives of proteins and mRNA, and complicated signal transduction pathways with multiple points of regulation. On the basis of these results, it was determined that the simultaneous analysis of responses of the transcriptome and proteome would be necessary to obtain a more complete understanding of the molecular mechanisms of toxicity of TBCO in embryos.

Changes in Transcript and Protein Abundances and an Explanation of Observed Effects of TBCO on Embryos: Hatching of Embryos. One goal of this study was to use enrichment analysis of transcripts and proteins whose abundances were different in embryos exposed to TBCO to identify mechanisms of physiological and pathological effects readily observed during exposure. Exposure to TBCO did not increase incidences of mortality or deformities of embryos, but a concentration-dependent increase in number of days to hatch and decrease in success of hatching was observed (Figure 3). Effects of exposure to TBCO on time to hatch is reported as days needed for 50% of embryos to hatch. This is similar to the use of LC_{50} to report effects of chemicals on survival.[34] Embryos started to hatch after 7 days of exposure, and approximately 50% of embryos hatched after 7.8 and 8.0 days of exposure to solvent control and the least concentration of TBCO, respectively. However, time required for 50% of embryos exposed to medium and greatest concentrations of TBCO to hatch were significantly increased to 8.8 ($p = 0.0003$) and 9.7 days ($p < 0.0001$), respectively. After 13 days of exposure, cumulative hatching of eggs exposed to solvent control and least or medium concentrations of TBCO were approximately 81.6 ± 9.5%, 77 ± 9.3%, and 72.4 ± 10.1%, respectively. However, only 54.3 ± 10% ($p < 0.001$) of eggs exposed to the greatest concentration of TBCO hatched after 13 days of exposure. Effects similar to these have been reported for PBDEs at a similar range of concentrations.[35,36]

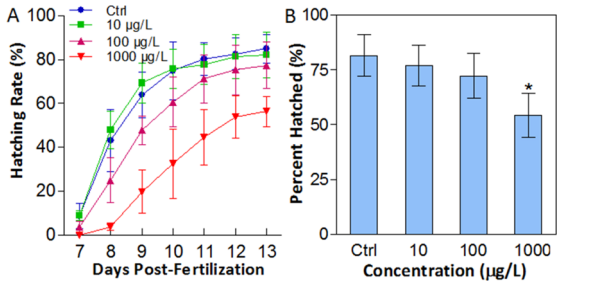

Figure 3. Effects of TBCO on hatching of embryos of Japanese medaka. (A) Time to hatch and (B) percentage of embryos hatched at day 13 were determined. Embryos were exposed to a control solution of acetone (0.1% v/v) or 10, 100, or 1000 µg/L TBCO, beginning approximately at 2 h postfertilization. The assay was performed seven times ($n = 7$), each with a separate batch of embryos. Data are expressed as the mean ± standard deviation. An asterisk (*) indicates a significant difference from the solvent control ($p \leq 0.05$).

Because embryos exposed to the medium concentration of TBCO exhibited moderately adverse effects, this group was selected for analysis of responses of the transcriptome and proteome to evaluate molecular mechanisms of toxicity. Consistent with the effects of TBCO on hatching of embryos, significantly down-regulated proteins were enriched in the pathway "embryo development ending in birth or egg hatching" at the level of the proteome (Figure 2B). Among the five proteins enriched in this pathway, abundances of four subunits of ribosomal proteins (RPS4x, RPS3, RPL11, and RPS19) were significantly lesser relative to controls (0.04−0.40-fold), suggesting that TBCO impaired biosynthesis of proteins. In fact, pathways of translation and protein folding (Figure 2) also were significantly down-regulated in medaka exposed to TBCO. These results are consistent with those of another study, which reported that expression of highly abundant ribosomal proteins is essential for normal development of zebrafish.[37]

Changes in Transcript and Protein Abundances and Predictions of Apical Effects. Perturbations of gene expression often are critical events in toxicity pathways of adverse effects at higher levels of biological organization.[38,39] Therefore, bioassays were utilized to determine if changes in abundances of transcripts and proteins were predictive of adverse biological outcomes in ELS of medaka exposed to TBCO. Several phenotype-related pathways, including phototransduction, cardiac-muscle contraction, and oocyte meiosis were enriched in embryos exposed to TBCO (Figure 2). Therefore, targeted bioassays were used to determine if phenotypes related to these pathways were affected in ELS of medaka exposed to TBCO.

Visual Perception. Several transcripts that had significantly lesser abundances in embryos exposed to TBCO than in embryos exposed to the solvent control were enriched in pathways related to eye development, phototransduction, and sensory perception of light stimulus, which suggested that exposure to TBCO might impaired visual performance (Figure 4A). Abundances of transcripts of *atoh7*, *atp6v1f*, *col15a1b*,

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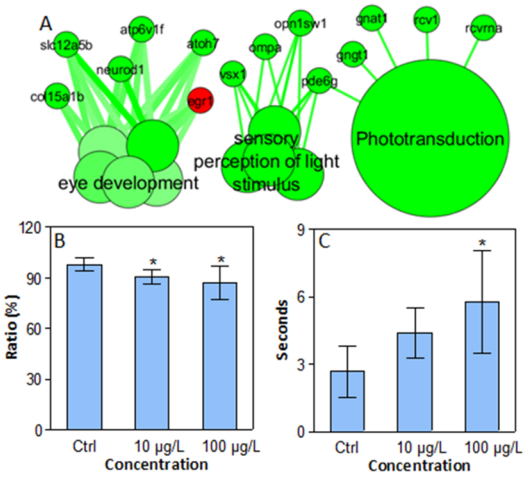

Figure 4. Effects of TBCO on visual performance of larvae. (A) Cytoscape visualization of the results of ClueGO clustering of significantly decreased expression of genes important for development and visual performance of the eye (eye development, sensory perception of light stimulus, and phototransduction). The significance of GO terms (p value corrected with the Benjamini–Hochberg procedure) is expressed by the size of the node. Down-regulated genes and GO terms are shown in green, and up-regulated genes are colored in red. (B) Percentage of larvae that swam to the white side of the vessel within 30 s. (C) Average time required to swim to the white side of the vessel. Data in panels B and C are expressed as the mean ± standard deviation. Visual performance was determined with early life stages that had been exposed to a control solution of acetone (0.1% v/v) or 10 or 100 µg/L TBCO from approximately 2 h postfertilization until 14 days post-hatch. There were 20 larvae per trial, and trials were conducted on four separate batches of larvae (n = 4). Data in panels B and C are expressed as the mean ± standard deviation. An asterisk (*) indicates a significant difference from the solvent control (p < 0.05).

neurod, and *slc12a5b*, which are important for development of the eye were significantly less (0.51–0.73-fold), and the abundance of transcripts of *egr1* was significantly greater (1.43-fold) in embryos exposed to the medium concentration of TBCO. The protein product of *col15a1b* is a component of the internal limiting membrane (ILM) of the retina, and knockdown of this gene in zebrafish resulted in smaller eyes.[40] The protein product of *atoh7* is a basic helix–loop–helix transcription factor that is important for the formation of retinal ganglion cells that form the optic nerve.[41] Lesser expression of this gene has been associated with nonsyndromic congenital retinal nonattachment, a consequence of which is insensitivity to light. Abundances of transcripts of *rcvrna*, *gngt1*, *rcv1*, *pde6g*, and *gnat1*, which are important for phototransduction, were significantly less (0.57–0.71-fold) in embryos exposed to TBCO (Figure 4A). Guanine nucleotide-binding protein G(t) subunit α (*gnat1*) and recoverin (*rcv1*) are expressed in the photoreceptor layer of the retina, and reduced expression of these genes lead to fewer signals being received by the transmembrane receptor.[42] Abundances of transcripts of *ompa*, *opn1sw1*, *vsx1*, and *pde6g* that are important for sensory perception of light stimulus were significantly less (0.67–0.71-fold) in embryos exposed to TBCO (Figure 4A). This neurological process is responsible for converting light stimuli into a molecular signal. cGMP phosphodiesterase type 6 (*pde6*) is the primary effector enzyme in the phototransduction cascade.[43] Stimulation of photoreceptor visual pigments by light activates phosphodiesterase, which hydrolyses cGMP to GTP,[44] and insufficient breakdown of cGMP leads to cell death in cones and rods. The cone opsin gene 1, which is short-wave-sensitive (*opn1sw1*), is responsible for perception of short wavelengths (ultraviolet) of daylight,[45] and lesser expression of this gene would affect perception of color. Visual system homeobox 1 (*vsx1*) regulates proliferation and differentiation of retinal progenitor cells and maintains function of bipolar cells in vertebrates.[46]

On the basis of reduced expression of genes important for eye development, phototransduction, and sensory perception of light stimulus, it was hypothesized that visual function would be negatively affected by exposure to TBCO. To test this hypothesis, we used a background-color preference assay[47] to investigate visual function in medaka exposed to the least and medium concentrations of TBCO until 14 days post-hatch. Almost all larvae exposed to the solvent control (96.3 ± 5.2%) gathered over the white background, whereas significantly fewer larvae exposed to the least (90.6 ± 4.2%, p = 0.013) and medium (86.9 ± 10%, p = 0.028) concentrations of TBCO gathered over the white background (Figure 4B). Also, most larvae exposed to the solvent control swam directly to the white side of the testing apparatus, whereas no pattern of swimming was observed for larvae exposed to the medium concentration of TBCO. Thus, it took an average of 2.7 ± 1.1 s for larvae exposed to the solvent control to gather over the white background portion of the tank, and it took 4.4 ± 1.1 s (p = 0.073) or 5.8 ± 2.3 s (p = 0.05) for larvae exposed to the least or medium concentration of TBCO, respectively, to move to the white background (Figure 4C). These results indicated that exposure to TBCO during early stages of development might impair visual performance by inhibiting expression of genes required for development and performance of the eye.

Cardiac-Muscle Contraction. The cardiovascular system in vertebrates is a target of some environmental pollutants. Responses of the proteome showed that exposure to TBCO significantly altered abundances of several proteins that are important for development and activity of the cardiovascular system. Specifically, abundances of cox4i, vmhc, uqcrc, and cmlc, which are important for development of the cardiovascular system, were significantly less (0.14–0.50-fold) in embryos exposed to TBCO. Ventricular and cardiac isoforms of myosin (vmhc and cmlc) have distinct roles during cardiogenesis[48] and it has been shown that expression of these genes was affected by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a chemcial known to affect cardiac function.[49] Two cytochrome c oxidase proteins, cox4i (0.14-fold less) and cox6b2 (3.71-fold greater), were differentially expressed in embryos exposed to TBCO (Figure 5A). Altered expression of these proteins can cause metabolic disorders and impair energy metabolism in cardiac tissue.[49] Results of the RNA-seq did not identify pathways related to the cardiovascular system as being enriched with transcripts of increased or decreased abundance in embryos exposed to TBCO. Enrichment at proteome level for cardiac function was different from those of visual perception, which was enriched at transcriptome level. Such results highlight the advantages of analyzing the transcriptome and proteome for different apical end points. But abundance of a single transcript (calcium channel gamma, *cacng6a*) was significantly less (0.61-fold) in embryos exposed to TBCO (Figure 5A). The protein product of this gene is critical for excitation and contraction of cardiac muscle.[50]

On the basis of effects of TBCO on abundances of proteins that are important for development and function of the cardiovascular system, it was hypothesized that cardiac function

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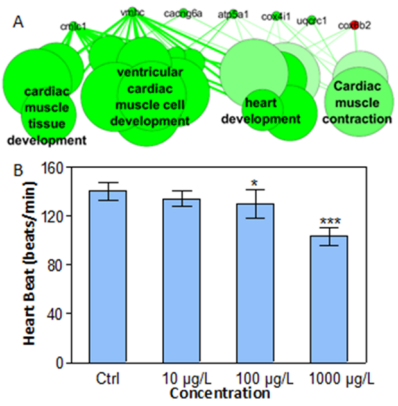

Figure 5. Effects of TBCO on the heart rates of embryos of Japanese medaka. (A) Cytoscape visualization of ClueGO clustering results of significantly altered pathways related to the development of the heart and the contraction of cardiac muscle. With the exception of one transcript (*cacng6a*) that was less abundant in embryos exposed to TBCO, the effect was evident at the proteome level. Significance of GO terms (p value corrected with the Benjamini−Hochberg procedure) is expressed by the size of the node. Reduced abundant proteins and enriched GO terms are shown in green, and increased abundant proteins are colored in red. (B) Effects of TBCO on heart rate in medaka embryos at day 5. Heart rates were determined with embryos that had been exposed from approximately 2 h postfertilization until 5 days postfertilization to a control solution of acetone (0.1% v/v)or 10, 100, or 1000 μg/L TBCO. A total of three embryos were randomly selected from each Petri dish for the quantification of the heartbeat, and there were four Petri dishes for each exposure (n = 4). Data are expressed as the mean ± standard deviation. An asterisk (*) indicates a significant difference from the solvent control (* = p < 0.05; ** = p < 0.01; *** = p < 0.001).

would be impaired in ELS of medaka exposed to TBCO. To verify this putative adverse outcome, heart rates were quantified in embryos exposed to TBCO until 5 days postfertilization, which is within the critical period for development of this organ in medaka.[24] Heart rates were reduced by exposure to TBCO in a concentration-dependent manner (Figure 5B). Heart rates of embryos exposed to the solvent control were 140 ± 7.6 beats per min. However, heart rates of embryos exposed to the least, medium, and greatest concentrations of TBCO were 134 ± 6.3 (p = 0.335), 130 ± 11.7 (p = 0.014), and 103 ± 7.4 (p < 0.001) beats per min, respectively. This represents a decrease of 26.4% in embryos exposed to the greatest concentration of TBCO. Previously, it has been determined that a reduction of heart rate by 20.2% is associated with malformation of embryos, and a reduction of heart rate by 37.8% is associated with death of embryos.[51] Although malformations were not observed in embryos exposed to the greatest concentration of TBCO, more than 50% of them did not survive exposure to TBCO until 14 days post-hatch, when embryos were raised for the background-color-preference assay. Lesser heart rate might have contributed to this adverse effect. Although the species of fish are different, the effect of TBCO on heart beat is in contrast to the faster heart rate of embryos of marine medaka (*Oryzias melastigma*) or zebrafish exposed to 5−50 μg/L of HBCD[30,52] or Japanese medaka exposed to 0.1−1 μg/L of TCDD[53] and might represent differences in toxic modes of actions of these chemicals. Differences in effects of TBCO and HBCD highlight the need for toxicity testing of replacements for current use BFRs.

Oocyte Meiosis. In medaka, oocytes undergo asynchronous development. Oogenesis begins early in development and then is arrested during meiosis I, during which time oocytes undergo a lengthy period of accumulation of yolk and other substances necessary for early embryogenesis.[54,55] Proteins that regulate oocyte meiosis were significantly less abundant in embryos exposed to TBCO (Figure 2B). In particular, abundances of proteins in the tyrosine 3-monooxygenase−tryptophan 5-monooxygenase activation family (ywha, also known as the 14−3−3 family), including ywhaqba, ywhabl, ywhaqb, ywhag, and ywhab (0.04−0.32-fold decreased), and proteins in the calcium- and calmodulin-dependent protein kinase 2 family (camk2), including camk2g, camk2a, camk2b, camk2g1, camk2b1, and camk2d2 (0.34-fold decreased) were significantly less in embryos exposed to TBCO. Ywha proteins are important for a variety of cellular processes, including meiosis. For example, these proteins bind to the cell cycle control protein Cdc25 in G2-arrested *Xenopus* oocytes.[56] Camk2 proteins are activated by calcium and calmodulin in oocytes, and the camk2-dependent signaling pathway is involved in the emission of the first polar body and pronuclear formation during oocyte meiotic maturation.[57] Inhibition of oocyte meiosis would disturb development of oocytes and might result in reduced fecundity. Although oogenesis was not assessed in embryos, fecundity of medaka exposed to TBCO as adults was reduced compared to the control group,[11] but it is not known if this effect was caused by effects on meiosis. However, the fecundity of medaka exposed to TBCO only by maternal transfer was significantly reduced compared to that in fish not exposed to TBCO (unpublished data). Lesser fecundity of fishes exposed to BFRs has been reported, and the effect was attributed to reduced male reproductive fitness[58] or to disruption of steroidogenesis.[11] Results of this study suggest that impairment of oocyte meiosis might be another mechanism by which chemicals can impair reproductive capacity.

Implications. Characterization of effects of TBCO by use of open-format, high-throughput "omics" technologies, including transcriptomics and proteomics applied in this study, allows for identification of toxicity pathways that can be used to establish models for predicting apical outcomes of regulatory relevance.[38,39,59,60] One such model that has gained increasing momentum and acceptance as a tool in regulatory toxicology is that of adverse outcome pathways (AOPs). AOPs are conceptual frameworks that organize knowledge about predictive and causal linkages (termed "key event relationships", KERs) between biological alterations (termed "key events", KEs) necessary for progression from a molecular initiating event (MIE) to an adverse outcome of regulatory relevance.[61−63] Because changes in gene expression are a key event in many toxicity pathways, transcriptomics and proteomics have been proposed as effective "middle-out" strategies for development of AOPs when MIEs or apical effects are not known.[57] Findings of the present study, which provided a comprehensive and mechanistic understanding of effects of TBCO on ELS of medaka, suggest that information garnered from the simultaneous use of transcriptomics and proteomics provides a comprehensive understanding of potential mechanisms of toxicity of chemicals and can be used in concert to predict apical effects on attributes that contribute to fitness, thereby illustrating the power of omics technologies as a "middle-out" approach for the development of AOPs. Finally, this study demonstrated the applicability of

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using mechanistic information from assays with ELS of a model species of fish for predicting biologically relevant outcomes and therefore strongly supports the use of ELS for toxicity testing in a research and regulatory context.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.6b01249.

Additional details on RNA-seq, protein preparation and identification, identification of proteins by mass spectrometry, analysis of TCBO, visual performance assessment, and cardiac function assessment. A table showing concentrations of TBCO in exposure media. Figures showing structures of HBCD and TBCO, a heatmap of significantly altered transcripts and proteins in embryos exposed to control and 100 μg/L TBCO, and biplots illustrating distance-based redundancy analysis (dbRDA) of transcriptome and proteome, and regression between transcripts and proteins from control samples. (PDF)

A table showing the fragments per kilobase of transcript per mapped reads, fold change, *p* values of significantly changed transcripts, and the spectral counts, fold change, and *p* values of significantly changed proteins. (XLSX)

■ AUTHOR INFORMATION

Corresponding Authors

*H.P. tel: +1 306 966 8733; fax: +1 306 966 4796; e-mail: huisci@gmail.com.

*S.W. tel: (403) 329-2320; fax: (403) 329-2082; e-mail: steve.wiseman@uleth.ca.

Present Address

[∇]Department of Biology, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Jianxian, S.; Hui, P.; Jianying, H. Temporal trends of polychlorinated biphenyls, polybrominated diphenyl ethers, and perfluorinated compounds in Chinese Sturgeon (*Acipenser sinensis*) eggs (1984−2008). *Environ. Sci. Technol.* **2015**, *49* (3), 1621−1630.

(2) Stapleton, H.; Dodder, N.; Kucklick, J.; Reddy, C. M.; Schantz, M.; Becker, P.; Gulland, F.; Porter, B.; Wise, S. Determination of HBCD, PBDEs and MeO-BDEs in California sea lions (*Zalophus californianus*) stranded between 1993 and 2003. *Mar. Pollut. Bull.* **2006**, *52* (5), 522−531.

(3) Schecter, A.; Haffner, D.; Colacino, J.; Patel, K.; Päpke, O.; Opel, M.; Birnbaum, L. Polybrominated diphenyl ethers (PBDEs) and hexabromocyclodecane (HBCD) in composite US food samples. *Environ. Health Persp.* **2009**, *118* (3), 357.

(4) De Wit, C. A.; Kierkegaard, A.; Ricklund, N.; Sellström, U. Emerging brominated flame retardants in the environment. *Environ. Chem.* **2010**, *16*, 241−286.

(5) Appendix 3 on Physical Chemical Properties of Brominated Flame Retardants. http://www2.mst.dk/udgiv/Publications/1999/87-7909-416-3/html/bil03_eng.htm (accessed 1998/99).

(6) Dodson, R. E.; Perovich, L. J.; Covaci, A.; Van den Eede, N.; Ionas, A. C.; Dirtu, A. C.; Brody, J. G.; Rudel, R. A. After the PBDE phase-out: a broad suite of flame retardants in repeat house dust samples from California. *Environ. Sci. Technol.* **2012**, *46* (24), 13056−13066.

(7) Sühring, R.; Busch, F.; Fricke, N.; Kötke, D.; Wolschke, H.; Ebinghaus, R. Distribution of brominated flame retardants and dechloranes between sediments and benthic fish—A comparison of a freshwater and marine habitat. *Sci. Total Environ.* **2016**, *542*, 578−585.

(8) Fisk, P. R.; Girling, A. E.; Wildey, R. J.*Prioritisation of flame retardants for environmental risk assessment*; UK Environment Science Agency: Rotherham, England, 2008; pp 1−129.

(9) Saunders, D. M.; Higley, E. B.; Hecker, M.; Mankidy, R.; Giesy, J. P. *In vitro* endocrine disruption and TCDD-like effects of three novel brominated flame retardants: TBPH, TBB, & TBCO. *Toxicol. Lett.* **2013**, *223* (2), 252−259.

(10) Mankidy, R.; Ranjan, B.; Honaramooz, A.; Giesy, J. P. Effects of novel brominated flame retardants on steroidogenesis in primary porcine testicular cells. *Toxicol. Lett.* **2014**, *224* (1), 141−146.

(11) Saunders, D. M.; Podaima, M.; Wiseman, S.; Giesy, J. P. Effects of the brominated flame retardant TBCO on fecundity and profiles of transcripts of the HPGL-axis in Japanese medaka. *Aquat. Toxicol.* **2015**, *160*, 180−187.

(12) Embry, M. R.; Belanger, S. E.; Braunbeck, T. A.; Galay-Burgos, M.; Halder, M.; Hinton, D. E.; Léonard, M. A.; Lillicrap, A.; Norberg-King, T.; Whale, G. The fish embryo toxicity test as an animal alternative method in hazard and risk assessment and scientific research. *Aquat. Toxicol.* **2010**, *97* (2), 79−87.

(13) Volz, D. C.; Belanger, S.; Embry, M.; Padilla, S.; Sanderson, H.; Schirmer, K.; Scholz, S.; Villeneuve, D. Adverse outcome pathways during early fish development: a conceptual framework for identification of chemical screening and prioritization strategies. *Toxicol. Sci.* **2011**, *123* (2), 349−358.

(14) Villeneuve, D.; Volz, D. C.; Embry, M. R.; Ankley, G. T.; Belanger, S. E.; Léonard, M.; Schirmer, K.; Tanguay, R.; Truong, L.; Wehmas, L. Investigating alternatives to the fish early-life stage test: A strategy for discovering and annotating adverse outcome pathways for early fish development. *Environ. Toxicol. Chem.* **2014**, *33* (1), 158−169.

(15) EU Monitor. *Proposal for a Directive of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes*; Commission of the European Communities: Brussels, Belgium, 2008, 543−5.

(16) Belanger, S. E.; Balon, E. K.; Rawlings, J. M. Saltatory ontogeny of fishes and sensitive early life stages for ecotoxicology tests. *Aquat. Toxicol.* **2010**, *97* (2), 88−95.

(17) Doering, J. A.; Tang, S.; Peng, H.; Eisner, B. K.; Sun, J.; Giesy, J. P.; Wiseman, S.; Hecker, M. High conservation in transcriptomic and proteomic response of white sturgeon to equipotent concentrations of 2, 3, 7, 8-TCDD, PCB 77, and benzo [a] pyrene. *Environ. Sci. Technol.* **2016**, *50* (9), 4826−4835.

(18) Kasahara, M.; Naruse, K.; Sasaki, S.; Nakatani, Y.; Qu, W.; Ahsan, B.; Yamada, T.; Nagayasu, Y.; Doi, K.; Kasai, Y.; et al. The medaka draft genome and insights into vertebrate genome evolution. *Nature* **2007**, *447* (7145), 714−719.

(19) Kim, D.; Pertea, G.; Trapnell, C.; Pimentel, H.; Kelley, R.; Salzberg, S. L. TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol.* **2013**, *14* (4), R36.
(20) Goecks, J.; Nekrutenko, A.; Taylor, J.; The Galaxy Team. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.*; **2010**, *11*, 8, R86; 10.1186/gb-2010-11-8-r86.
(21) Liu, X.; Hu, Y.; Pai, P.-J.; Chen, D.; Lam, H. Label-free quantitative proteomics analysis of antibiotic response in *Staphylococcus aureus* to oxacillin. *J. of Proteome Res.* **2014**, *13* (3), 1223−1233.
(22) Bindea, G.; Mlecnik, B.; Hackl, H.; Charoentong, P.; Tosolini, M.; Kirilovsky, A.; Fridman, W.-H.; Pagès, F.; Trajanoski, Z.; Galon, J. ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* **2009**, *25* (8), 1091−1093.
(23) Shannon, P.; Markiel, A.; Ozier, O.; Baliga, N. S.; Wang, J. T.; Ramage, D.; Amin, N.; Schwikowski, B.; Ideker, T. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* **2003**, *13* (11), 2498−2504.
(24) Iwamatsu, T. Stages of normal development in the medaka *Oryzias latipes*. *Mech. Dev.* **2004**, *121* (7), 605−618.
(25) Kondo, T.; Yamamoto, H.; Tatarazako, N.; Kawabe, K.; Koshio, M.; Hirai, N.; Morita, M. Bioconcentration factor of relatively low concentrations of chlorophenols in Japanese medaka. *Chemosphere* **2005**, *61* (9), 1299−1304.
(26) Villalobos, S. A.; Hamm, J. T.; Teh, S. J.; Hinton, D. E. Thiobencarb-induced embryotoxicity in medaka (*Oryzias latipes*): stage-specific toxicity and the protective role of chorion. *Aquat. Toxicol.* **2000**, *48* (2), 309−326.
(27) Sanz-Landaluze, J.; Pena-Abaurrea, M.; Muñoz-Olivas, R.; Cámara, C.; Ramos, L. Zebrafish (*Danio rerio*) eleutheroembryo-based procedure for assessing bioaccumulation. *Environ. Sci. Technol.* **2015**, *49* (3), 1860−1869.
(28) Lietti, E.; Marin, M.; Matozzo, V.; Polesello, S.; Valsecchi, S. Uptake and elimination of 4-nonylphenol by the clam *Tapes philippinarum*. *Arch. Environ. Contam. Toxicol.* **2007**, *53* (4), 571−578.
(29) Peng, H.; Zhang, S.; Sun, J.; Zhang, Z.; Giesy, J. P.; Hu, J. Isomer-specific accumulation of perfluorooctanesulfonate from (N-ethyl perfluorooctanesulfonamido) ethanol-based phosphate diester in Japanese Medaka (*Oryzias latipes*). *Environ. Sci. Technol.* **2014**, *48* (2), 1058−1066.
(30) Hong, H.; Li, D.; Shen, R.; Wang, X.; Shi, D. Mechanisms of hexabromocyclododecanes induced developmental toxicity in marine medaka (*Oryzias melastigma*) embryos. *Aquat. Toxicol.* **2014**, *152*, 173−185.
(31) Pratt, J. M.; Petty, J.; Riba-Garcia, I.; Robertson, D. H.; Gaskell, S. J.; Oliver, S. G.; Beynon, R. J. Dynamics of protein turnover, a missing dimension in proteomics. *Mol. Cell. Proteomics* **2002**, *1* (8), 579−591.
(32) Lubzens, E.; Young, G.; Bobe, J.; Cerdà, J. Oogenesis in teleosts: how fish eggs are formed. *Gen. Comp. Endocrinol.* **2010**, *165* (3), 367−389.
(33) Pillai, S.; Behra, R.; Nestler, H.; Suter, M. J.-F.; Sigg, L.; Schirmer, K. Linking toxicity and adaptive responses across the transcriptome, proteome, and phenotype of *Chlamydomonas reinhardtii* exposed to silver. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111* (9), 3490−3495.
(34) Morandi, G. D.; Wiseman, S.; Pereira, A. D. S.; Mankidy, R.; Gault, I. G.; Martin, J. W.; Giesy, J. P. Effects-directed analysis of dissolved organic compounds in oil sands process affected water (OSPW). *Environ. Sci. Technol.* **2015**, *49* (20), 12395−12404.
(35) Timme-Laragy, A. R.; Levin, E. D.; Di Giulio, R. T. Developmental and behavioral effects of embryonic exposure to the polybrominated diphenylether mixture DE-71 in the killifish (*Fundulus heteroclitus*). *Chemosphere* **2006**, *62* (7), 1097−1104.
(36) Fernie, K. J.; Marteinson, S. C.; Bird, D. M.; Ritchie, I. J.; Letcher, R. J. Reproductive changes in American kestrels (*Falco sparverius*) in relation to exposure to technical hexabromocyclododecane flame retardant. *Environ. Toxicol. Chem.* **2011**, *30* (11), 2570−2575.
(37) Danilova, N.; Sakamoto, K. M.; Lin, S. Ribosomal protein S19 deficiency in zebrafish leads to developmental abnormalities and defective erythropoiesis through activation of *p53* protein family. *Blood* **2008**, *112* (13), 5228−5237.
(38) Ankley, G. T.; Daston, G. P.; Degitz, S. J.; Denslow, N. D.; Hoke, R. A.; Kennedy, S. W.; Miracle, A. L.; Perkins, E. J.; Snape, J.; Tillitt, D. E.; et al. Toxicogenomics in regulatory ecotoxicology. *Environ. Sci. Technol.* **2006**, *40* (13), 4055−4065.
(39) Schirmer, K.; Fischer, B. B.; Madureira, D. J.; Pillai, S. Transcriptomics in ecotoxicology. *Anal. Bioanal. Chem.* **2010**, *397* (3), 917−923.
(40) Gonzalez-Nunez, V.; Nocco, V.; Budd, A. Characterization of *drCol 15a1b*: a novel component of the stem cell niche in the zebrafish retina. *Stem Cells* **2010**, *28* (8), 1399−1411.
(41) Gao, Z.; Mao, C. A.; Pan, P.; Mu, X.; Klein, W. H. Transcriptome of *Atoh7* retinal progenitor cells identifies new *Atoh7*-dependent regulatory genes for retinal ganglion cell formation. *Dev. Neurobiol.* **2014**, *74* (11), 1123−1140.
(42) Hensley, M. R.; Emran, F.; Bonilla, S.; Zhang, L.; Zhong, W.; Grosu, P.; Dowling, J. E.; Leung, Y. F. Cellular expression of Smarca4 (Brg1)-regulated genes in zebrafish retinas. *BMC Dev. Biol.* **2011**, *11* (1), 45.
(43) Ionita, M. A.; Pittler, S. J. Focus on molecules: rod cGMP phosphodiesterase type 6. *Exp. Eye Res.* **2007**, *84* (1), 1−2.
(44) Stearns, G.; Evangelista, M.; Fadool, J. M.; Brockerhoff, S. E. A mutation in the cone-specific *pde6* gene causes rapid cone photoreceptor degeneration in zebrafish. *J. Neurosci.* **2007**, *27* (50), 13866−13874.
(45) Yokoyama, S. Molecular evolution of vertebrate visual pigments. *Prog. Retinal Eye Res.* **2000**, *19* (4), 385−419.
(46) Ohtoshi, A.; Wang, S. W.; Maeda, H.; Saszik, S. M.; Frishman, L. J.; Klein, W. H.; Behringer, R. R. Regulation of retinal cone bipolar cell differentiation and photopic vision by the CVC homeobox gene *Vsx1*. *Curr. Biol.* **2004**, *14* (6), 530−536.
(47) Viczian, A. S.; Zuber, M. E. A simple behavioral assay for testing visual function in *Xenopus laevis*. *J. Visualized Exp.* **2014**, *88*, e51726−e51726.
(48) Chen, Z.; Huang, W.; Dahme, T.; Rottbauer, W.; Ackerman, M. J.; Xu, X. Depletion of zebrafish essential and regulatory myosin light chains reduces cardiac function through distinct mechanisms. *Cardiovasc. Res.* **2008**, *79* (1), 97−108.
(49) Handley-Goldstone, H. M.; Grow, M. W.; Stegeman, J. J. Cardiovascular gene expression profiles of dioxin exposure in zebrafish embryos. *Toxicol. Sci.* **2005**, *85* (1), 683−693.
(50) Neely, A.; Hidalgo, P. Structure-function of proteins interacting with the α1 pore-forming subunit of high-voltage-activated calcium channels. *Front. Physiol.* **2014**, *5*, 209.
(51) Marentette, J. R.; Frank, R. A.; Bartlett, A. J.; Gillis, P. L.; Hewitt, L. M.; Peru, K. M.; Headley, J. V.; Brunswick, P.; Shang, D.; Parrott, J. L. Toxicity of naphthenic acid fraction components extracted from fresh and aged oil sands process-affected waters, and commercial naphthenic acid mixtures, to fathead minnow (*Pimephales promelas*) embryos. *Aquat. Toxicol.* **2015**, *164*, 108−117.
(52) Wu, M.; Zuo, Z.; Li, B.; Huang, L.; Chen, M.; Wang, C. Effects of low-level hexabromocyclododecane (HBCD) exposure on cardiac development in zebrafish embryos. *Ecotoxicology* **2013**, *22* (8), 1200−1207.
(53) Dong, W.; Matsumura, F.; Kullman, S. W. TCDD induced pericardial edema and relative COX-2 expression in medaka (*Oryzias latipes*) embryos. *Toxicol. Sci.* **2010**, *118* (1), 213−223.
(54) Solt, L. A.; Burris, T. P. Action of RORs and their ligands in (patho) physiology. *Trends Endocrinol. Metab.* **2012**, *23* (12), 619−627.
(55) Tyler, C.; Sumpter, J. Oocyte growth and development in teleosts. *Rev. Fish Biol. Fisher.* **1996**, *6* (3), 287−318.

DOI: 10.1021/acs.est.6b01249
Environ. Sci. Technol. 2016, 50, 7781−7790

(56) Yang, J.; Winkler, K.; Yoshida, M.; Kornbluth, S. Maintenance of G_2 arrest in the *Xenopus* oocyte: a role for 14−3-3-mediated inhibition of Cdc25 nuclear import. *EMBO J.* **1999**, *18* (8), 2174−2183.
(57) Fan, H.-Y.; Huo, L.-J.; Meng, X.-Q.; Zhong, Z.-S.; Hou, Y.; Chen, D.-Y.; Sun, Q.-Y. Involvement of calcium/calmodulin-dependent protein kinase II (CaMKII) in meiotic maturation and activation of pig oocytes. *Biol. Reprod.* **2003**, *69* (5), 1552−1564.
(58) Muirhead, E. K.; Skillman, A. D.; Hook, S. E.; Schultz, I. R. Oral exposure of PBDE-47 in fish: toxicokinetics and reproductive effects in Japanese medaka (*Oryzias latipes*) and fathead minnows (*Pimephales promelas*). *Environ. Sci. Technol.* **2006**, *40* (2), 523−528.
(59) Snape, J. R.; Maund, S. J.; Pickford, D. B.; Hutchinson, T. H. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. *Aquat. Toxicol.* **2004**, *67* (2), 143−154.
(60) Fent, K.; Sumpter, J. P. Progress and promises in toxicogenomics in aquatic toxicology: is technical innovation driving scientific innovation? *Aquat. Toxicol.* **2011**, *105* (3), 25−39.
(61) Ankley, G. T.; Bennett, R. S.; Erickson, R. J.; Hoff, D. J.; Hornung, M. W.; Johnson, R. D.; Mount, D. R.; Nichols, J. W.; Russom, C. L.; Schmieder, P. K.; et al. Adverse outcome pathways: a conceptual framework to support ecotoxicology research and risk assessment. *Environ. Toxicol. Chem.* **2010**, *29* (3), 730−741.
(62) Villeneuve, D. L.; Crump, D.; Garcia-Reyero, N.; Hecker, M.; Hutchinson, T. H.; LaLone, C. A.; Landesmann, B.; Lettieri, T.; Munn, S.; Nepelska, M.; et al. Adverse outcome pathway (AOP) development I: strategies and principles. *Toxicol. Sci.* **2014**, *142* (2), 312−320.
(63) Villeneuve, D. L.; Crump, D.; Garcia-Reyero, N.; Hecker, M.; Hutchinson, T. H.; LaLone, C. A.; Landesmann, B.; Lettieri, T.; Munn, S.; Nepelska, M.; et al. Adverse outcome pathway development II: best practices. *Toxicol. Sci.* **2014**, *142* (2), 321−330.

DOI: 10.1021/acs.est.6b01249
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Supporting Information

For

A combined transcriptomic and proteomic approach to identify and predict toxicity pathways in early-life stages of Japanese medaka (*Oryzias latipes*) exposed to 1,2,5,6-tetrabromocyclooctane (TBCO)

Jianxian Sun[a], Song Tang[b], Hui Peng*[,a], David M.V. Saunders[a], Jon A. Doering[a], Markus Hecker[a,b], Paul D. Jones[a,b], John P. Giesy[a,c,d,e,f], and Steve Wiseman*[,a]

[a] Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B3, Canada
[b] School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5C8, Canada
[c] Department of Veterinary Biomedical Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B4, Canada
[d] Zoology Department, Center for Integrative Toxicology, Michigan State University, East Lansing, Michigan 48824, USA
[e] School of Biological Sciences, University of Hong Kong, Hong Kong Special Administrative Region 999077, People's Republic of China
[f] State Key Laboratory of Pollution Control and Resource Reuse, School of the Environment, Nanjing University, Nanjing 210093, People's Republic of China

***Corresponding author:** Hui Peng, huisci@gmail.com, Tel.: +1 306 966 8733; Fax: +1 306 966 4796. Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5B3, Canada; Steve Wiseman: steve.wiseman@uleth.ca, Tel: (403) 329-2320, Fax: (403) 329-2082. Department of Biology, University of Lethbridge, Lethbridge, Alberta T1K 3M4, Canada.

Words	1,798
Tables	1
Figure	3

This supporting information provides text of details, tables and figures addressing (1) RNA sequencing; (2) Protein preparation and identification; (3) Identification of proteins by mass spectrometry; (4) Analysis of TBCO; (5) Visual performance assessment; (6) Cardiac function assessment; (7) Concentrations of TBCO in exposure media prior to media-change; (8) Structures of HBCD and TBCO; (9) Heatmap of significantly altered transcripts and proteins in embryos exposed to control and 100 μg/L TBCO; (10) Biplots illustrating diatance-based redundancy analysis (dbRDA) of transcriptome and proteome, and regression between transcripts and proteins from control samples.

RNASeq. To determine responses of the transcriptome to TBCO, 5 embryos hatched at day 8 post-fertilization were selected randomly from each petri dish and were pooled. Total RNA was extracted from each of the seven pools of embryos exposed to the solvent control and medium concentration of TBCO by use of a RNeasy Plus Mini Kit according to the protocol provided by the manufacturer (Qiagen, Mississauga, ON, Canada). Concentrations of RNA were determined by use of a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). Integrity of RNA was determined by use of an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Three samples of RNA from medaka exposed to the solvent control (n=3) and three samples of RNA from medaka exposed to the medium concentration of TBCO (n=3), each of which had the highest RNA integrity (RIN) value (greater than 8), were selected for sequencing. One RNA-Seq library per sample was prepared by use of the Tru-Seq RNA Sample Prep Kit (*Illumina*, San Diego, CA, USA), according to the protocol provided by the manufacturer. Quality of libraries was confirmed by use of a 2100 Bioanalyzer Agilent Technologies). Each library was loaded onto a separate MiSeq v3 150 cycle cartridge (*Illumina*) and run as 2×75 base-pair (bp) paired-end reads on a MiSeq sequencer (*Illumina*) at the Toxicology Centre (University of Saskatchewan, Saskatoon, SK, Canada).

Sequencing reads were assessed for quality using FastQC (v0.52) on the Galaxy public server (http://galaxy-qld.genome.edu.au).[1] A median of 99.9% (range from 83.3 to 99.9%) of reads had a Phred score of > 30 across all samples. Various FASTQ quality formats were converted by FASTQ Groomer (v1.0.4). Reads were mapped independently for each sample against the reference genome of medaka[2] (Ensembl, v1.78) by use of TopHat2 (v0.6)[3] with

default settings on Galaxy. Across all samples, 80.4%-86.8% of reads were mapped to the reference genome. Alignments from TopHat2 were provided to Cufflinks (v2.02) to assemble transcript for each condition using a reference annotation as a guide.[4] Cuffmerge (v0.0.6) was used to combine all assemblies into a consensus assembly in GTF file format, which provided a uniform basis for calculating changes in abundances of transcripts in each condition. The reads and the merged assembly were fed to Cuffdiff (v0.0.7), and expression levels were calculated and statistical significance of changes in abundances of transcripts was determined.

Protein preparation and identification. Pools of 50 embryos per exposure were homogenized on ice in lysis buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 100 mM KCl, 420 mM NaCl, 20% Glycerol, and protease inhibitor cocktail, pH=7.4) by use of a model-100 sonic dismembrator (Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation at 15,000 × g for 15 min at 4 °C, the supernatant was recovered and total concentration of proteins were quantified by use of the Bradford protein assay with bovine serum albumin (BSA) as a standard.[5] Filter-aided sample preparation (FASP) was used for protein digestion by use of a 30 kDa molecular weight cutoff spin ultrafiltration filter. Briefly, an aliquot of each lysate, containing approximately 100 μg of protein, was transferred to a YM-30 microcon filter unit (Cat No. MRCF0R030, Millipore, Etobicoke, ON, Canada). After centrifugation at 14,000 × g for 30 min, salt and other interferences in the buffer were removed from samples by washing three times with 0.1 M Tris-HCl. Proteins were reduced with 50 μL of 5 mM DTT for 60 min at 37 ^{o}C, and then carboxymethylated for 30 min in the dark, with 15 mM of iodoacetamide. Samples were further digested overnight with 5 μg

trypsin and gentle shaking. Digestion was terminated by adding formic acid to a final concentration of 1% (v/v). The final sample was collected by centrifugation at 14,000 × g for 30 min.

Identification of Proteins by Mass Spectrometry. Each sample was loaded onto a 75 mm inner diameter fused silica microcapillary column (Polymicron Technologies, Phoenix, AZ, USA) packed with 10 cm of Luna 3-mC18 100 Å, reversed phase particles (Phenomenex, Torrance, CA, USA) and placed in-line with a nano-LC-electrospray ion source (Proxeon, Mississauga, ON, USA) interfaced to a LTQ Orbitrap Velos hybrid mass spectrometer (Thermo Fisher Scientific) at the University of Toronto (Toronto, ON, Canada). The organic gradient was driven by the EASY-nLC system at 300 nL/min. Mobile phases were (A) 95% acetonitrile with 0.1% formic acid and (B) 5% acetonitrile. Initially, 2% of B was increased to 6% in 2 min, then increased to 24% in 62 min, followed by an increase to 90% in 26 min and held static for 5 min, and then decreased to initial conditions of 2% of B and held for 8 min for equilibration. Positive precursor ions (400 - 2000 *m/z*) were subjected to data-dependent collision-induced dissociation as the instrument cycled through one full mass scan at 60,000 full-width at half maximum followed by 17 successive MS / MS scans targeting the most intense precursors with dynamic exclusion and +2 / +3 charge state selection enabled. The MS proteomics data have been deposited to the ProteomeXchange Consortium[6] via the Proteomics Identifications (PRIDE) partner repository with the dataset identifier PXD003823.

Raw MS files were analyzed by use of MaxQuant software (v1.5.1.2).[7] MS/MS spectra were searched against the database for medaka protein (UniProt) containing forward and

reversed (decoy) sequences, allowing for variable modifications of methionine oxidation and N-terminal acetylation and fixed cysteine carbamidomethylation. Parent mass and fragment ions were matched using a maximal initial mass deviation of 7 p.p.m and 0.5 Th, respectively. The false discovery rate for proteins was set to 0.01. Spectral count was used for label-free quantification of proteins and only proteins with spectral counts greater than 4 were used for data analysis. Proteins were defined as being differentially expressed if the fold-change in abundance was ±1.5 fold, and if the *p*-value was less than 0.05; as conducted previously.[8] Details of significantly changed transcripts and proteome are provided in Supporting Data Sheet.

Analysis of TBCO. Concentrations of TBCO in embryos and in exposure media were quantified. To determine concentrations of TBCO in unhatched embryos/eggs, 3 eggs that were not hatched were selected randomly from each petri dish on days 1, 3, 5, 7 and 9, and eggs from two petri dishes were pooled as one sample so that the number of replicates analyzed per treatment was 3 (n=3). Also on day 9 of the exposure, three embryos that had hatched also were collected and embryos from 2 petri dishes were pooled as one sample so that the number of replicates was 3. F-BDE-47 was spiked to samples of embryos and media as an internal control. Eggs were crushed and 4 mL of nano pure water was added to each sample. Liquid-liquid extraction was conducted twice with a solution of *n*-hexane/methyl tert-butyl ether (1:1 v/v) by shaking for 20 min at 300 × rpm, followed by sonication for 20 min in a 15 mL centrifuge tube and centrifugation for 15 min at 4000 × g. Both supernatants were pooled, evaporated to dryness under a gentle stream of nitrogen, and redissolved in 1

mL of toluene for analysis (approximately 200× dilution). Three laboratory blanks and matrix spikes were extracted for quality assurance purposes. Extracts were analyzed for TBCO by use of an Agilent (Santa Clara, CA, USA) 7890A gas chromatograph (GC) system coupled to an Agilent 5975C mass spectrometer (MS) operating in electron impact ionization mode (EI).

One μL of each sample was injected at an injection port temperature of 280 °C in the splitless mode. Chromatographic separation was achieved with a 15 m × 250 μm i.d. Rtx-1614 fused silica capillary GC column, which had a 0.1 μm film thickness (Restek Corporation, Bellefonte, PA, USA). The carrier gas (helium) was kept at a constant flow rate of 1.5 mL/min. The following GC oven temperature program was used: 100 °C for 1 min, 5 °C/min to 190 °C for 2 min, 20 °C/min to 220 °C for 2 min, and 40 °C/min to 300 °C for 4 min. The GC/MS transfer line was maintained at 280°C. Selected ion monitoring of *m/z* 267/187 and 343/234 was used for quantification/confirmation of TBCO and F-BDE-47, respectively. TBCO was quantified by use of the internal standard method using F-BDE-47. Concentration of TBCO in blanks was less than the limit of detection. Limit of detection of TBCO were 0.12 μg/L and 0.072 μg/g ww in exposure medium and embryos, respectively. The mean and standard error for recovery of TBCO were 95.9 ± 22.0%.

Visual Performance Assessment. To assess adverse effects of TBCO on visual performance, new batches of embryos were exposed to solvent control, 10, 100 and 1,000 μg/L TBCO from 2 hours post-fertilization until 14 days post-hatch. There were four petri dishes for each concentration of TBCO and there with 100 embryos per dish. Visual performance of medaka exposed to TBCO was determined by use of a background color preference assay that was

developed to assess visual performance of *Xenopus laevis*.[14] For the assay, a transparent tank was placed inside another tank that was half black and half white to provide contrasting background colors. For the assay, groups of 20 larvae per replicate were gently moved into the inner tank and allowed to acclimate for 10 s, and then the inner tank was placed into the outer tank. Behaviour of fry was observed for 30 s. Then the inner tank was lifted and the outer tank was rotated 180° to change the position of white and black background so that fry were positioned over the black area of the outer tank. Behaviour of fry were recorded with a camera for another 30 s. Percentages of fry in the black and white areas were determined, as was the time for all fry to move to the white side of the tank. Swimming routs were traced by use of ZooTracer software (Microsoft ResearchCambridge, MA, USA).[9] Suitability of the assay for assessing visual function was first optimized with control fry. Similarly to *Xenopus laevis*, fry of Japanese medaka exposed to the solvent control swam from the side of the tank that overlayed the black backgrouns to the side of the tank that overlayed the white background (96.3 ± 5.2%) within a short period of time (average = 2.7 s).

Cardiac Function Assessment. To assess adverse effects of TBCO on heartbeat, new batches of embryos were exposed to solvent control, 10, 100 and 1000 µg/L TBCO from 2 hours post-fertilization until 5 days post-fertilization. There were four petri dishes for each concentration (n=4) and there were 100 embryos per dish. Effects of TBCO on cardiac function were determined by quantifying heart rate of embryos viewed at 4× magnification by use of an inverted microscope (Zeiss, Toronto, ON, Canada). The number of beats per minute was determined in 3 embryos from each of the four petri dishes per exposure (n=4).

Table S1. Concentrations of TBCO in exposure media prior to media-change. Approximately 5 ml of solution was sampled on days 2, 4 and 6 from each of the 7 petri dishes per exposure group. Data represent mean ± standard deviation.

	Nominal Concentration (μg/L)	Measured Concentration (μg/L)
Control	0	ND
Low	10	2.7±0.4
Medium	100	29.6±7.5
High	1000	151.3±81.9

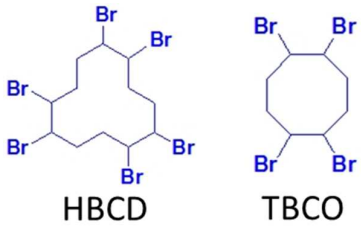

Figure S1. Structures of hexabromocyclododecane (HBCD) and 1,2,5,6-tetrabromocyclooctane (TBCO).

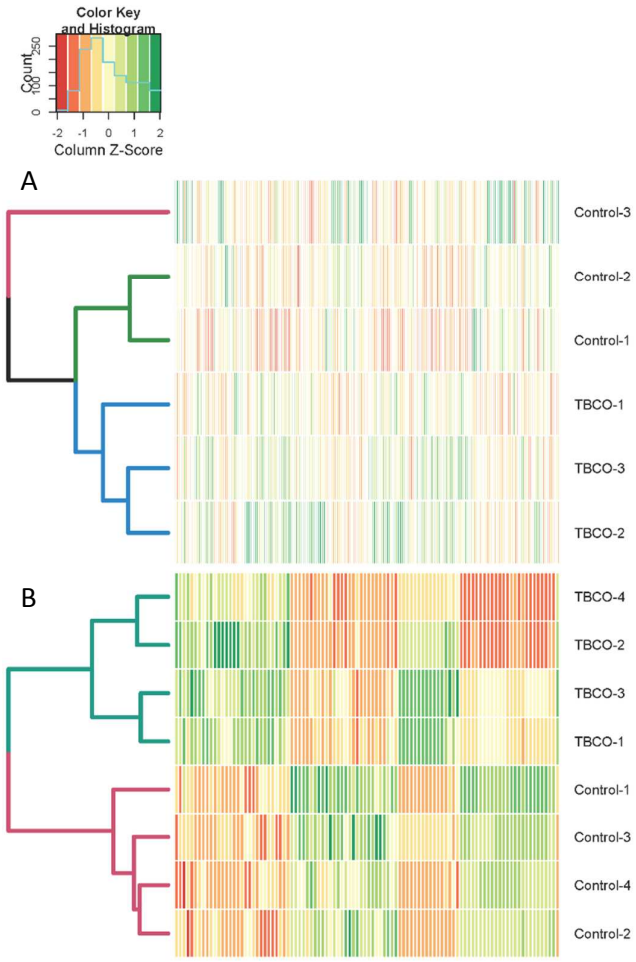

Figure S2. Heatmap of significantly altered transcripts (A) and proteins (B) in embryos exposed to the solvent control and 100 µg/L TBCO.

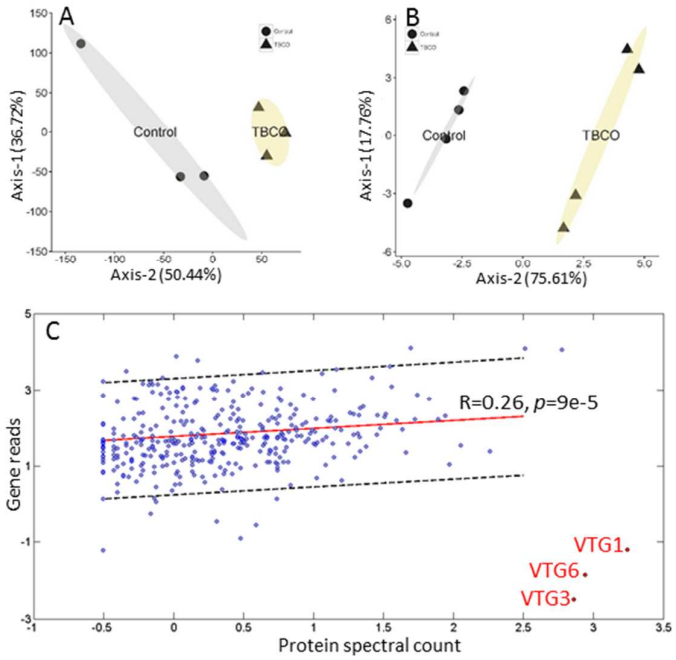

Figure S3. Biplots illustrating diatance-based redundancy analysis (dbRDA) of measured responses of (A) transcriptome (n=3) and (B) proteome (n=4) of embryos of Japanese medaka exposed to the solvent control or 100 µg/L of TBCO. Each data point represents one sample, and proximity of points in the biplots is an approximation of the similarity of samples with respect to their response. Ovals represent the 95% confidence ellipse around the group centroids. In (A), 50.44% of variation in measured responses of the transcriptome is accounted for by the X-axis and 36.72% of variation is accounted for by the Y-axis; In (B), 75.61% of variation in measured responses of the proteome is accounted for by the X-axis and 17.76% of variation is accounted for by the Y-axis. Regression between transcripts and proteins (C) in embryos exposed to the solvent control. Black dashed lines indicate 95% confidence limits.

References

(1) Goecks, J.; Nekrutenko, A.; Taylor, J., Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol* **2010,** *11*, (8), R86.

(2) Kasahara, M.; Naruse, K.; Sasaki, S.; Nakatani, Y.; Qu, W.; Ahsan, B.; Yamada, T.; Nagayasu, Y.; Doi, K.; Kasai, Y., The medaka draft genome and insights into vertebrate genome evolution. *Nature* **2007,** *447*, (7145), 714-719.

(3) Kim, D.; Pertea, G.; Trapnell, C.; Pimentel, H.; Kelley, R.; Salzberg, S. L., TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* **2013,** *14*, (4), R36.

(4) Trapnell, C.; Roberts, A.; Goff, L.; Pertea, G.; Kim, D.; Kelley, D. R.; Pimentel, H.; Salzberg, S. L.; Rinn, J. L.; Pachter, L., Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. protoc.* **2012,** *7*, (3), 562-578.

(5) Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976,** *72*, (1), 248-254.

(6) Vizcaíno, J. A.; Deutsch, E. W.; Wang, R.; Csordas, A.; Reisinger, F.; Ríos, D.; Dianes, J. A.; Sun, Z.; Farrah, T.; Bandeira, N., ProteomeXchange provides globally coordinated proteomics data submission and dissemination. *Nat. Biotechnol.* **2014,** *32*, (3), 223-226.

(7) Cox, J.; Mann, M., MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. *Nat. Biotechnol.* **2008,** *26*, (12), 1367-1372.

(8) Liu, X.; Hu, Y.; Pai, P.-J.; Chen, D.; Lam, H., Label-free quantitative proteomics analysis of antibiotic response in *Staphylococcus aureus* to oxacillin. *Journal of proteome research* **2014,** *13*, (3), 1223-1233.

(9) A. Buchanan, A. F., Zoo tracer. 〈*http://research.microsoft.com/zootracer*〉 **2014**.