Alterations to the intestinal microbiome and metabolome of *Pimephales promelas* and *Mus musculus* following exposure to dietary methylmercury

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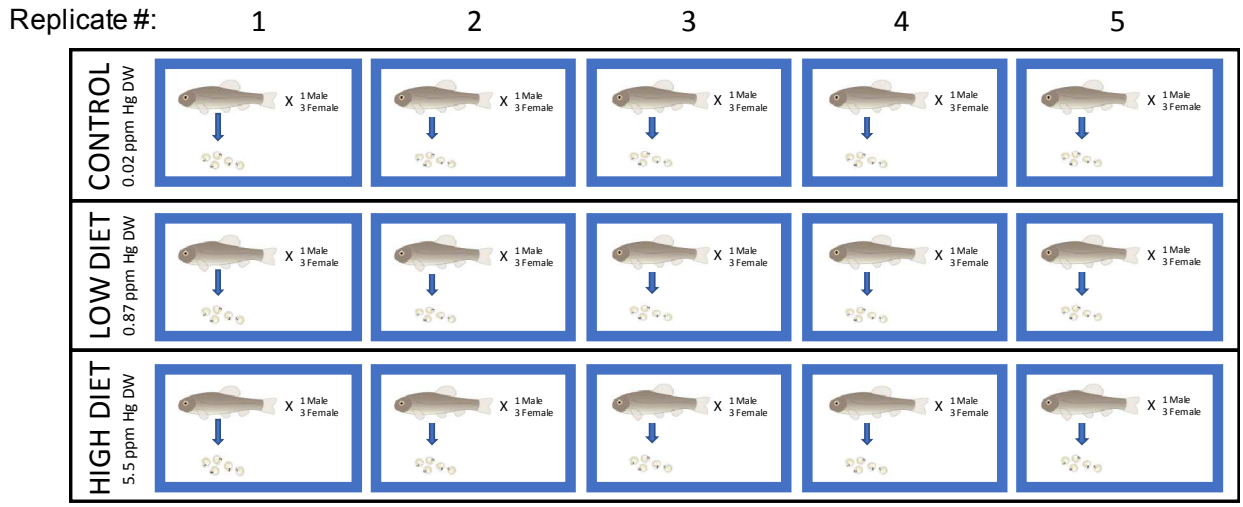
SUPPORTING INFORMATION

COHORT 1: Behavior & hatch (*offspring*)[10], gut microbiome sequencing (*adults*).



COHORT 2: DA analysis *(adults and offspring)* & behavior *(offspring)* [12], metabolomics *(offspring),* gene expression *(offspring).*

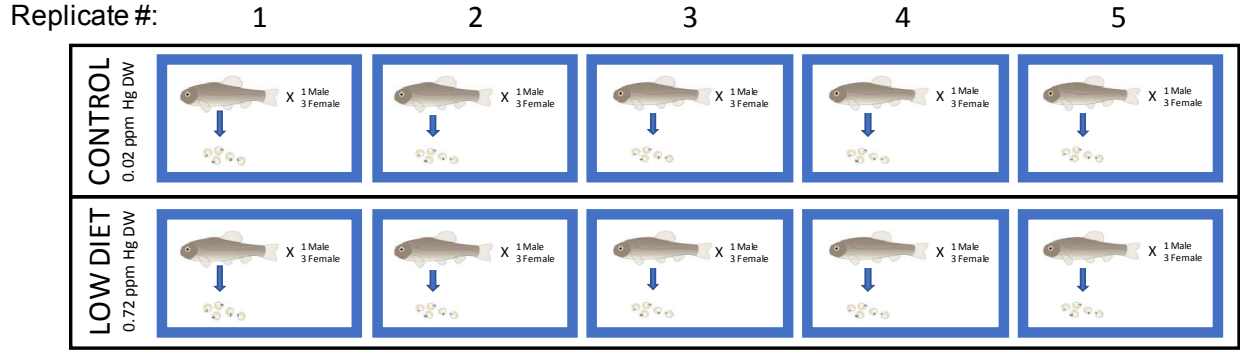


Figure S1. Fathead minnow exposure design, with tissue use and dietary concentration specifications. Both cohorts of fish were maintained under identical environmental conditions at the University of North Texas (IACUC 1303-3), and were obtained from Aquatic Biosystems (Fort Collins, CO). Diets were administered twice daily, for 30 days, and remaining food/waste/debris was siphoned from tanks after each feeding [4, 5]. Fish (3F:1M per tank) were maintained in 21-L glass aquaria on a 16:8 (light: dark) photoperiod, at 23°C. Each tank contained an activated carbon filtration system, heater, and 2 breeding tiles (consisting of halved PVC pipe sections).

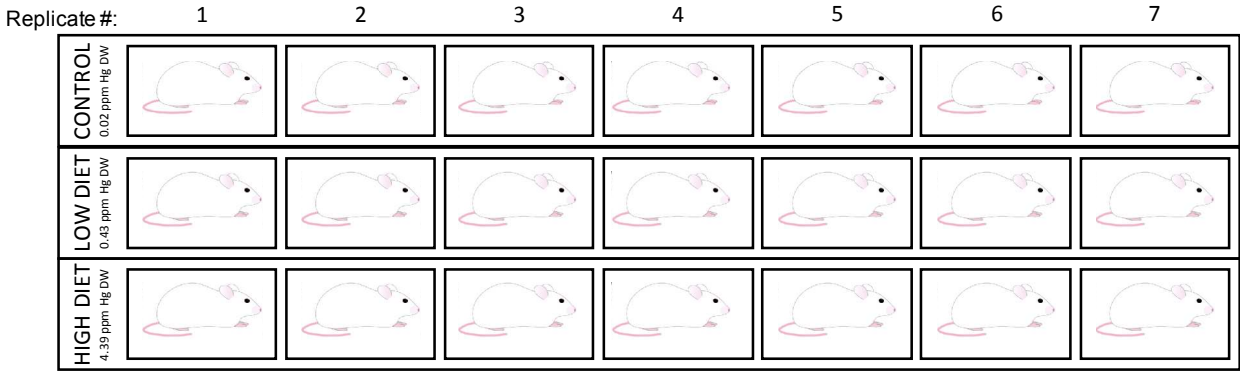


Figure S2. Male CD-1 mice were maintained at the animal care facility at University of North Texas Health Science Center (IACUC 2014/15-36-A04). Diets were administered twice daily, for 30 days, and water was available *ad libitum.* Mice were maintained on a 12:12 (light: dark) photoperiod, at 23°C, with one mouse per cage.

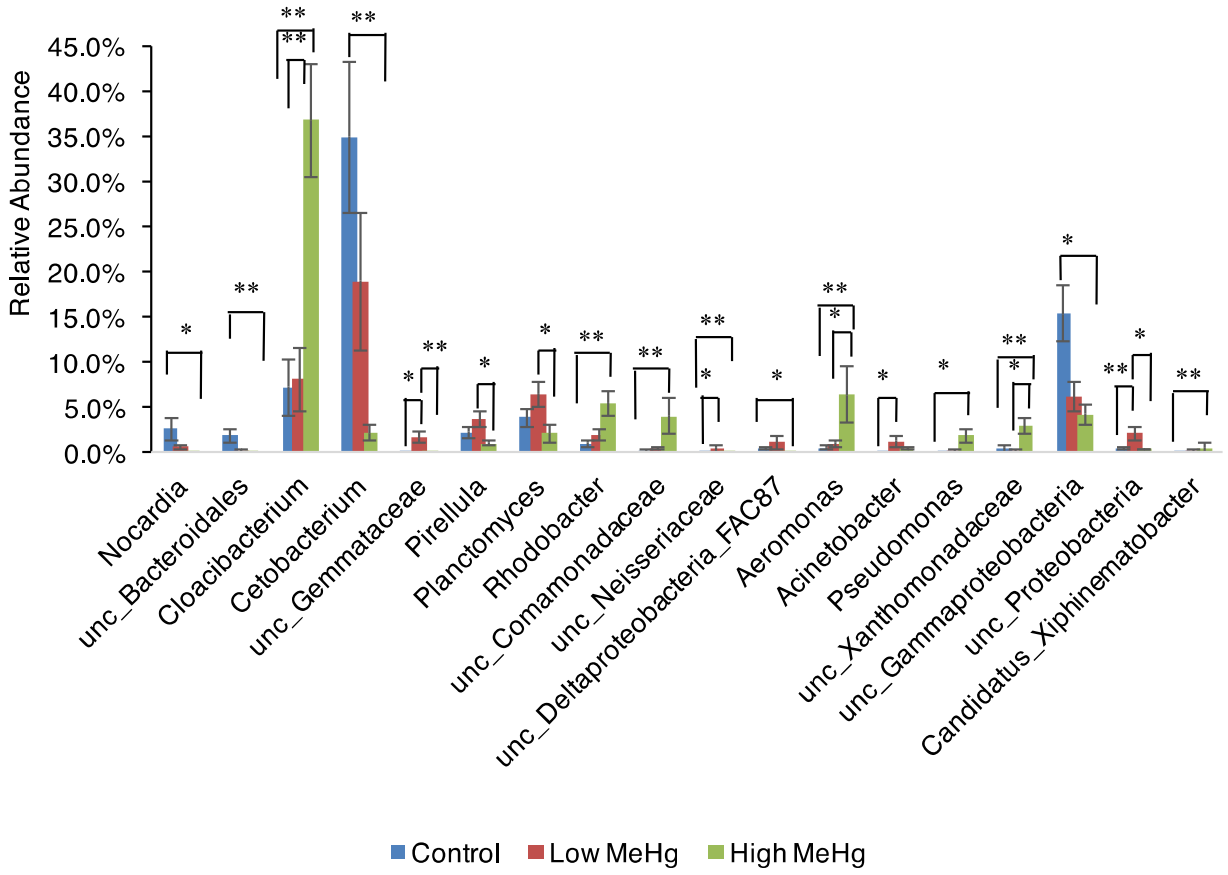


Figure S3. The relative abundance of many gut bacteria genera were significantly altered by exposure to dietary MeHg (* p <0.05, ** p <0.01).

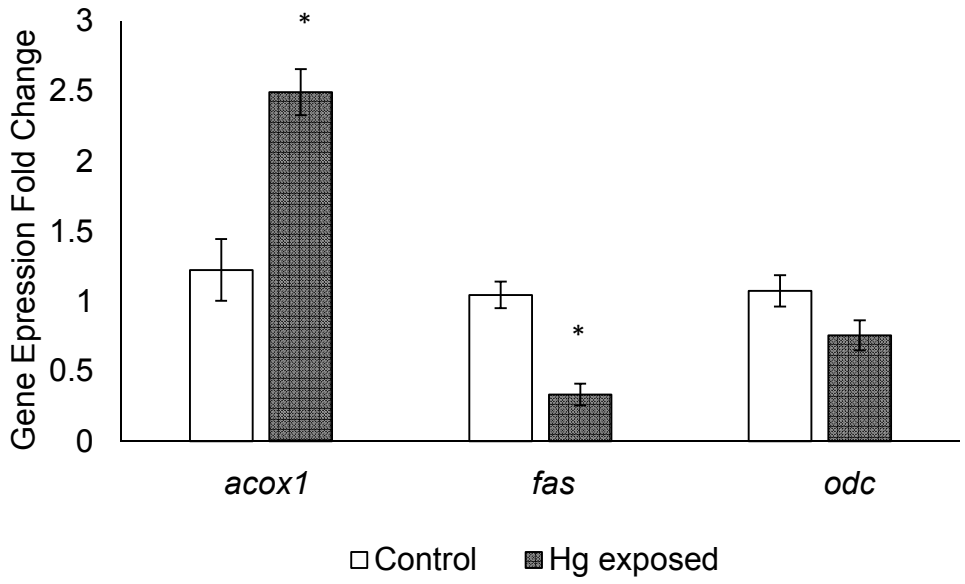


Figure S4. Mean fold change (± 1 SE) in gene expression between Hg exposed and control larvae, with an asterisk * denoting significantly altered gene expression relative to controls ($p < 0.05$, n= 5 clutches/ treatment).

Table S1. Mean wet weight Hg tissue concentrations (± 1 SD) from A) fathead minnows and B) mice administered control and Hg spiked diets (all values report wet weight, with the exception of fecal pellets from mice).

A) Fathead Minnows	CONTROL (µg/g)	LOW Hg DIET (µg/g)	
FEMALE MUSCLE	0.084 ± 0.02 (n = 8)	0.804 ± 0.08 (n = 8)	
MALE MUSCLE	0.084 ± 0.02 (n = 5)	0.506 ± 0.04 (n = 5)	
BRAIN TISSUE	0.07 ± 0.01 (n = 5)	0.46 ± 0.23 (n = 5)	
LARVAE	0.012 ± 0.01 (n = 7)	0.64 0.04 (n = 7)	
B) *Mice*	CONTROL (µg/g)	LOW Hg DIET (µg/g)	HIGH Hg DIET (µg/g)
BRAIN	0.002 ± 0.0004 (n = 5)	0.24 ± 0.08 (n = 5)	1.69 ± 0.27 (n = 5)
FECES (Dry Weight)	0.019 ± 0.004 (n = 15)	1.14 ± 0.41(n = 17)	8.84 ± 3.66 (n = 17)

Table S2. Full list of metabolites detected in fathead minnow larvae from both treatments. An * is used to denote significance ($p < 0.05$) between control and mercury exposed (Hg) larvae metabolite response

factors (relative to IS).

METABOLITE	N control	Control Diet Mean (± 1 SD)	N Hg	Low Diet Mean (± 1 SD)	Hg/Control
*putrescine	7	74.3 ± 18.9	9	147.7 ± 50	1.99
*L-serine	5	31.8 ± 15.1	7	66.2 ± 27.2	2.08
*glycerol	7	250.1 ± 41.1	9	401.2 ± 156.3	1.60
*stearic acid	7	1126 ± 363.5	9	767.2 ± 193.9	0.68
*palmitic acid	7	860.6 ± 236	9	621.8 ± 170.8	0.72
*oleic acid	7	357.1 ± 158	9	197.2 ± 135.5	0.55
cholesterol	7	11931 ± 3791	9	8017.2 ± 5617.7	0.67
2-hydroxypyridine	5	40.4 ± 20.9	8	56.2 ± 20.3	1.39
L-mimosine	5	19.3 ± 15.1	6	11.3 ± 5.1	0.59
Sucrose	5	14.5 ± 8.8	9	39.5 ± 69.6	2.73
myristic acid	7	965.9 ± 445.7	9	772.3 ± 349.1	0.80
L-glutamic acid	7	210.1 ± 84.6	9	263.1 ± 140.1	1.25
Phenylalanine	6	420.7 ± 214	9	316 ± 224.5	0.75
D-malic acid	7	88.2 ± 33.4	9	70.9 ± 45.4	0.80
L-threonine	5	35.9 ± 13.4	8	45.7 ± 27.8	1.27
D-mannose	6	122.1 ± 51.9	8	97.8 ± 55.2	0.80
glycerol 1-phosphate	6	36.8 ± 25.5	9	55.8 ± 60.4	1.52
pyrophosphate	7	220.5 ± 246	9	146.8 ± 194.4	0.66
L-allothreonine	5	150.1 ± 50.3	5	130.6 ± 50.8	0.87
DL-isoleucine	5	230.2 ± 155.1	7	188.6 ± 100.1	0.82
Creatinine	7	775.3 ± 551.9	9	656.6 ± 522.9	0.85
L-(+) lactic acid	7	566.6 ± 305.6	8	499.6 ± 311.1	0.88
urea	7	221.2 ± 206.7	8	248.9 ± 153.2	1.13
succinic acid	7	38.2 ± 27.5	7	34.3 ± 22.7	0.90
L-pyroglutamic acid	5	241.6 ± 122	6	280.3 ± 294.9	1.16
glycine	7	466 ± 250.8	9	434.7 ± 206.3	0.93
L-(+) lactic acid	7	566.6 ± 305.6	7	521.4 ± 329.4	0.92
O-phosphocolamine	6	118.7 ± 39.6	9	125.6 ± 71.4	1.06
citric acid	7	327.7 ± 155	9	346.9 ± 166.7	1.06
phosphoric acid	7	7765.7 ± 3143	9	7393.3 ± 3235.9	0.95
N-acetyl-L-aspartic acid	6	168.4 ± 132.4	9	157.7 ± 91	0.94
L-valine	6	197.8 ± 89.3	6	191.4 ± 119.3	0.97
glycolic acid	7	22.5 ± 11	8	22.1 ± 9.6	0.98
L-valine	6	197.8 ± 89.3	7	194.1 ± 109.1	0.98
glycolic acid	7	22.5 ± 11	7	22.8 ± 10.2	1.01

Table S3. Full list of metabolites detected in midbrains of mice. An * is used to denote significance ($p < 0.05$) between control and mercury exposed (Hg) metabolite response factors (relative to IS).

METABOLITE	N control	Control Diet Mean ± (1 SD)	N Hg	Low Diet Mean ± (1 SD)	Hg/Control
L-glutamine*	7	2554.29 ± 1186.34	7	1044.43 ± 286.22	0.41
O-phosphocolamine*	7	2778.57 ± 747.96	7	1857 ± 429.44	0.67
Dopamine*	7	35.58 ± 23.76	6	10.49 ± 7.81	0.29
Tagatose*	6	37.18 ± 18.67	6	15.62 ± 3.59	0.42
Hydroquinone*	7	6.22 ± 2.17	7	3.88 ± 1.65	0.62
L-ascorbic acid*	7	1051.27 ± 98.02	7	198.43 ± 114.26	0.19
Inosine 5'-monophosphate*	6	64.58 ± 7.85	6	39.82 ± 19.84	0.62
Uracil *	7	75.94 ± 31.31	7	46.09 ± 13.84	0.61
L-glutamic acid	7	8902.86 ± 2633.53	7	6397.1 ± 1534.8	0.72
Aspartic acid	7	4754.29 ± 1409.53	7	3494.28 ± 601.57	0.73
Stearic acid	7	2217.14 ± 704.72	7	1605.71 ± 241.58	0.72
L-homoserine	6	6.60 ± 3.13	6	3.81 ± 0.77	0.58
N-methyl-DL-glutamic acid	6	34.40 ± 22.60	6	14.38 ± 4.18	0.42
Allo-inositol	7	11000.00 ± 3213.12	7	8507.14 ± 1046.22	0.77
Xylitol	7	66.69 ± 48.31	7	29.62 ± 21.6	0.44
2,3-dihydroxypyridine	6	9.11 ± 2.40	5	6.56 ± 2.23	0.72
L-threonine	7	769.14 ± 398.47	7	459.8 ± 223.36	0.60
Malonic acid	6	61.85 ± 33.83	7	34.78 ± 11.22	0.56
Pantothenic acid	7	71.43 ± 24.76	7	50.01 ± 23.37	0.70
Thymine	7	7.28 ± 1.85	6	5.27 ± 2.33	0.72
Putrescine	7	12.39 ± 6.85	7	7.8 ± 0.99	0.63
Glycine	7	2506.57 ± 794.17	7	1908.57 ± 539.67	0.76
Adenosine-5-monophosphate	7	1040.86 ± 407.85	7	736.28 ± 283.23	0.71
Guanosine	5	23.42 ± 10.87	7	14.07 ± 6.34	0.60
Palmitic acid	7	1345.29 ± 637.75	7	928.85 ± 164.03	0.69
L-serine	7	1493.71 ± 891.89	7	912.42 ± 336.58	0.61
L-valine	7	164.97 ± 73.27	6	118.05 ± 19.95	0.72
D-glucose	7	58.34± 21.84	5	44.2 ± 7.1	0.76
Creatinine	7	2458.57± 816.89	7	1770 ± 877.15	0.72
2-hydroxypyridine	7	209.86 ± 74.65	7	157.82 ± 55.59	0.75
N-acetyl-L-aspartic acid	7	10221.43 ± 2901.43	7	8418.571 ± 1243.64	0.82
Urea	7	3852.86± 783.02	7	3387.14 ± 436.64	0.88
Arachidic acid	5	23.66 ± 14.36	7	13.9 ± 2.98	0.59

Adenine	7	80.80 ± 22.64	7	67.92 ± 12.09	0.84
Adenosine	7	604.86 ± 672.16	7	251.428 ± 186.67	0.42
Methyl-beta-D-galactopyranoside	7	20.51 ±11.03	7	13.98 ± 7.44	0.68
Beta-glycerolphosphate	7	90.81 ± 65.31	7	57.45 ± 14.436	0.63
L-pyroglutamic acid	7	2679.80 ± 1806.05	7	1633.85 ± 1263.35	0.61
Fumaric acid	7	151.86 ± 64.67	7	117.47 ± 31.72	0.77
L-(+) lactic acid	7	8748.57 ± 1770.10	7	7800 ± 935.62	0.89
Glycerol 1-phosphate	7	5834.29 ± 1795.80	7	4892.85 ± 836.09	0.84
Sucrose	7	11.71± 17.17	7	3.29 ± 1.596	0.28
DL-isoleucine	6	66.38 ± 25.35	6	51.61 ± 14.46	0.78
4-guanidinobutyric acid	7	6528.57 ± 1640.63	7	5734.28 ± 489.34	0.88
Inosine	7	1561.43 ± 704.83	7	1214.71 ± 318.51	0.78
Succinic acid	7	1685.71 ± 710.91	6	1346.66 ± 278.83	0.80
L-cysteine	7	87.34 ± 39.70	7	63.75 ± 39.01	0.73
Serotonin	5	3.05 ± 1.74	6	2.13 ± 0.33	0.70
Tyrosine	7	56.39 ± 22.57	6	43.783 ± 19.87	0.78
Hypoxanthine	7	222.57 ± 82.65	7	180.57 ± 64.16	0.81
Xanthine	7	32.29 ± 16.21	5	25.56 ± 3.27	0.79
2-hydroxybutyric acid	5	144.60 ± 31.09	6	116.3 ± 60	0.80
Dehydroascorbic acid	7	996.57 ± 272.08	7	1177 ± 428.12	1.18
Beta- alanine	7	303.29 ± 165.67	7	238.42 ± 73.68	0.79
D-ribose-5-phosphate	7	315.29 ± 55.10	7	380.14 ± 179.29	1.21
D-malic acid	7	1085.00 ± 275.46	7	937.57 ± 341.45	0.86
L-mimosine	7	27.74 ± 30.37	7	17.27 ± 11.61	0.62
Pyruvic acid	7	22.35 ± 26.86	7	13.578 ± 9.35	0.61
Maltose	7	8.51 ± 3.69	7	7.037 ± 3.37	0.83
L-lysine 2	7	110.74 ± 46.38	6	95.23 ± 28.18	0.86
L-glutathione reduced	5	20.08 ± 6.31	6	36.19 ± 59.87	1.80
Benzoic acid	6	36.70 ± 18.93	6	43.55 ± 22.03	1.19
uridine 5'-monophosphate	7	12.31 ± 7.43	5	9.78 ± 8.5	0.80
D-xylose	5	36.80 ± 18.29	6	31.75 ± 11.94	0.86
Phosphoric acid	7	9735.71 ± 3661.04	7	10228.57 ± 1533.74	1.05
Cytindine-5'-monophosphate	7	117.26 ± 86.69	5	136.52 ± 137.91	1.16
1-methyl nicotinamide	7	101.21 ± 27.12	7	97.5 ± 27.25	0.96
Glycolic acid	7	136.16 ± 96.28	7	124.54 ± 73.05	0.91
Norepinephrine	7	9.46 ± 2.95	7	9.35 ± 1.91	0.99
Glycerol	7	633.57 ± 336.18	7	647.57 ± 365.3	1.02
Ribulose-5-phosphate	7	14.26 ± 2.90	5	13.88 ± 12.29	0.97
L-ornithine	7	44.12 ± 34.15	6	43.43 ± 31.89	0.98

Table S4. Primers used for qPCR analysis.

Gene name	Primer sequences	Annealing T (ºC)
Ornithine decarboxylase (*odc*)[44]	F: TTGACATTGGAGGAGGCTTT R: GATGACCTTTTTGGCGATG	50
Fatty acid synthase (*fas*)[43]	F: ATTCTGCTGGACGCTTTGTT R: GTCTCCTCTGAACGGACCTG	52
Peroxisomal acyl-coenzyme A oxidase 1 (*acox1*)[43]	F: GTTGACGGGCATTGTTTCTT R: TGTTGTTCCAAGCATCCTCA	51
β-actin (*actb*)[45]	F: CACCATGTACCCTGGCATTG R: GATCTTAATTTTCATGGTGGAAGGA	52

Materials and Methods.

Mercury determination

The standard deviation of seven replicates multiplied by the corresponding t-value (for the 95% confidence limit), was used to calculate the method detection limit (MDL), which was determined to be 0.001 µg/g in a 0.02 g sample (all blanks were below the MDL). For samples pertaining to the FHM exposures, certified reference materials (MESS-3, DORM and DOLT-4) were analyzed every 10 samples with a mean percent recovery for each of: MESS-3 102.52 ± 5.6 % (n = 9), DORM 104.38 ± 2.49 % (n = 6) and DOLT-4 102.64 ± 5.86 % (n = 8). The same reference materials were used to QAQC all samples from the mouse exposure, with the following mean percent recoveries: MESS-3 100.5 ± 0.42% (n= 4), TORT-2 93.7 ± 1.15 % (n = 4), and DOLT-4 104.9 ± 4.2 % (n= 4).

16S rRNA gene library preparation

Genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) following the manufacturer's instructions with minor modifications. Briefly, gastrointestinal tissue samples were thawed on ice, and pulverized with a sterilized pestle in a 1.5-mL microcentrifuge tube, before adding ATL buffer. DNA was eluted twice using 100-µL of buffer AE, following a 2-minute incubation at room temperature. 16S rRNA was amplified using universal bacterial primers, which target the V4 hypervariable region [38]. The forward primer was labeled with a 10-base barcode (unique to each sample) following the Ion Xpress barcode design (Life Technologies, Carlsbad, CA; catalog

#4471250). Each sample was prepared in duplicate 25-µL PCR reaction containing AccuPrime™ PCR Buffer II, 200µM dNTPs, 1 U AccuPrime™ Taq DNA Polymerase High Fidelity (Life Technologies), 0.2 µM each of forward IonA-515F and reverse IonP1-806R primers, 2 mM additional $MgSO_4$, 0.1 mg/mL BSA, and 10-100 ng template DNA. Thermocycler conditions for PCR were as follows: denaturation at 94 °C for 2-min; 30 cycles of denaturation at 94 °C for 15-sec, annealing at 52 °C for 15-sec, extension at 68 °C for 20-sec; and a final extension at 68 °C for 5-min. PCR products were then examined by gel electrophoresis (1.5% agarose gel), and remaining duplicate PCR samples were combined prior to purification via Agencourt® AMPure® XP magnetic beads (Beckman Coulter Inc, Brea, CA, product #A63882), using the manufacturer's specifications. The purified amplicon libraries were examined for size and quantity on a Bioanalyzer 2100 instrument using the Agilent DNA 7500 kit (Agilent Technologies, Santa Clara, CA, catalog #5067-1506). The 16S rRNA gene libraries were then combined in equimolar ratios, and diluted according to the manufacturer's recommendation for emulsion PCR on the Ion OneTouch™ 2 instrument (Life Technologies).

Metabolomics

Subsamples of larvae, or mouse midbrains from the low diet were transferred to a clean micro-centrifuge tube and flash frozen in liquid nitrogen. Samples were then homogenized in cold 2:5:2 chloroform:methanol:Mili-Q™ water solution with a motorized pellet pestle, and then centrifuged at 14000 rpm at 4° C for 8 minutes. Supernatant was removed and spiked with 10 µL of 150 µg/g D-27 myristate internal standard (IS) before evaporation under a gentle stream of nitrogen. Evaporated samples were derivatized with 50 µL of 15 mg/mL methoxyamine in pyridine solution, vortexed, and heated at 50° C for 30 minutes. This was followed by a second derivatization step with the addition of 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (TMCS), after which samples were vortexed and heated again at 70° C for 30 minutes. Samples were then analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 6890 GC and 5973 MS).

The Agilent Fiehn Retention Time Locking Library (Agilent part # G1676-90000) was used according to the manufacturer's recommendations for analysis and metabolite identification. Briefly, chromatographic (.D) files were processed using the Automated Mass Spectral Deconvolution &

Identification System (AMDIS; National Institute of Standards and Technology, Gaithersburg, Maryland), then submitted to the Agilent Fiehn library to generate semi-quantitative relative response factors ratioed against the response of the IS. A suite of fatty acid methyl ester standards was injected into every 5th sample (n=3) to provide data for the AMDIS Retention Index library used to track slight variations in predicted metabolite retention times.

qPCR Gene Expression Analysis

Entire clutches of larval fish (n = 5 clutches) from both Hg and control tanks were collected immediately post hatch, and frozen in a – 80° C freezer prior to RNA extraction. Larvae were homogenized with motorized pestles, and RNA was isolated on a Promega Maxwell™16 Automated Nucleic Acid Extraction system (Promega, Madison, WI), using a Promega Maxwell™16 Total RNA Purification Kit. Concentrations of DNase-treated RNA were determined spectrometrically (Nanodrop®, Thermo Scientific, Wilmington, DE). First-strand cDNA was synthesized from 1 µg RNA using an iScript™ Reverse Transcription Supermix for RT-qPCR (Bio-Rad, Hercules, CA) and a Biometra Thermocycler (LABREPCO, Göttingen, Germany), following manufacturer's instructions. The cDNA concentrations in all samples were determined spectrometrically and stored at – 20° C if not immediately used in quantitative polymerase chain reaction (qPCR).

Genes chosen for qPCR analysis were selected based on literature availability, and their relevance to the metabolomics data set [42-45]. Primers were made by Integrated DNA Technologies (Coralville, IA). Reactions were prepared using a QuantiTect™ SYBR™ Green PCR Kit (Qiagen, Louisville, KY) according to the manufacturer's guidelines. A Rotor-Gene (Corbett Research, Mortlake, Australia) was used for qPCR analysis, using the following protocol: a 15-minute activation step at 95°C, followed by 40 cycles of a 15 second denaturing step at 94°C, a 30 second annealing step at set temperatures for each primer (Supplementary Table S1), and a 30 second synthesis step at 72°C. Non-template controls were run for all primers to monitor contamination and primer-dimer formation. All samples were run in triplicate, and normalized to β-actin transcript levels. Differences in gene expression between treatments were evaluated via statistical analysis of ΔΔCt values.