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Hepatic metabolite profiling of polychlorinated biphenyl (PCB)-resistant and sensitive populations of Atlantic killifish (*Fundulus heteroclitus*)

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Abstract

Atlantic killifish inhabiting polluted sites along the east coast of the U.S. have evolved resistance to toxic effects of contaminants. One such contaminated site is the Acushnet River estuary, near New Bedford Harbor (NBH), Massachusetts, which is characterized by very high PCB concentrations in the sediments and in the tissues of resident killifish. Though killifish at this site appear to be thriving, the metabolic costs of survival in a highly contaminated environment are not well understood. In this study we compared the hepatic metabolite profiles of resistant (NBH) and sensitive populations (Scorton Creek (SC), Sandwich, MA) using a targeted metabolomics approach in which polar metabolites were extracted from adult fish livers and quantified. Our results revealed differences in the levels of several metabolites between fish from the two sites. The majority of these metabolites are associated with one-carbon metabolism, an important pathway that supports multiple physiological processes including DNA and protein methylation, nucleic acid biosynthesis and amino acid metabolism. We measured the gene expression of DNA methylation (DNA methyltransferase 1, *dnmt1*) and demethylation genes (Ten-Eleven Translocation (TET) genes) in the two populations, and observed lower levels of *dnmt1* and higher levels of TET gene expression in the NBH livers, suggesting possible differences in DNA methylation profiles. Consistent with this, the two populations differed significantly in the levels of 5-methylcytosine and 5-hydroxy methylcytosine nucleotides. Overall, our results suggest that the unique hepatic metabolite signatures observed in NBH and SC reflect the adaptive mechanisms for survival in their respective habitats.

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Keywords

Atlantic killifish; polychlorinated biphenyls; metabolomics; mass spectrometry

1. Introduction

Populations of Atlantic killifish inhabiting highly contaminated waters along the North Atlantic coast of the United States have evolved resistance to persistent organic pollutants such as polynuclear aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) (Bello et al., 2001; Hahn, 1998; Nacci et al., 1999). One such heavily contaminated site is the Acushnet River estuary located near New Bedford Harbor (NBH), Massachusetts, which is characterized by high concentrations of PCBs and PAHs in estuarine sediments and fish tissues (Bello et al., 2001; Nacci et al., 2002). The PCB and PAH levels in NBH sediment are extremely high and range from 2.8–3,240 µg/g and 11–2,360 µg/g dry weight, respectively, and with similarly high levels in fish and shellfish tissues (Subedi et al., 2014).

Killifish inhabiting NBH are resistant to the biochemical and toxic effects of dioxin-like compounds compared to fish from non-polluted ecosystems (Nacci et al., 2010). There has been intense focus to understand the mechanistic basis of this heritable resistance to dioxin-like compounds in this species (Hahn et al., 2004; Proestou et al., 2014; Reitzel et al., 2014). Investigators have hypothesized that altered aryl hydrocarbon receptor (AHR) functioning due to allelic variation in the resistant population is a potential mechanism of resistance. AHR is a ligand-activated transcription factor, well known for its role in response to exposure to dioxin-like compounds such as planar PCBs (Fernandez-Salguero et al., 1996). One of the widely used indicators of AHR activation is an increase in the expression of the gene encoding cytochrome P4501A1 (CYP1A) or enzyme activity as measured by ethoxyresorufin-o-deethylase (EROD) activity. NBH embryos from both field-caught and laboratory killifish showed decreased sensitivity to 3,3',4,4',5-pentachlorobiphenyl (PCB126) compared to the embryos from fish collected from non-polluted environments, and this resistance is heritable for multiple generations (Nacci et al., 2010). More recently, using whole genome sequencing of a large number of individuals, Reid et al. (2017) have demonstrated that variants in genes associated with the AHR signaling pathway are responsible for rapid adaptation to dioxin-like PCBs in NBH.

In addition to xenobiotic metabolism, AHR regulates other physiological processes such as energy metabolism, immune function, and development (Gutierrez-Vazquez and Quintana, 2018; Linden et al., 2010; Schneider et al., 2014). Most of our understanding of these physiological processes comes from laboratory-based *in vivo* studies under acute exposure regimes or *in vitro* cell culture systems. Very little is known about the metabolic effects of longterm multigenerational exposure to toxicants. NBH killifish provide a unique opportunity to study the metabolic effects of multigenerational exposure to contaminants. Recently, a few studies have explored the energy metabolism of Atlantic killifish embryos and adults from other pollutant-tolerant populations. For example, Lindberg et al. (2017) have shown that killifish from a PAH-contaminated site exhibited higher oxygen consumption rates during embryonic development and reduced metabolic plasticity as adults

than those from pristine sites. Similarly, Du et al. (2016) have shown that hepatic oxidative phosphorylation was significantly higher in fish collected from the PAH-contaminated Elizabeth River (Virginia) in comparison to fish from a pristine environment (King's Creek, Virginia). These studies suggest that fish inhabiting polluted environments have altered metabolic capacity. However, there are no reports comparing cellular metabolite levels in fish inhabiting contaminated and pristine environments.

The objective of this study was to determine the hepatic metabolite profiles of PCB-resistant Atlantic killifish sampled from the highly contaminated New Bedford Harbor site, and compare them with similar profiles from PCB-sensitive killifish from Scorton Creek, a pristine site in Sandwich, Massachusetts. We used targeted metabolomics to focus on liver metabolites, because the liver is an important organ involved in xenobiotic and energy metabolism. Unlike genes and proteins, the functions of which are subject to epigenetic regulation and post-translational modifications, respectively, metabolites serve as direct signatures of biochemical activity and are therefore easier to correlate with phenotype (Viant, 2008). The metabolomics approach is widely used to study the impact of toxicant exposure in aquatic organisms, including fish (Ekman et al., 2018; Mosley et al., 2018; Simmons et al., 2017). Our results demonstrate differences in the levels of metabolites associated with one-carbon metabolism, a critical pathway involved in epigenetic regulation.

2. Materials and Methods

2.1. Fish collection

Adult Atlantic killifish (*Fundulus heteroclitus*) were collected in June 2014 from PCB-contaminated Acushnet River Estuary in New Bedford Harbor (NBH) and from pristine Scorton Creek (SC). The fish from both collection sites were maintained for two weeks in the laboratory at ambient temperature and photoperiod before dissecting the tissues. Fish were fed once daily with a commercial pellet feed (Skretting Inc. Utah, USA). All animal husbandry practices were according to the regulations of the Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

2.2. Liver dissection and extraction of metabolites

Fish were euthanized by anaesthetizing the fish in Triacine (MS-222) and then by cervical transection. Livers were quickly removed and their total weights were recorded. From each fish, a small piece of liver tissue was immediately ground into a fine powder (in liquid nitrogen) using a mortar and pestle, and transferred into pre-weighed glass vials (Table 1). Remaining tissue was snap-frozen in liquid nitrogen and stored at -80°C . Polar (hydrophilic) metabolites were extracted using methanol/water:chloroform following a previously published method (Hines et al., 2007b), with some modifications. Briefly, metabolites were extracted in 1 ml of methanol and 212.5 μl of deionized water (followed by 60 s vortex) and 0.5 ml of chloroform (followed by 60 s vortex). The samples were then placed on ice for 10 min and gently mixed every 30–60 s. Additional volumes of 0.5 ml chloroform and 0.5 ml deionized water were added (followed by 60 s vortex), and the mixture was centrifuged (15 min, 1000 X g, 4°C). The top (polar) layer was carefully removed into clean glass vials,

dried in a centrifugal vacufuge and stored at -20°C until analysis. Prior to the analysis, samples were reconstituted in 95:5 water:acetonitrile (v:v) and diluted as needed (Table 1).

2.3. Targeted metabolomics

Samples were analyzed using a liquid chromatography system (Thermo Accela Open Autosampler and Accela 1250 Pump) coupled via a heated electrospray ionization (H-ESI) source to a triple quadrupole mass spectrometer (Thermo TSQ Vantage) operated in polarity switching mode. Instrument calibration, selected reaction monitoring (SRM) conditions, metabolite standard preparation, instrument operation, and data collection were all conducted as described previously (Kido Soule et al., 2015). Because the range of concentrations for the different metabolites in our samples was unknown, all samples were measured three times: undiluted, $5 \times$ dilution and $10 \times$ dilution in 95:5 water:acetonitrile (v:v). Data were analyzed using the Xcalibur Quan Browser software version 2.1.0 (Thermo Scientific). Standard curves were manually curated based on figures of merit such as extracted ion chromatograms (EIC) peak quality and curve linearity. Peak integrations for all measured metabolites in all samples were also manually curated. Following curation, five- to nine-point standard curves ($0.5\text{--}500\text{ ng ml}^{-1}$) were used to determine relative concentrations of each metabolite. The resulting quality-checked metabolite concentrations were then exported to Microsoft Excel. For each sample, the calculated metabolite concentrations were normalized using tissue weight to obtain the mass (ng) of metabolite contained per milligram of each sample. Normalized metabolite concentrations were analyzed with unpaired t-tests to determine significant differences between NBH and SC metabolic contents, and a 10% false discovery rate (FDR, q -value) was applied. Adjusted p-value less than or equal to 0.05 was considered statistically significant. Statistical analysis of the targeted metabolomics data was performed using GraphPad Prism (GraphPad Software Inc., version 6.07).

2.4. Isolation of genomic DNA

Isolation of genomic DNA was performed using the ZR-Duet™ DNA isolation kit (Zymo Research, CA). DNA was quantified using the Nanodrop Spectrophotometer. The quality of DNA was checked using Agilent 2200 TapeStation system. The DNA integrity numbers of all samples were between 8 and 10.

2.5. Quantification of 5-methyl cytosine and 5-hydroxymethyl cytosine levels

We quantified 5-methyl cytosine (5-mC) and 5-hydroxymethyl cytosine (5-hmC) levels using ELISA kits from Zymo Research (Irvine, CA) following manufacturer's instructions. Briefly, 100 ng of genomic DNA was mixed with 5-mC or 5-hmC coating buffer and added to the 96-well plate and incubated at 37°C for an hour. The DNA was discarded and incubated with ELISA buffer for 30 minutes prior to the addition of antibody mix consisting of primary antibody (monoclonal Anti-5-mC or anti-5-hmC) and HRP-conjugated secondary antibody. The samples were incubated for 1 hour and the wells were washed several times with ELISA buffer. HRP developer was added and absorbance was measured at 450 nm using a spectrophotometer (Molecular Devices Inc.). Standard curves were generated by serial dilution of 100% methylated DNA and methylation levels in the samples are expressed as percent methylation. A negative control sample (0% methylation) was included in each

run. All the samples and standard curves were run in duplicate wells. All the samples were assayed twice using kits with different lot numbers.

2.6. Total RNA isolation, cDNA synthesis and quantitative real-time PCR

DNase-treated total RNA was isolated using the BioRad Aurum kit (BioRad, Hercules, CA). RNA was quantified using the Nanodrop Spectrophotometer (ThermoFisher Scientific, CA). Complementary DNA was synthesized from 1 µg total RNA using the iScript cDNA Synthesis Kit (Bio-Rad, CA). Quantitative PCR was performed with iQ SYBR Green Supermix in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, CA). Real-time PCR primers used to amplify different genes of interest are provided in Table 2. The PCR conditions used were 95°C for 3 min (1 cycle) and 95°C for 15 s/65°C for 1 min (40 cycles). At the end of each PCR run, a melt curve analysis was performed to ensure that only a single product was amplified. Three technical replicates were used for each sample. A no-template control was included on each plate to ensure the absence of background contamination. Relative expression was normalized to that of β -actin (2^{-Ct} ; where $Ct = [Ct_{(GOI)} - Ct_{(housekeeping\ gene)}]$ and GOI is the gene of interest]. An unpaired t-test was used to determine the differences in expression between the two sites (GraphPad Prism version 5.3). A probability level of $p < 0.05$ was considered statistically significant.

2.7. Oil Red O staining

Frozen liver tissues from NBH and SC fish were cryosectioned (50 µm thickness) and stained for neutral lipid content using oil Red O as previously described (Mehlem et al., 2013). These were the same livers from which metabolites were extracted. Cryosectioning and staining were performed by Mass Histology Services (Worcester, MA). Sections were visualized under a Zeiss Axiovert 200 Inverted Microscope (Carl Zeiss Group, NY, USA) at 200X magnification. Images were taken with an AxioCam MRc (Carl Zeiss Group, NY, USA). Qualitative analysis of Oil Red O staining was performed with assistance from a certified veterinary pathologist.

3. Results

3.1. Targeted metabolomic analysis

Out of a total of 72 metabolites that were analyzed in the targeted metabolomics experiment, 40 were detected and quantified in our liver samples. Among them, the levels of 15 metabolites were found to be significantly different between the two sites (Table S1). Ten metabolites showed higher levels in NBH compared to SC. These included choline, sarcosine, arginine, ornithine, leucine/isoleucine, tyrosine, inosine, inosine monophosphate, pantothenic acid and ribose 5-phosphate (Fig. 1). The remaining 5 metabolites, glycine betaine, serine, threonine, adenosine and S-(1,2-carboxyethyl)-glutathione, were at lower levels in NBH fish livers compared to ones from SC (Fig. 2). Pathway analysis revealed that most of the significantly altered metabolites are part of one-carbon metabolic pathway (Fig. 3).

3.2. Global 5-mC and 5-hmC levels

NBH fish had significantly lower global 5-mC in comparison to SC fish (Fig. 4A). Average methylation was ~53% in the NBH livers vs. ~64% in SC samples. Levels of 5-hmC (Fig. 4B) were significantly higher in NBH (average ~32%) as compared to SC (average ~22%).

3.3. *dnmt1* and TET expression levels

NBH killifish had significantly lower *dnmt1* and higher *tet1* and *tet3* expression in comparison to SC fish (Fig. 5). The *dnmt1* expression was two-fold lower in NBH, whereas *tet1* and *tet3* expression were two-fold and three-fold higher respectively, in NBH. No significant differences in *tet2* expression between SC and NBH were observed.

3.4. Oil Red O staining

We decided to do Oil Red O staining for neutral lipids based on our visual observation that the livers of NBH fish were fatty in comparison to SC fish. Oil Red O staining of liver tissue showed that the majority of fish from NBH and SC had minimal to moderate amounts of lipid accumulation (Fig. 6). We did not observe any site-specific or sex-specific differences in staining. However, there was more variability in lipid accumulation in NBH fish livers in comparison to SC livers. Punctate-lipid droplets of variable size were found mostly in the hepatocytes surrounding the hepatic portal vein. In a couple of fish from NBH, the staining is in larger variable-sized globules, which are found throughout the cytoplasm of the hepatocytes that appear to be swollen.

4. Discussion

In this study we demonstrated differences in the hepatic metabolite levels associated with the one-carbon metabolic pathway between pollutant-tolerant and pollutant-sensitive Atlantic killifish. These two populations of killifish also exhibited differences in global 5-methyl cytosine levels and expression of genes involved in DNA methylation. These combined results suggest that altered one-carbon metabolism is one of the strategies for survival in polluted environments.

Metabolites serve as direct signatures of biochemical activity downstream of transcription. Metabolomics approaches are increasingly used for characterizing the effects of environmental toxicants on cellular metabolite levels in aquatic organisms (Ekman et al., 2018; Hines et al., 2007a; Mosley et al., 2018; Simmons et al., 2017; Viant, 2008; Viant et al., 2009; Viant et al., 2003). Several studies have investigated the transcriptional changes associated with PCBs in NBH Atlantic killifish (Oleksiak et al., 2011; Whitehead et al., 2012; Whitehead et al., 2010), but the biochemical and metabolic changes following transcription have not been investigated in this population. In this study, we found significant differences in 15 metabolites, most of which are associated with the one-carbon metabolic pathway. One-carbon metabolism is considered an integrator of the cellular nutritional status by converting nutrients (e.g., glucose, amino acids) into metabolites that feed into diverse biological functions, including cellular biosynthesis, maintaining cellular redox status, and regulation of epigenetic status through protein and nucleic acid methylation (Locasale,

2013). Differences in these metabolite levels suggest that fish inhabiting polluted and pristine sites adopt different physiological strategies for survival in their environment.

Higher levels of choline and sarcosine and lower levels of glycine betaine were detected in the livers of fish inhabiting the highly contaminated NBH in comparison to SC fish. Choline is an essential nutrient and important for normal liver function (Corbin and Zeisel, 2012). For example, phosphorylated choline is used for making phospholipids, an essential component of cell and mitochondrial membranes, while oxidized choline is a donor for methyl groups, an important player in epigenetic modification of DNA and proteins. High availability of choline is generally linked to increased production of glycine betaine, a downstream metabolite. However, we observed reduced levels of glycine betaine in NBH fish, suggesting either a decrease in the availability of choline for glycine betaine production or increased turnover of glycine betaine. Low glycine betaine levels could result in decreased methylation of homocysteine to form methionine, a precursor for synthesis of S-adenosylmethionine (SAM), the universal methyl donor needed for methylation of nucleic acids and proteins (Blom and Smulders, 2011). However, we did not observe any significant differences in SAM, S-adenosyl-L-homocysteine (SAH) or methionine levels between NBH and SC fish. The SAM/SAH ratio is widely used as an indicator of DNA and protein methylation capacity in the cell (Cantoni, 1985; Caudill et al., 2001); the lack of any differences in fish from the two sites suggests that methylation substrate is not limiting. Nevertheless, we observed differences in the DNA methylation machinery between NBH and SC fish.

NBH fish showed lower *dnmt1* gene expression and global 5-methyl cytosine levels. In addition, the *tet1* and *tet3* genes had higher expression in NBH fish. Provided that the transcriptional changes are mirrored in protein activity, our results indicate that NBH and SC fish could have differences in DNA methylation patterns. In a few loci (*ahr* and *cyp1a*), we and others have not detected any differences in methylation patterns between the fish from these two sites (Aluru et al., 2011; Timme-Laragy et al., 2005). Future studies should include genome-wide DNA methylation profiling in order to determine the differences in the epigenetic landscape between resistant and sensitive fish. Lower *dnmt1* expression and higher expression of TET genes in NBH fish livers suggest that there is increased metabolism of 5-methylcytosine. This is also reflected in the lower global 5-methylcytosine levels and higher 5-hydroxymethyl cytosine levels observed in the NBH fish. With the availability of genomic resources, it is possible to profile DNA methylation and hydroxymethylation at base-pair resolution and determine the role of epigenetic variation in these populations.

In addition to the effects on epigenetic regulation, differences in one-carbon metabolism could also impact nucleic acid biosynthesis. Our results show differences in purine nucleotide pools between NBH and SC fish livers. Purine nucleotides are the metabolic end products of folate metabolism (Locasale, 2013). We observed low levels of adenosine (A) and high levels of both inosine (I) and inosine monophosphate in NBH fish compared with SC, suggesting increased deamination of adenosine (and conversion to inosine) in NBH (Alseth et al., 2014). Previous studies have shown that environmental toxicants can induce deamination (Hu et al., 2015; Lin et al., 2011). DNA deamination is a pre-mutagenic event,

and in normal cells DNA repair enzymes (Base Excision Repair) recognize and remove inosines. Defective DNA deamination is also seen in a number of human diseases (Alseth et al., 2014). In contrast to DNA deamination, which is considered mutagenic, incorporation of inosine into RNA is a normal and essential modification that is necessary to increase transcriptome diversity. Studies in humans have determined that A-to-I editing of RNA occurs mainly in the non-coding regions containing repetitive elements, and in 5' and 3' untranslated regions (UTRs) (Ramaswami et al., 2012). Similar analysis of the killifish genome in general and NBH fish population in particular should be conducted in order to determine the impact of chronic exposure to PCBs on A-to-I editing.

We also observed differences in the levels of leucine/isoleucine, tyrosine and 5-ribose phosphate levels between NBH and SC fish. Leucine/isoleucine and tyrosine are key amino acids involved in the generation of fatty acids, whereas ribose 5-phosphate is an important intermediate metabolite in the pentose phosphate pathway. It is unclear if any of these individual metabolite changes have a significant impact on the fish physiology, but in concert with the changes seen in one-carbon metabolism, it is possible that NBH fish have higher energetic costs to maintain metabolic homeostasis. For example, amino acid imbalance, particularly of leucine and tyrosine, is linked to non-alcoholic fatty liver disease (NAFLD). It has been recently demonstrated that dioxin and dioxin-like PCBs can cause NAFLD in rodents (Fader et al., 2015; Jeanneret et al., 2014; Matsubara et al., 2012; Nault et al., 2015; Ruiz-Aracama et al., 2011). The differences in hepatic metabolite levels between NBH and SC fish resemble some seen in NAFLD, suggesting NAFLD-like symptoms in fish. We have consistently observed fatty livers in NBH fish. However, oil Red O staining did not reveal any significant differences and this could be due to the high degree of variability between individual fish and between sexes. Nevertheless, based on the differences in the metabolite levels these the two populations of killifish would be a good “natural” model for understanding the long-term effects of exposure to dioxin-like chemicals on metabolic dysfunction.

5. Conclusions

Overall our results demonstrate differences in the metabolite levels between fish collected from polluted and pristine sites. The differences in metabolites related to the one-carbon metabolic pathway suggest that cellular metabolism is different in fish from these two sites. This could potentially affect epigenetic regulation of gene expression, nucleic acid biosynthesis and metabolic disturbances. However, we cannot completely rule out the possibility that the differences between NBH and SC observed in this study could be partly due to ecological differences (e.g., habitat, food availability) at these two sites. In order to determine if the differences in the metabolites are adaptive mechanisms for survival in highly contaminated environment, future studies should be conducted in laboratory-reared animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Hepatic metabolite profiling of PCB-resistant and sensitive fish revealed differences in metabolites associated with one-carbon metabolism, a major donor of methyl groups for DNA methylation.
2. There are also differences in global DNA methylation levels as well as *dnmt1*, *tet1* and *tet3* gene expression between PCB-resistant and sensitive fish.
3. Differences in the one carbon metabolism and DNA methylation suggest that fish inhabiting polluted and pristine sites adopt different physiological strategies for survival in their environment.

Figure 1.

Box plot representation of metabolites that showed significantly higher levels in NBH fish livers compared to SC fish livers. Concentrations of metabolites are presented as ng/mg wet weight of liver tissue. Statistical significance was determined by unpaired t-test with 10% false discovery rate (q -value; Benjamini-Hochberg test). Adjusted p -value less than or equal to 0.05 was considered statistically significant. Fold change values (NBH:SC ratio) and the corresponding adjusted p -values of the metabolites are given in supplementary table 1.

Figure 2.

Box plot representation of metabolites that showed significantly lower levels in NBH fish livers in comparison to SC. Concentrations of metabolites are presented as ng/mg wet weight of liver tissue. Statistical significance was determined by unpaired t-test with 10% false discovery rate (q -value; Benjamini-Hochberg test). Adjusted p -value less than or equal to 0.05 was considered statistically significant. Fold change values (NBH:SC ratio) and the corresponding adjusted p -values of the metabolites are given in supplementary table 1.

Figure 3.

Overview of the one-carbon metabolism pathway. Significantly altered metabolites in this are highlighted. Metabolites highlighted in blue and red represent those that are significantly higher and lower in NBH respectively. THF – tetrahydrofolate; me-THF – N5N10-methylenetetrahydrofolate; m-THF – N5-methyl-tetrahydrofolate; F-THF – N10-formyl-tetrahydrofolate; DMG – dimethylglycine; SAM – S-adenosylmethionine; SAH – S-adenosylhomocysteine; SAHH – S-Adenosyl-L-homocysteine hydrolase.

Figure 4. Global 5-methylcytosine (A; 5mC) and 5-hydroxymethyl cytosine (B; 5-hmC) levels in NBH and SC fish livers. 5mC and 5hmC levels were determined using a standard curve. * represents significant difference in the levels between the two sites (unpaired t-test; $p < 0.01$).

Figure 5. DNA methylation and demethylation gene expression patterns in NBH and SC liver. *dnmt1* (A) and *tet* (B-D) expression was quantified using qPCR and their relative expression was calculated using the delta Ct method. β -actin was used as a reference gene. * represents significant difference in expression between the two sites (unpaired t-test; $p < 0.01$).

Figure 6.

Lipid accumulation in fish livers from NBH (A-D) and SC (E-H). Liver tissues were cryosectioned and stained for neutral lipids with Oil Red O. The lipid droplets around the hepatic portal vein are stained in red. Representative images from NBH (A-D) and SC (E-H) fish are shown. Top and bottom panel of images are from the male and female fish respectively.

Table 1.

Information on the experimental animal and sample preparation. The whole weight of the fish, sex, total liver weight and weight of the liver tissue used in solvent extraction are shown. Final concentration for injection of all samples in targeted metabolomics method was 0.1 mg/ μ l.

Sample name	Whole fish weight (g)	Sex	Total liver weight (mg)	Sample weight (mg)	Reconstitution volume (μ l)
SC 1	4.3	F	221	31.3	313
SC 2	4.3	F	158	14.3	143
SC 3	4.8	M	288	67.2	672
SC 4	3.9	M	144	11.8	118
SC 5	4.9	M	219	37.6	376
SC 6	4.3	F	187	60.3	603
SC 7	2.5	M	63	12.6	126
SC 8	3.3	M	98	18.1	181
NBH 1	6.2	M	266	22.4	224
NBH 2	5.8	M	148	28.8	288
NBH 3	6.2	M	157	17.5	175
NBH 4	6.5	M	298	51.2	512
NBH 5	7.7	F	204	22.4	224
NBH 6	10.6	M	252	44.2	442
NBH 7	7.0	M	182	85.4	854
NBH 8	8.0	F	273	44.8	448
NBH 9	8.0	M	179	34.2	342
NBH 10	10.0	F	203	41.6	416

Table 2.

List of quantitative PCR primers and their annealing temperatures (T_m; °C) used in this study.

Gene	Primer Sequence (5'.....3')	T _m (°C)
<i>dnmt1</i>	Forward - CTGACCAGTGGCGTTTTTGCCAGG Reverse - TCAGGCTGTAGTCGGCGAAAGACG	65
<i>tet1</i>	Forward - GGCGCCAAAGGAAATGCAGTGAG Reverse - CACCCATTTAGCTATGGGACACC	62
<i>tet2</i>	Forward - CGCCTCGTGCCACTGCGTCGATCA Reverse - GGCTCGCCCGCTGAGGCCAGACCTT	62
<i>tet3</i>	Forward - GGACAGAAGGGGAAGCAGTTCGG Reverse - CTCGCTGCTTCGACGAATCACCCA	62
<i>β-actin</i>	Forward - TGGAGAAGAGCTACGAGCTCC Reverse - CCGCAGGACTCCATTCCGAG	62
<i>18S</i>	Forward - TGGTTAATTCCGATAACGAACGA Reverse - CGCCACTGTGTCCTCTAAGAA	65