Published in final edited form as:

Aquat Toxicol. 2019 May ; 210: 30–43. doi:10.1016/j.aquatox.2019.02.011.

Altered lipid homeostasis in a PCB-resistant Atlantic killifish (*Fundulus heteroclitus*) population from New Bedford Harbor, MA, U.S.A.

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Abstract

Sentinel species such as the Atlantic killifish (*Fundulus heteroclitus*) living in urban waterways can be used as toxicological models to understand impacts of environmental metabolism disrupting compound (MDC) exposure on both wildlife and humans. Exposure to MDCs is associated with increased risk of metabolic syndrome, including impaired lipid and glucose homeostasis, adipogenesis, appetite control, and basal metabolism. MDCs are ubiquitous in the environment, including in aquatic environments. New Bedford Harbor (NBH), Massachusetts is polluted with polychlorinated biphenyls (PCBs), and, as we show for the first time, tin (Sn). PCBs and organotins are ligands for two receptor systems known to regulate lipid homeostasis, the aryl hydrocarbon receptor (AHR) and the peroxisome proliferator-activated receptors (PPARs), respectively. In the current study, we compared lipid homeostasis in laboratory-reared killifish

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from NBH (F2) and a reference location (Scorton Creek, Massachusetts; F1 and F2) to evaluate how adaptation to local conditions may influence responses to MDCs. Adult killifish from each population were exposed to 3,3',4,4',5-pentachlorobiphenyl (PCB126, dioxin-like), 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153, non-dioxin-like), or tributyltin (TBT, aPPAR γ ligand) by a single intraperitoneal injection and analyzed after 3 days. AHR activation was assessed by measuring *cyp1a* mRNA expression. Lipid homeostasis was evaluated phenotypically by measuring liver triglycerides and organosomatic indices, and at the molecular level by measuring the mRNA expression of *pparg* and *ppara* and a target gene for each receptor. Acute MDC exposure did not affect phenotypic outcomes. However, overall NBH killifish had higher liver triglycerides and adiposomatic indices than SC killifish. Both season and population were significant predictors of the lipid phenotype. Acute MDC exposure altered hepatic gene expression only in male killifish from SC. PCB126 exposure induced *cyp1a* and *pparg*, whereas PCB153 exposure induced *ppara*. TBT exposure did not induce *ppar*-dependent pathways. Comparison of lipid homeostasis in two killifish populations extends our understanding of how MDCs act on fish and provides a basis to infer adaptive benefits of these differences in the wild.

Keywords

Fundulus heteroclitus; polychlorinated biphenyl (PCB); tributyltin (TBT); liver triglyceride; tin (Sn); metabolism disrupting compound (MDCs)

1. Introduction

The dramatic rise in obesity worldwide has drawn considerable clinical and research attention. One conclusion from obesity-related research is that diet and exercise cannot entirely explain the global obesity epidemic. Rather, a complex array of genetic, epigenetic, environmental and behavioral factors contribute to an individual's risk of obesity (e.g., Burgio et al., 2015). This discovery has led to the identification of environmental metabolism disrupting compounds (MDCs), a class of compounds that alter metabolic homeostasis, including lipid and glucose homeostasis, adipogenesis, appetite control, and basal metabolism (e.g., Grün & Blumberg, 2006, 2009; Heindel et al., 2017). MDCs, also referred to as “obesogens,” are structurally diverse and act through multiple molecular pathways. Among MDCs are pollutants common in aquatic environments such as organotins (e.g., tributyltin, TBT; Grün and Blumberg, 2006; Heindel et al., 2017) and dioxin- and non-dioxin-like polychlorinated biphenyls (PCBs; Baker et al., 2013; Gadupudi et al., 2015; Wahlang et al., 2013).

Three ligand-activated transcription factors play particularly important roles in metabolic homeostasis and can be activated by environmental MDCs: peroxisome proliferator activated receptor (PPAR) gamma and alpha (PPAR γ and PPAR α , respectively), and the aryl hydrocarbon receptor (AHR). Activation of the PPAR γ pathway induces adipogenesis, hypertrophy and hyperplasia of adipocytes, and lipogenic gene expression in adipose tissue and liver (Grün et al., 2006; Heindel et al., 2017; Kim et al., 2018; Pereira-Fernandes et al., 2013; Tontonoz et al., 1994; Wang et al., 2017; Watt and Schlezinger, 2015). PPAR α pathway activation stimulates fatty acid transport and β -oxidation during periods of energy

deprivation, including between meals (Kersten et al., 2000; van Raalte et al., 2004), countering lipid-sequestering effects of PPAR γ . AHR plays a central role in xenobiotic metabolism (Nebert, 2017), as well as regulation of lipid accumulation and inflammation in adipocytes, glucose tolerance, insulin sensitivity (Arsenescu et al., 2008; Baker et al., 2013; N. A. Baker et al., 2015; Kerley-Hamilton et al., 2012; La Merrill et al., 2009), and proper development of pancreatic β -cells (Timme-Laragy et al., 2015). The potential for the AHR to suppress PPAR γ gene expression and activity has been suggested (e.g., Gadupudi et al., 2015), but remains poorly understood.

Ligands for both AHR (dioxin-like PCBs) and PPAR γ (TBT) are found in urban waterways, near major population centers in the United States. New Bedford Harbor (NBH) is a marine Superfund site, located in southeastern Massachusetts (Figure 1). PCBs were used in electronics manufacturing facilities around NBH, and their discharge into the environment has led to widespread, severe contamination throughout the harbor (sediment PCBs were measured in excess of 100,000 parts per million (ppm dry weight, as Aroclor 1254; Weaver, 1982) and remain elevated despite ongoing remediation; US EPA, 2019). Organotins were used as an antifouling agent in marine boat paints and are common sediment contaminants in prominent harbors around the world (Antizar-Ladislao, 2008). NBH has long served as a major fishing and shipping port in the region, suggesting that concentrations of tin compounds may be elevated, though tin has not previously been measured in NBH.

NBH is home to wildlife, including the Atlantic killifish (*Fundulus heteroclitus*), which is both an ecological sentinel and a vertebrate model amenable to mechanistic toxicology studies (Burnett et al., 2007). Seasonal climate variability results in dramatic changes in feeding behavior, body composition and energetics in killifish. During the summer months, energy resources are allocated to growth and reproduction, whereas in the fall and winter, energy storage and maintenance become the respective priorities (e.g., Berg & Bremset, 1998; Kandemir, Polat, & Turk, 2007; Sheridan, 1994). Killifish have been studied extensively for their response to industrial chemicals, most notably PCBs and polycyclic aromatic hydrocarbons (PAHs) acting through the AHR (e.g., Nacci et al. 1999, 2010; Oleksiak et al. 2011; Reid et al. 2016). The small home ranges of killifish have led to the evolution of genetically distinct populations along the US eastern seaboard. Killifish living in polluted estuaries, like NBH, demonstrate dramatic heritable resistance (evolved tolerance) to AHR pathway activation (e.g., Bello et al. 2001; Meyer and Di Giulio 2003; Nacci et al. 1999, 2010; Reid et al. 2016) and have emerged as an important example of convergent evolution (Reid et al., 2016; Whitehead et al., 2012).

In this study, we examined liver lipid homeostasis in NBH killifish in comparison to killifish from a reference location, Scorton Creek (SC), Massachusetts. We took advantage of intraspecific variation in these two killifish populations (NBH, SC) to evaluate the effects of MDCs. Exposure to MDCs is associated with increased risk of developing metabolic syndrome, yet little is currently known about how multi-generational exposures to MDCs may have affected NBH killifish. We tested the hypothesis that exposure to select environmental MDCs (e.g., PCBs and TBT) would increase liver triglyceride levels and perturb molecular signaling related to lipid homeostasis across seasons. We also tested the hypothesis that SC and NBH killifish would respond differently to MDC exposure due to

population-specific exposure histories and adaptations. Although it is inherently difficult to attribute phenotypic differences observed between killifish populations (e.g., SC and NBH) to site-specific conditions in their respective estuaries, this study provides novel insights into intra-specific and seasonal variability in lipid homeostasis in killifish. This study is also the first to report Sn in NBH sediments, which may be associated with historic applications of organotins in marine paints in the harbor (Supplementary Materials).

2. Methods

2.1 Animal Care and Selection

All activities were performed in accordance with U.S. Environmental Protection Agency Atlantic Ecology Division Laboratory animal care protocols and every effort was made to minimize pain and distress. Adult, reproductively-active *F. heteroclitus* (F0) were collected from NBH and SC (reference) using baited traps as described in Nacci et al. (2002). Killifish were held in 300- or 600-liter flow-through aquaria receiving 5µM filtered, ambient-temperature seawater from Narragansett Bay, Rhode Island. Variable light:dark cycles represented ambient conditions throughout the year. Killifish were fed *ad libitum*, as described in Nacci et al. (2002). F0 killifish spawned with semi-lunar periodicity throughout summer months, during which time embryos (F1) were harvested and reared to reproductive maturity (1-2 years) under the conditions described above. F2 embryos were then harvested from F1 adults and raised to reproductive maturity in the same manner.

Weight-matched killifish from NBH and SC (Table 1) were selected for use in four replicate intraperitoneal (IP) injection exposure experiments, referred to herein by their date: October 2015, March and October 2016 and May 2017. Given seasonal changes in resource allocation in killifish, two experimental replicates were conducted in the fall and spring to capture periods of high and low lipid reserves. At least two weeks prior to each experiment, water in the flow-through aquaria was warmed to and held at 23°C, the approximate ambient summer temperature in Narragansett Bay. NBH F2 adults were used to ensure complete depuration of persistent organic pollutants (POPs) from NBH and to evaluate heritable effects of adaptation to NBH contaminants. Since SC fish represent the reference site, maternal transfer of POPs is not of concern. F2 killifish from SC were selected when possible (October 2016 and May 2017). When F2 size matching between NBH and SC animals was not possible, F1 killifish from SC were utilized (October 2015 and March 2016).

2.2 Chemical Exposure by Intraperitoneal Injection

An even number of male and female killifish from each population were divided among tanks, with 6-10 individuals per tank, depending on the experimental replicate (n=10, 5 males and 5 females in October 2015; n=6, 3 males and 3 females in all others). Killifish were distributed to achieve similar mean weight across tanks (Table 1) and were allowed to acclimate for one week prior to IP injection. In each experimental replicate, all killifish administered a given chemical treatment were housed in one tank per population. On the seventh day, killifish were anesthetized in tricaine methanesulfonate (MS222) and administered chemicals by IP injection. While IP injection is not representative of natural

routes of killifish exposure to MDCs (e.g., ingestion, inhalation, dermal absorption), this route of exposure allowed for precise administration of known, environmentally-relevant doses of MDCs.

3,3',4,4',5-Pentachlorobiphenyl (PCB126) and 2,2',4,4',5,5'-hexachlorobiphenyl (PCB153) used in the first experiment (October 2015) were purchased from AccuStandard (New Haven, CT, USA). PCB126 and PCB153 used in all other experimental replicates were purchased from Ultra Scientific (North Kingstown, RI). Tributyltin chloride (TBT) was purchased from Sigma Aldrich (St. Louis, MO, USA). S26948, a non-thiazolidinedione (TZD) selective PPAR γ agonist and antidiabetic pharmaceutical agent, was purchased from Tocris Bioscience (Minneapolis, MN, USA). Chemicals were suspended in DMSO or acetone. Exposures consisted of vehicle (0.03% DMSO or acetone), PCB126 (29 ng/g), PCB153 (2,120 ng/g), TBT (10 μ g/g), or S26948 (100 μ g/g) in corn oil (1 μ g corn oil solution per g fish). PCB doses were selected with the goal of achieving concentrations comparable to those measured in liver of wild-caught, undepurated killifish from NBH (Black et al., 1998). We measured total tin (Sn) in NBH sediments (Supplementary Materials, Figure S1a-b), but organotin concentrations have not been evaluated in NBH killifish. Therefore, TBT as well as S26948 doses were selected based on EC₅₀ concentrations noted in other studies (Carmona et al., 2007; Grün, 2014).

Chemical exposures differed among experimental replicates and are summarized in Table S1. In October 2016 and May 2017, naïve (uninjected) killifish from the same cohort were sampled to confirm that outcome measures of interest were not altered relative to vehicle-treated animals. Three days after IP injections, killifish were euthanized in MS222. Animal weight (g, wet weight (ww)) and standard length were measured for all animals except those euthanized in October 2015. Liver, adipose tissue, gonad and brain were resected and weighed (g, ww).

2.3 Killifish Morphology

Condition factor (CF), analogous to body mass index (BMI), was calculated using killifish weight (ww) and standard length at euthanasia. Hepatosomatic (HSI), adiposomatic (ASI) and gonadosomatic (GSI) indices were calculated using resected organ and killifish weight (ww) at euthanasia. Killifish characteristics, including phenotypic parameters are summarized in Table 1 for each experimental replicate.

2.4 Liver Triglyceride (TAG) Analysis

Liver TAG (mg TAG per g liver) were measured as described in Norris et al. (2003). Briefly, approximately 45mg (ww) of liver was digested in ethanolic potassium hydroxide. Following neutralization with magnesium chloride, glycerol was measured spectrophotometrically at 540nm using Free Glycerol Reagent (Sigma Aldrich). TAG concentration was calculated from a triolein standard curve included on the same plate.

2.5 Gene Expression Analysis

Total RNA was extracted from approximately 30mg (ww) of liver using RNA STAT-60™ (Tel-Test Inc., Friendswood, TX, USA). Genomic DNA was removed using the Aurum™

Total RNA Mini Kit (BioRad). cDNA was prepared from total RNA using the GoScript™ Reverse Transcription System (Promega), using equal parts random and oligo (dT)₁₅ primers. qPCR reactions were performed using iTaq Universal SYBR® Green Supermix (BioRad) with 400nM forward and reverse primers (Table S2). Thermocycling parameters for all genes were 95°C for three minutes to activate iTaq DNA polymerase, followed by 40 cycles of melting at 95°C for 15 seconds and annealing at 66°C for 60 seconds. A manually-selected threshold in the linear phase of the amplification curve was used to determine threshold cycle (C_T) values to standardize across multiple plates.

Gene expression was normalized by the Pfaffl method (Pfaffl, 2001), using primer-specific amplification efficiency and the expression of constitutively expressed (housekeeping) genes. Three housekeeping genes were evaluated for stability across population, sex and chemical treatment. Variability was greatest in *β-actin* (overall mean (standard deviation) = 1.157 (0.798)), with differences between populations (SC > NBH) and sexes (male > female). Expression of *efl α* was less variable (mean (SD) = 0.874 (0.317)) with no difference between populations, though expression differed slightly between sexes (female > male). *Rn18s* was most stably expressed (mean (SD) = 0.997 (0.172)) with no discernable pattern of variability between populations, sexes or chemical treatment groups. Thus, all gene expression data presented herein are normalized to *rn18s* and reported as a relative expression ratio (relative to a consistent set of naive liver samples harvested from an equal number of SC and NBH killifish at the same time and under identical conditions as those killifish used for chemical exposures).

2.6 Data and Statistical Analyses

All statistical analyses were performed using R version 3.3.2, including lmerTest and lme4 packages. A comparative approach was taken to statistically evaluate phenotypic outcomes (liver TAG, ASI, GSI and HSI) across all experimental replicates. Since normality and homogeneity of variance assumptions were met, parametric measures, including ANOVA and t-tests, were used to compare means among populations, experimental replicates, sexes and chemical treatments. To better account for predictors of each phenotypic outcome and seasonal oscillations in killifish lipid reserves, we also used multiple linear regression (MLR) and linear mixed effects regression (LMER) models, which are important, yet underutilized, tools in toxicological research. LMER is an extension of standard linear models and is frequently used when there is potential non-independence in experimental units due to hierarchical structures in the data generating process. Both types of models are robust despite modest deviations from parametric assumptions (Everitt and Hothorn, 2010; Wooldridge, 2006). The use of regression modeling techniques was possible due to a relatively large overall sample size (n=225) compared to many toxicological studies. We included fixed effects for population (NBH, SC), sex, fish weight (g), season (fall, spring) and chemical treatment as covariates. Based on knowledge of killifish physiology, season was considered a random effect in LMER. The parsimonious MLR and LMER models for each phenotypic outcome are referred to herein as “fully adjusted.”

When evaluating liver TAG, we also included ASI to account for potential confounding and compared results to unadjusted models to evaluate whether ASI is on the causal pathway to

TAG. To evaluate the potential for overfitting the MLR model with ASI, we conducted a sensitivity analysis by comparing MLR models with and without ASI. Although effect estimates and p-values changed slightly depending on whether ASI was included in MLR for liver TAG, changes were minor and did not alter model interpretation. Together with *a priori* knowledge of lipid homeostasis in killifish, we chose to include ASI in the MLR models presented herein.

Intra-class correlation coefficients (ICCs) were calculated to evaluate within group correlations. Statistical significance was evaluated at $\alpha = 0.05$ for all analyses. Outliers were identified as points falling more than 1.5 times the interquartile range below or above the first or third quartiles, respectively. All data points, including possible outliers, were used in analyses due to the small sample size in each group (n=3 per group).

Gene expression was analyzed using ANOVA and t-tests to evaluate differences in mean relative expression among populations, sexes and treatment groups for killifish in the May 2017 IP injection study. No differences were found in the expression of housekeeping or target genes between naive (uninjected) and control (DMSO) killifish livers. As a result, the naive treatment group was eliminated from the dataset for statistical analyses.

3. Results

This study evaluated the effects of acute exposure to MDCs on lipid homeostasis in adult killifish at the phenotypic and molecular levels. We also quantified concentrations of total tin (Sn) in NBH sediments at multiple sites (Supplementary Methods, Figure S1a) and found that levels of Sn in NBH (ranging from 3.2 to 38 ug/g, dry weight; mean = 17 ug/g) exceeded those measured in a reference sample collected outside the harbor (mean = 2.2 ug/g), Buzzards Bay (Figure S1b).

3.1 Fish Characteristics

Condition factor was calculated for killifish in all but the first experiment, in which standard length and resected organ weights were not recorded at euthanasia. Killifish weight (g, ww) and CF are summarized in Table 1. Weight and CF were consistent for each sex, across populations, within all experimental replicates. However, due to size class limitations in F1 SC killifish available to be paired with F2 NBH animals in March 2016, weight and CF were significantly higher in females than males (two-way ANOVA: $p_{\text{weight}} = 0.0332$; $p_{\text{CF}} = 0.0170$) within each population. Weight-matching was maintained for males and females across the two populations. There were no differences in weight or CF between control and chemical treatment groups.

3.2 Population and Season, but not Acute MDC Exposure, Determine Lipid Phenotypes

Next, we tested the hypothesis that MDCs affect lipid homeostasis in killifish. The liver is a significant storage site for lipids in many fish (reviewed in Nanton et al., 2003; Sheridan, 1988, 1994) so we began by analyzing liver lipids (triglycerides, TAG). Liver TAG was not affected by acute exposure to either PCBs or TBT (Figure 2a). We took advantage of the unusually large sample size (n=225) to evaluate additional predictors (season, population, sex, treatment, ASI) of liver TAG using multiple approaches: two-way ANOVA, MLR and

LMER. Results from adjusted models are included in Table 2. No differences in liver TAG were observed between sexes (mg TAG/g liver (mg/g): female = 88.86, male = 88.44; t-test, $p = 0.9379$), as reported in other fish species (e.g., Craig et al., 2000; Scott, Fåhræus-Van Ree, & Parrish, 2002), so we did not control for sex in TAG linear regression models. Both killifish populations exhibited marked seasonal variability in liver TAG (Figure 2b, Table 2; MLR, $\beta_{\text{fall}} = 40.86$ mg/g, $p_{\text{fall}} = <0.0001$). NBH killifish consistently had higher TAG levels than SC individuals (Figure 2b, Table 2; MLR, $\beta_{\text{NBH}} = 32.05$ mg/g, $p_{\text{NBH}} = <0.0001$). Liver TAG was significantly negatively associated with killifish weight (Table 2; MLR, $\beta_{\text{NBH}} = -4.29$ mg/g, $p_{\text{NBH}} = <0.0014$). Liver TAG was significantly positively associated with ASI (Table 2; MLR, $\beta_{\text{NBH}} = 3.12$ mg/g, $p_{\text{NBH}} = <0.0086$), and the interpretation of linear regression models did not change when a sensitivity analysis was performed by removing ASI from the model (Table S3). We also evaluated HSI by ANOVA and linear regression modeling techniques. In agreement with liver TAG analyses, HSI tended to be higher in NBH killifish relative to SC animals (Figure 3a) and to a significant degree after adjusting for other covariates (Table 2; MLR, $\beta_{\text{NBH}} = 0.85$, $p_{\text{NBH}} = 0.0001$). HSI was significantly higher in female killifish relative to males (Table 2; MLR, $\beta_{\text{male}} = -0.50$, $p_{\text{male}} = 0.0131$) as reported in Pait & Nelson (2009), and was higher in the fall than the spring after adjusting for other covariates (Table 2; MLR, $\beta_{\text{fall}} = 1.83$, $p_{\text{fall}} = <0.0001$). HSI was significantly positive associated with weight and ASI (Table 2; MLR, $\beta_{\text{weight}} = 0.15$ g, $p_{\text{weight}} = 0.0132$; $\beta_{\text{ASI}} = 0.12$, $p_{\text{ASI}} = 0.0337$). HSI was not affected by MDC exposure.

Adipose tissue and gonads can also be sites of lipid accumulation in fish. ASI was significantly higher in NBH killifish compared to SC killifish (Figure 3b, Table 2; MLR, $\beta_{\text{NBH}} = 1.67$, $p_{\text{NBH}} = <0.0001$). This pattern persisted regardless of whether SC fish were from the F1 or F2 generation. Females had higher ASI than males, after controlling for other covariates (Table 2; MLR, $\beta_{\text{male}} = -0.66$, $p_{\text{male}} = 0.0200$). Season was not a significant predictor of ASI and as a result, LMRE models using season as a mixed effect were not used to analyze ASI. Season does, however, affect ASI in SC killifish differently than NBH animals (Table 1, Figure 3b). In the spring, ASI in NBH killifish was significantly higher than in SC killifish (MLR, $\beta_{\text{NBH*fall}} = -4.34$, $p_{\text{NBH*fall}} = <0.0001$). This difference diminishes over the course of the summer, such that ASI was comparable between the populations in the fall. Killifish weight and chemical treatment were not significant predictors of ASI. GSI was higher in females than males (Table 2; MLR, $\beta_{\text{male}} = -4.43$, $p_{\text{male}} = <0.0001$), especially during spring experiments (Table 1, Figure 4; March 2016 and May 2017) as has been shown previously (Fernández-Delgado, 1989; Greytak, et al., 2005). GSI was lower in NBH killifish relative to those from SC (Table 2; MLR, $\beta_{\text{NBH}} = -2.79$, $p_{\text{NBH}} = <0.0001$), which is also consistent with earlier studies (Greytak et al., 2005). Weight was positively associated with GSI in MLR (Table 2; MLR, $\beta_{\text{weight}} = 0.55$, $p_{\text{weight}} = 0.0015$). GSI did not differ between treatment groups in either MLR or LMRE models (Table 2).

Overall, these results support anecdotal field and laboratory observations, and prior findings (Greytak et al., 2005) that suggest greater lipid storage in NBH killifish relative to SC individuals.

3.3 Population and Sex are Important Modulators of MDC-Induced Changes in Gene Expression

Expression of *cyp1a* is a biomarker of AHR pathway activation that differentiates sensitive and tolerant killifish populations (e.g., Bello et al., 2001; Oleksiak et al., 2011; Whitehead, Clark, Reid, Hahn, & Nacci, 2017). There was no difference in basal *cyp1a* expression between control SC and NBH killifish (Figure 5a). PCB126 significantly induced *cyp1a* expression in male SC killifish (Figure 5b; two-way ANOVA, Dunnett's, $p = 0.0009$). As expected, PCB126 did not induce *cyp1a* expression in NBH killifish (Figure 5b). Relative expression of *cyp1a* in SC animals administered PCB126 tended to be greater in male than female animals (t-test, $p = 0.1045$). The same was true in NBH killifish (t-test, $p = 0.0026$), though *cyp1a* expression tended to be higher in male controls compared to female controls in this population.

The PPAR family of nuclear receptors plays important roles in maintaining lipid homeostasis in vertebrates. PPAR γ regulates adipocyte differentiation and lipid storage. mRNA expression of *pparg* in control killifish from SC and NBH can be thought of as each population's basal expression; no population-level differences were observed in basal *pparg* expression (Figure 6a). Unexpectedly, *pparg* was significantly upregulated in male SC killifish treated with PCB126 (Figure 6b; $p = 0.0208$, ANOVA, Dunnett's). *pparg* was not upregulated in female SC killifish or NBH killifish. Fatty acid binding proteins (FABPs) are a class of transport proteins for fatty acids and lipophilic substances. Using a sequence homology approach based on the zebrafish orthologue known to be under selective transcriptional control of *pparg* (Laprairie et al., 2016), we identified *fabp1b* in killifish. *Fabp1b* expression tended to be higher in control killifish from SC compared to those from NBH, but the difference was not statistically significant (Figure 6c; t-test, $p = 0.1079$). Like *pparg*, *fabp1b* expression was highest in SC males exposed to PCB126 (Figure 6d). However, despite the large increase in *fabp1b* expression among PCB126-treated SC killifish, the change was not statistically significant (ANOVA, Dunnett's, $p = 0.308$) due to high variability and small sample size ($n=3$ per group). To the best of our knowledge, S26948, a mammalian PPAR γ -selective synthetic ligand, has not previously been studied in fish and does not appear to activate the *pparg* pathway in killifish under the current exposure scenario.

PPAR α regulates liberation of fatty acids and their use as an energy source. We measured mRNA expression of *ppara* and its target gene, *cpt1b* (Liu et al., 2008). Basal expression of both genes in control animals is equivalent between populations (Figures 6e and 6g). No significant changes in *ppara* or *cpt1b* expression were observed following any chemical exposure in SC or NBH killifish. However, PCB153 caused slight increases in both *ppara* and *cpt1b* expression in SC male killifish, suggesting a possible effect of PCB153 on the pathway.

In summary, SC killifish are more responsive to MDC exposure at the transcriptional level than NBH killifish. Males are more susceptible to perturbations of nuclear receptor pathways by MDCs than females. This pattern was observed in genes involved with the AHR, PPAR γ , and PPAR α pathways. PCB126 significantly upregulated *cyp1a* and *pparg*

expression in male SC killifish, yet small sample size and high inter-individual variability confounded changes in the expression of other genes following MDC exposure.

4. Discussion

With increasing recognition of the role MDCs may play in the epidemic of obesity worldwide, we sought to characterize the effects of exposures to MDCs on lipid homeostasis in an ecological sentinel species, Atlantic killifish, which is likely exposed to multiple MDCs in its home habitat. MDCs can cause transgenerational effects (e.g., effects evident in generations not directly exposed to the chemical; Xin et al. 2015). Therefore, we tested the hypothesis that F2 killifish from NBH would exhibit altered lipid homeostasis (e.g., increased adiposity and hepatic triglycerides, changes in expression of genes involved in lipid homeostasis) compared to F1 or F2 killifish from SC (reference) because of heritable adaptations to persistent organic pollutants in NBH. We evaluated SC and NBH killifish under uncontaminated common garden conditions to evaluate whether the observation of elevated levels of adipose tissue in field-caught NBH killifish was better explained as a response to a resource-rich or complexly contaminated environment. We also exposed F1 and F2 SC and NBH killifish to MDCs with the expectation that NBH killifish might display tolerance by mechanisms related or unrelated to those already identified. We also provided novel measurements of total Sn in NBH sediments, which has not previously been measured in the harbor.

4.1 Evidence of Lipid Disruption in NBH Killifish: A Cost of Adaptation?

ASI and liver TAG were elevated in NBH relative to SC killifish, with greatest differences occurring in the spring. Population-level differences observed in ASI and liver TAG corroborate field observations that F0 NBH killifish have more abdominal adipose tissue than SC individuals, our original motivation for this study. Our results show that the liver is an important and dynamic lipid storage depot in killifish. Mean (range) liver TAG comprised 7.0% (0.18-15%) of overall liver mass in SC and 11% (3.0-20%) in NBH killifish. This is consistent with literature demonstrating that teleost fishes tend to store more lipids in their liver (*ca* 10-20%, up to 67% in teleost species such as cod; reviewed in Sheridan, 1988, 1994; haddock: Nanton et al., 2003; red drum: Craig et al., 2000; tilapia: reviewed in Sheridan, 1988) than do mammals (*ca* 3%; e.g., Nam et al., 2015). The relative apportionment of lipids to adipose tissue, muscle, and liver is species-specific (Sheridan, 1988). Despite the fact that the liver plays a more significant role in lipid storage in teleosts than mammals under normal conditions (reviewed in Lowe, O’Rahilly, & Rochford, 2011), several studies have shown that excess liver lipids can have deleterious effects on fish health, akin to those seen in mammals (e.g., Bolla et al., 2011; Lu et al., 2013; Nanton et al., 2003).

Population-level differences in lipid homeostasis in F1 and F2 killifish held under “common garden” laboratory conditions are noteworthy. These variations suggest that there are genetic differences in lipid homeostasis between SC and NBH killifish. Killifish are a well-known vertebrate example of evolved tolerance to environmental pollutants (reviewed in Whitehead et al. 2017). These adaptations are driven by genetic mechanisms through selective pressure on the AHR signaling pathway (Reid et al., 2016) and enable killifish to survive in highly

polluted estuaries. Conventional scientific theory of evolution and adaptation to environmental pollutants suggests that fitness costs ensue because the original, and presumably optimal, phenotype has been altered (Coustau, Chevillon, & ffrench-Constant, 2000; reviewed in Whitehead et al., 2017). A few examples that have been identified in some pollution-adapted killifish populations (but not including NBH) include altered bioenergetics (Jayasundara et al., 2017; Lindberg et al., 2017) and increased susceptibility to hypoxia (Meyer and Di Giulio, 2003), oxidative stress (Harbeitner et al., 2013), and infectious disease (Frederick et al., 2007).

Relative differences in ASI and liver TAG between NBH and SC fish are consistent across generations F0, F1, and F2. Differences in lipid homeostasis between NBH and reference killifish populations likely have underpinnings in adaptation to their respective environments. Since excess liver TAG is associated with adverse health outcomes in fish, which is also well documented in humans and mammalian toxicological models, this phenotype could indicate a fitness cost of adaptation to contaminants in NBH. Additionally, greater levels of lipid accumulation in storage depots such as adipose tissue and liver could be associated with changes in resource allocation to somatic growth, reproduction, and maintenance (e.g., Martin, Heintz, Danner, & Nisbet, 2017; Roff, 1983), although our studies were not designed to explore questions of energy budgeting. However, killifish in other studies have shown higher HSI, condition factor, fecundity and successful reproduction (e.g., Greytak et al., 2005; Nacci et al., 2001), suggesting that a fitness cost associated with changes in lipid resource apportionment are likely subtle, involving molecular mechanisms, site-specific environmental conditions and the adaptive phenotype.

While chemical contamination in NBH is an obvious distinction between the ecological conditions in NBH and SC, there are other differences between the two locations. NBH is a low-energy estuary, characterized by low wave height, finer sediment and vegetative growth in the intertidal zone (Jackson et al., 2002), and a more robust microbenthic community (Wildish and Kristmanson, 1979). The water is more turbid, reducing risk of predation on killifish by shore birds and larger fish (Kneib, 1986), and there is greater food availability for scavengers such as killifish, which feed predominantly on salt marsh detritus, copepods, diatoms, polychaetes, and insects (James-Pirri et al., 2001; Kneib, 1986; McMahon et al., 2005). In contrast, SC is a high energy-estuary, characterized by clear, fast-moving water and scoured substrate. By extension, SC killifish may be more vulnerable to predation and likely have less food availability. Mammalian toxicology and epidemiologic literature shows that both malnutrition and overnutrition during developmentally sensitive windows are associated with metabolic syndrome, including liver steatosis (Brumbaugh and Friedman, 2014; Burgio et al., 2015; El Hajj et al., 2014; Sookoian et al., 2013; Wang et al., 2016). Multiple complex mechanisms are likely responsible for the connection between nutritional status during development and metabolic syndrome later in life. Heritable responses to ecological conditions between SC and NBH could explain the differences in lipid homeostasis we observed in laboratory-raised killifish of F1 and F2 generations.

Laboratory-raised killifish were held in filtered ambient water from Narragansett Bay except during our actual experimental periods, when water was artificially warmed to 23°C. Natural fluctuations in water temperature in the bay caused F1 and F2 killifish to exhibit natural

seasonal variability in body bioenergetics and feeding behavior. Our data clearly demonstrate the importance of season as a predictor of lipid homeostasis in SC and NBH killifish. This pattern is consistent with seasonal variability in liver triglycerides (fall > spring) reported in other fish species (e.g., Kandimer & Polat, 2007, Berg & Bremset, 2005) and can be explained by seasonal changes in feeding behavior and metabolic demands (Sheridan, 1994).

ASI was also significantly positively associated with TAG, suggesting that after controlling for population, season and chemical treatment, killifish with greater lipid levels store these energy reserves in multiple depots.

PPAR nuclear receptors are important in maintaining lipid homeostasis. We hypothesized that either basal expression of the PPARs or their target genes (*pparg*, *fabp1b*; *ppara*, *cpt1b*) would show differences between populations, which may inform differences in lipid homeostasis observed at the phenotypic level. The similarity of basal expression of these genes between the two populations suggests that heritable differences in lipid homeostasis observed at the phenotypic level may not involve these nuclear receptor pathways. Additional evaluation of hepatic gene expression by targeted qPCR or transcriptomic analyses is recommended in subsequent studies to further characterize killifish PPAR pathways. Since we evaluated PPAR pathways at the transcriptional level, subsequent studies would be needed to confirm whether gene expression is positively correlated with protein activity.

4.2 Effects of MDCs on Regulators of Lipid Homeostasis

Lipid homeostasis was not expected to be significantly affected by administration of MDCs for three days, and therefore we also assessed gene (*pparg*, *fabp1b*, *ppara*, and *cpt1b*) expression as a potentially more sensitive measure of exposure. We hypothesized that SC and NBH would respond differently to MDC exposure. PCB126 significantly upregulated *cyp1a* expression in the livers of SC killifish but not those from NBH, consistent with previous studies (e.g., Bello et al., 2001; Oleksiak et al., 2011; reviewed in Whitehead et al., 2017). *Cyp1a* was more strongly induced by PCB126 in male killifish than females from each population. Greater responsiveness of male fish to AHR pathway induction has been shown previously, both in killifish (Meyer, Nacci, & Di Giulio, 2002) and other fish species (e.g., flounder: Elskus, Pruell, & Stegeman, 1992) and may be attributed to an inhibitory relationship between the estrogen receptor (ER) and AHR pathways under normal, endogenous conditions (Gräns et al., 2010; Navas and Segner, 2001). Alternatively, differences in *cyp1a*, *pparg* and *fabp1b* expression may have been due to the sex differences in chemical distribution following IP injection that were also found, resulting in sex-specific biologically effective concentrations in the liver (males > females). This may be explained by sex-specific patterns in the partitioning of lipophilic contaminants between liver and reproductive organs.

The most striking observation of PPAR pathway activation was the sex-specificity of response to PCB126 and PCB153. Both PPAR γ and PPAR α pathways were more responsive to PCBs in male compared to female killifish, regardless of the population. Sex-specific differences in lipid homeostasis and adiposity are known in humans and mammalian

animal models at the phenotypic (e.g., Montague, Prins, Sanders, Digby, & O’Rahilly, 1997; Varlamov, Bethea, & Roberts, 2015; Yessoufou & Wahli, 2010) and transcriptional (e.g., Yessoufou and Wahli, 2010; Leuenberger et al., 2009; Lane et al., 2003; Li et al., 2001) levels. Negative crosstalk between ER β and PPAR γ has emerged as a possible mechanism to explain sex specificity (Benz et al., 2012; Foryst-Ludwig et al., 2008; Wang and Kilgore, 2002). Studies showing sex-specificity to PPAR γ pathway activation in rodent models corroborate our findings, with greater responsiveness in males (Lane et al., 2003; Li et al., 2000). Similar sex-specificity has been shown in PPAR α pathway responsiveness, with evidence of estrogen-induced inhibition leading to suppression of genes critical for fatty acid beta-oxidation and lipid catabolism (reviewed in Yoon, 2010). Taken together, sexually dimorphic effects of PCB exposure on PPAR pathways are consistent with prior literature.

Perturbation of the PPAR γ pathway by PCB126 was unexpected as dioxin-like compounds are not typically considered PPAR γ agonists (e.g., Taxvig et al., 2012). In fact, AHR activation has been shown to negatively regulate the PPAR γ pathway through crosstalk mechanisms that are not well understood (e.g., Gadupudi et al., 2015). Nevertheless, *pparg* was significantly induced by PCB126 in SC male killifish. The mRNA expression of a *pparg* target gene, *fabp1b*, was also increased in SC male killifish following PCB126 exposure, but high inter-individual variability and a small sample size confounded our ability to detect statistical significance. While we were surprised to find activation of the PPAR γ pathway by PCB126 in our study, the capacity for dioxin-like PCBs to induce PPAR γ is not unprecedented. The same pattern was observed when *pparg* expression was compared between PCB126-treated killifish embryos from NBH and a reference population using RNAseq (Reid et al., 2016, Table S3). Further, Arsenescu et al. (2008) found that low doses of dioxin-like PCB77 induced *pparg* expression and promoted adipogenesis in 3T3-L1 murine preadipocytes.

At the population level, reduced AHR pathway transcriptional responsiveness to dioxin-like PCBs in NBH killifish is a hallmark of evolved tolerance amongst killifish from polluted estuaries (e.g., Bello et al., 2001; Joel N. Meyer & Di Giulio, 2003; D. Nacci et al., 1999; D. E. Nacci et al., 2010; Reid et al., 2016). Lower responsiveness of the PPAR γ pathway in NBH killifish following dioxin-like PCB exposure suggests that PPAR pathways may also be altered in NBH killifish, either as a direct or indirect result of selective pressure placed on the population from their environment. Since PPARs are central to regulating lipid homeostasis, population-level differences in their transcriptional activation by PCBs may provide clues into molecular mechanisms underlying the population-level differences we observed in liver TAG and ASI.

4.3 Killifish PPAR γ is unaffected by known mammalian ligands

Killifish were administered two compounds (TBT and S26948, a non-thiazolidinedione PPAR γ -selective pharmaceutical compound) known to bind and activate PPAR γ in mammals. Given that killifish PPAR γ has 76% sequence identity with human PPAR γ (NCBI genome BLAST), and PPAR γ is regarded as being highly conserved across vertebrates, we expected that TBT and S26948 would activate the killifish PPAR γ pathway, increasing the expression of *pparg* and *fabp1b*, and possibly altering lipid homeostasis,

though the latter was not expected following acute exposure. Surprisingly, exposure to TBT did not result in changes in lipid homeostasis or gene expression in our study, though both outcomes have been shown to be sensitive to TBT exposure in other teleost fish (reviewed in Capitão, Lyssimachou, Castro, & Santos, 2017). TBT is capable of binding and activating both PPAR γ and RXR in mammalian and fish models (Baker et al., 2015; Grim & Blumberg, 2006; Ouadah-Boussouf & Babin, 2016), yet has been shown to act most strongly through RXR (Baker et al., 2015; Balaguer, Delfosse, Grimaldi, & Bourguet, 2017). One possible explanation for the lack of TBT responsiveness in our study is the use of IP injection for acute exposure. Other studies reporting TBT effects on adiposity and the PPAR γ pathway in teleosts used water-borne (e.g., zebrafish: Capitão et al., 2017; Ouadah-Boussouf & Babin, 2016) and dietary (e.g., salmon: Meador, Sommers, Cooper, & Yanagida, 2011; Pavlikova, Kortner, & Arukwe, 2010) exposures. While the lack of PPAR γ responsiveness to S26948 was unexpected, we were less surprised since this synthetic compound has not previously been evaluated in teleosts.

Our findings align with an existing narrative about taxon-specific patterns of PPAR γ pathway responsiveness. PPAR γ has distinct differences in ligand specificity between fish and mammals (Grimaldi et al., 2015; He et al., 2015; Leaver et al., 2005; Wafer et al., 2017). Studies using teleosts for PPAR γ -related mechanistic toxicology research have reported that potent pharmaceutical ligands, such as rosiglitazone, did not activate PPAR γ as in mammalian models (Liu et al., 2005; Riu et al., 2014). However, subsequent studies have shown rosiglitazone to be efficacious at activating the PPAR γ -pathway in multiple fish species (e.g., rainbow trout: Sánchez-Gurmaches, Cruz-García, Gutiérrez, & Navarro, 2010; zebrafish: Ouadah-Boussouf & Babin, 2016; Tingaud-Sequeira et al., 2011). Since PPAR pathways have not previously been investigated in killifish, gene expression analyses should be expanded to include additional genes using targeted qPCR or transcriptomic approaches.

4.4 Applying Epidemiologic Methods to Wildlife Population Studies

Comparison of statistical approaches (ANOVA, MLR and LMER) highlights the utility of using more sophisticated statistical methods to evaluate toxicological data to accommodate complex data structures and covariate relationships. Regression modeling approaches allowed us to understand associations between GSI, HSI, ASI and liver TAG and covariates such as seasonality, killifish weight and sex that were not accounted for with two-way ANOVA comparisons of means. For example, MLR analysis confirmed that season is a significant predictor of liver TAG in killifish, and also revealed that liver TAG is significantly negatively associated with killifish weight and positively associated with ASI. Additionally, analysis of our data by ANOVA alone would have resulted in spurious interpretations of chemical treatments. For example, the two-way ANOVA evaluating liver TAG across populations and chemical treatments found that PCB126 significantly increased liver TAG, yet this association was attenuated after controlling for additional predictors (season, killifish weight, and ASI). Given that *pparg* and *fabp1b* were induced by PCB126 in gene expression analyses from our May 2017 experimental replicate, we would have concluded that PCB126 may be increasing liver TAG by way of PPAR γ pathway activation. However, after controlling for other covariates, such as killifish weight, population, season and ASI, we see that the effects of PCB126 on liver TAG are attenuated. Thus, linear

regression approaches allowed us to capture more sophisticated relationships between important predictors of liver TAG. As has been argued previously (Fox, 1991; Schmidt et al., 2016), regression modeling techniques are important and underutilized statistical tools in toxicology research and should be incorporated into analysis plans when sample sizes are adequate.

5. Conclusions

Killifish living in NBH are known to be exposed to PCBs. Exposure to other pollutants such as organotins is likely given elevated levels of Sn measured in NBH sediments relative to sediments from nearby areas. Laboratory-reared killifish from the NBH population had higher ASI and liver TAG than similarly raised killifish from SC. These differences were most pronounced in the spring, after a period of suppressed caloric consumption (winter) and reflect differences in lipid storage. Killifish were generally unresponsive to activation of the PPAR γ pathway by the classic environmental MDC, TBT, or the PPAR γ -specific synthetic compound, S26948. Surprisingly, exposure to PCB126 significantly increased liver *pparg* pathway gene expression in male SC killifish. This study has provided novel information that NBH killifish are resistant to short term treatment with MDCs, whereas SC killifish, particularly males, were more responsive. MLR models revealed significant associations between organosomatic indices and liver TAG and covariates, such as season, sex, and weight, which would have been overlooked with basic comparisons of means approaches (e.g., ANOVA). This highlights the necessity of using statistical tools such as regression modeling techniques to understand complex associations between outcomes and covariates in toxicological studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge Ian Roy, Joe Bishop and Ashley Bertrand for their excellent fish care, and Diana Franks for her technical expertise with gene expression analyses. This work was supported by the National Institute of Environmental Health Science (Boston University Superfund Research Program, P42ES007381; Boston University Environmental Epidemiology in Community Settings, T32ES014562).

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Highlights (REVISED):

- NBH killifish have more liver triglycerides and adipose tissue than fish from a reference population.
- Variation in lipid levels was most distinct in the spring, reflecting differences in lipid storage.
- PCB126 induced PPAR γ gene expression in livers of male reference (but not NBH) killifish.
- Regression analyses identified predictors of liver triglycerides not discernible by ANOVA.

Figure 1. Sites of parental killifish populations.

Offspring of killifish collected from New Bedford Harbor (B), a marine Superfund site located in southeastern Massachusetts, and a reference estuary, Scorton Creek (SC), in Sandwich, Massachusetts (A), were used in these analyses.

Figure 2. Liver triglyceride (TAG) was not affected by acute treatment with MDCs (A) but was substantially affected by season and population (B).

Vehicle (0.03% DMSO or acetone), PCB126 (29 ng/g), PCB153 (2,120 ng/g), or TBT (10 µg/g) in corn oil (1 µg corn oil solution per g fish) were administered by intraperitoneal (IP)

injection for a three-day duration. TAG concentrations were determined in saponified neutralized liver extracts. **(A)** Liver TAG data were pooled across spring (March 2016 and May 2017) and fall (October 2015 and October 2016) for each treatment group, with $n = 6$ killifish per chemical exposure for each population. Liver TAG was compared across chemical exposure, population and season using three-way ANOVA and Dunnett's post-hoc comparisons. No significant effect was detected. Red circles indicate outliers. **(B)** Liver TAG data were pooled within populations (SC (blue) and NBH (red)) for each experiment conducted between October 2015 and May 2017. Each replicate included $n = 30$ killifish per population, with equal numbers of males and females. Liver TAG was compared across seasons and populations using two-way ANOVA and Dunnett's post-hoc comparisons. Liver TAG varied seasonally, with higher levels in the fall than in the spring ($p_{\text{season}} = <0.0001$). The seasonal variability in liver TAG was greater in SC than NBH killifish ($p_{\text{season} \times \text{population}} = 0.0075$), suggesting SC killifish deplete a greater amount of their hepatic energy stores over the course of the winter than do NBH killifish.

Figure 3. Hepatosomatic index (HSI, A) and adiposomatic index (ASI, B), both important lipid storage sites in killifish, vary by population and season.

Killifish were treated as described in Figure 2. The liver and body weights were determined at euthanasia. Organ weights were not measured during the October 2015 experimental replicate. Each experimental replicate included $n = 30$ killifish per population, with equal numbers of males and females. HSI and ASI were compared across seasons and population using two-way ANOVA and Dunnett's post-hoc comparisons. HSI varied significantly by population and season ($p_{\text{population}} = <0.0001$ and $p_{\text{season}} = <0.0001$), whereas ASI varied significantly only by population ($p_{\text{population}} = <0.0001$). Red circles indicate outliers.

Figure 4. Gonadosomatic index (GSI), also an important lipid depot in killifish, varies by population, sex and season.

Killifish were treated as described in Figure 2. The gonad and body weights were determined at euthanasia. Organ weights were not measured during the October 2015 experimental replicate. Each experimental replicate included $n = 15$ killifish per population, per sex. GSI was compared across seasons and population using three-way ANOVA and Dunnett's post-hoc comparisons. GSI varied significantly by population, sex and season ($p_{\text{population}} = <0.0001$, $p_{\text{sex}} = <0.0001$ and $p_{\text{season}} = <0.0001$). Red circles indicate outliers.

Figure 5. *Cyp1a* is strongly induced by PCB126 in male SC killifish.

Vehicle (0.03% DMSO or acetone), PCB126 (29 ng/g), PCB153 (2,120 ng/g), TBT (10 µg/g), or S26948 (100 µg/g) in corn oil (1 µg corn oil solution per g fish) were administered by intraperitoneal (IP) injection for a three-day duration (May 2017). Liver *cyp1a* (a biomarker of AHR pathway activation) mRNA expression was measured using reverse transcriptase-qPCR. (A) Controls (Vh) in each population. *Cyp1a* expression was compared between populations using a t-test. (n=6 per population). No difference was detected. (B) Vh- vs. MDC-treated in each population. Basal *cyp1a* expression was compared between treatments and populations using ANOVA and Dunnett's post-hoc comparisons. (n=3 per population). Statistical significance is indicated by red asterisks (*) and p-values < 0.05 reported in the figure. *Cyp1a* was significantly induced by PCB126 in SC animals (p = 0.0009, ANOVA) and was higher in male than female animals (p = 0.1045, t-test).

Figure 6a-h. Acute PCB, but not TBT, exposure induces PPAR pathways in male SC killifish. Vehicle (0.03% DMSO or acetone), PCB126 (29 ng/g), PCB153 (2,120 ng/g), TBT (10 µg/g), or S26948 (100 µg/g) in corn oil (1 µg corn oil solution per g fish) were administered by intraperitoneal (IP) injection for a three-day duration (May 2017). mRNA expression of liver *pparg* and *ppara* as well as a target gene of each nuclear receptor (*fabp1b* and *cpt1b*, respectively) was measured using reverse transcriptase-qPCR. (A, C, E, G) Controls (Vh) in each population. Basal expression of each gene was compared between populations using a t-test. (n=6 per population). No significant differences were detected. (B, D, F, H) Vh- vs. MDC-treated killifish in each population. Expression was compared between treatments and populations using ANOVA and Dunnett's post-hoc comparisons. (n=3 per population). Statistical significance is indicated by red asterisks (*) and p-values < 0.05 reported in the figure. *Pparg* was significantly induced by PCB126 in SC males (p = 0.0208). *Fabp1b*, a *pparg* target, also tended to be higher in these animals, although not significantly. The *ppara* pathway was tended to be elevated in PCB153-treated SC males, yet not significantly.

Table 1.

Descriptive statistics for phenotypic outcomes.

Vehicle (0.03% DMSO or acetone), PCB126 (29 ng/g), PCB153 (2,120 ng/g), TBT (10 µg/g), or S26948 (100 µg/g) in corn oil (1 µg corn oil solution per g fish) were administered by intraperitoneal (IP) injection for a three-day duration. Organ and body weights and standard length were determined at euthanasia. Standard length and organ weights were not recorded at euthanasia during the October 2015. TAG concentrations were determined in saponified neutralized liver extracts. Since no statistically significant differences were detected between chemical treatments, summary statistics presented herein represent all killifish within an experimental replicate. All killifish and organ weights are reported as grams, wet weight (g, ww).

Date	Gen	Pop	Sex	N	Weight (g)		Condition Factor (CF)		Gonadosomatic Index (GSI)		Hepatosomatic Index (HSI)		Adiposomatic Index (ASI)		Triglycerides (mg TAG/g liver)	
					Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range	Mean (SD)	Range
Oct 2015	F1	SC	Overall	27	6.12 (0.99)	4.61 - 8.9									78.01 (25.87)	1.82 - 123.49
			F	14	5.84 (1.08)	4.61 - 8.9									78.29 (23.57)	35.86 - 123.49
			M	13	6.43 (0.81)	5.33 - 7.93									77.71 (29.13)	1.82 - 113.73
	F2	NBH	Overall	30	6.48 (0.96)	5.16 - 7.96									122.04 (27.5)	62.26 - 169.51
			F	15	6.46 (1.08)	5.17 - 7.96									126.45 (24.95)	80.55 - 169.51
			M	15	6.5 (0.86)	5.16 - 7.79									117.62 (30.04)	62.26 - 152.82
Mar 2016	F1	SC	Overall	24	7.35 (2.46)	4.36 - 14.11	2.11 (0.34)	1.64 - 3	7.77 (5.18)	2.14 - 17.89	3.09 (0.82)	1.81 - 5.17	0.78 (0.48)	0.08 - 1.71	34.25 (17.24)	7.6 - 68
			F	12	9.15 (2.08)	6.39 - 14.11	2.26 (0.22)	1.83 - 2.57	11.83 (4.28)	5.78 - 17.89	3.44 (0.85)	2.34 - 5.17	0.79 (0.5)	0.08 - 1.69	29.88 (17.81)	7.6 - 55.77
			M	12	5.55 (1.14)	4.36 - 7.47	1.95 (0.37)	1.64 - 3	3.72 (1.41)	2.14 - 6.85	2.73 (0.63)	1.81 - 3.68	0.78 (0.49)	0.2 - 1.71	38.62 (16.21)	17.45 - 68
	F2	NBH	Overall	24	7.14 (1.96)	4.34 - 10.55	2.16 (0.24)	1.67 - 2.7	4.76 (4.69)	0.79 - 19.57	4.79 (1.64)	2.1 - 9.55	3.4 (1.13)	1.68 - 5.54	86.98 (25.47)	29.53 - 133.94
			F	12	7.4 (1.61)	4.6 - 10.08	2.23 (0.2)	1.89 - 2.59	7.49 (5.39)	1.37 - 19.57	4.71 (0.9)	2.9 - 5.93	3.56 (1.17)	1.68 - 5.15	79.36 (28.54)	29.53 - 133.94
			M	12	6.87 (2.29)	4.34 - 10.55	2.1 (0.26)	1.67 - 2.7	2.02 (0.83)	0.79 - 4.13	4.86 (2.15)	2.1 - 9.55	3.23 (1.11)	1.76 - 5.54	94.61 (20.36)	60.63 - 129.11
Oct 2016	F2	SC	Overall	36	3.44 (0.39)	2.61 - 4.15	2.01 (0.23)	1.62 - 2.67	2.21 (2.7)	0.31 - 12.89	4.66 (1.12)	2.63 - 7.67	2.21 (2.7)	0.31 - 12.89	109.07 (23.71)	55.89 - 153.12
			F	18	3.48 (0.36)	2.92 - 4.13	2.01 (0.23)	1.69 - 2.49	3.73 (3.17)	1.21 - 12.89	4.79 (1.2)	2.68 - 7.67	3.73 (3.17)	1.21 - 12.89	111.22 (24.29)	59.41 - 150.74
			M	18	3.39 (0.43)	2.61 - 4.15	2 (0.23)	1.62 - 2.67	0.7 (0.32)	0.31 - 1.47	4.52 (1.05)	2.63 - 6.22	0.7 (0.32)	0.31 - 1.47	106.93 (23.62)	55.89 - 153.12
	F2	NBH	Overall	36	3.47 (0.49)	2.38 - 4.4	1.92 (0.15)	1.64 - 2.24	1.13 (0.62)	0.2 - 3.8	5.01 (1.32)	2 - 7.3	1.13 (0.62)	0.2 - 3.8	128.51 (18.81)	84.43 - 180.62
			F	18	3.61 (0.47)	2.76 - 4.4	1.93 (0.17)	1.68 - 2.24	1.45 (0.71)	0.7 - 3.8	5.61 (0.99)	3.59 - 7.3	1.45 (0.71)	0.7 - 3.8	128.39 (23.62)	84.43 - 180.62
			M	18	3.32 (0.47)	2.38 - 3.98	1.9 (0.13)	1.64 - 2.14	0.81 (0.27)	0.2 - 1.22	4.41 (1.36)	2 - 7.29	0.81 (0.27)	0.2 - 1.22	128.63 (13.07)	106.66 - 150.55
May 2017	F2	SC	Overall	36	4.55 (0.53)	3.35 - 5.77	2.03 (0.33)	1.48 - 3.35	7.82 (6.23)	0.52 - 26.56	2.37 (1.02)	0.84 - 5.07	0.47 (0.38)	0 - 1.58	47.89 (35.7)	5.63 - 125.56
			F	18	4.8 (0.48)	3.99 - 5.77	2.09 (0.37)	1.66 - 3.35	11.91 (6.54)	3.89 - 26.56	2.94 (1.03)	1.45 - 5.07	0.53 (0.41)	0 - 1.58	43.3 (35.12)	5.63 - 125.56
			M	18	4.3 (0.47)	3.35 - 5.26	1.96 (0.27)	1.48 - 2.54	3.73 (1.3)	0.52 - 5.62	1.81 (0.63)	0.84 - 3.61	0.41 (0.35)	0 - 1.14	52.49 (36.68)	5.66 - 109.43
	F2	NBH	Overall	36	4.72 (0.67)	3.53 - 6.07	1.91 (0.24)	1.6 - 2.64	3.71 (3.16)	0.86 - 16.75	3.4 (1.21)	1.77 - 8.12	4.12 (1.42)	0.1 - 6.89	94.21 (31)	34.95 - 201.53
			F	18	4.82 (0.76)	3.65 - 6.07	1.93 (0.25)	1.6 - 2.64	5.48 (3.67)	1.79 - 16.75	3.79 (1.53)	1.77 - 8.12	3.87 (1.57)	0.1 - 6.89	99.1 (37.7)	34.95 - 201.53
			M	18	4.61 (0.56)	3.53 - 5.63	1.9 (0.24)	1.62 - 2.64	1.94 (0.68)	0.86 - 4.01	3.02 (0.63)	2.09 - 4.23	4.37 (1.25)	2.68 - 6.68	89.32 (22.51)	51.97 - 126.88

Table 2.
Effect estimates (β) and standard errors (SE) for phenotypic outcomes (TAG, HSI, ASI and GSI).

ANOVA, multiple linear regression (MLR) and linear mixed effects regression (LMER) models were fit to evaluate associations between chemical treatment and phenotypic outcomes, adjusted for population, sex, season, and, in models of TAG, HSI, ASI and GSI. This exercise was made possible by a relatively large sample size ($n=225$) compared to many toxicological studies. Parsimonious MLR and LMRE models for each phenotypic outcome were selected and identified herein as “adjusted.” Statistical significance was evaluated at $\alpha = 0.05$ (bold, italicized values) for all analyses. For categorical covariates, the group being compared to the reference group is included in parentheses (e.g., New Bedford Harbor (NBH), Chemical Treatment, season (Fall), sex (Male)).

	TAG			GSI			ASI		HSI		
	2-Way ANOVA	Adjusted Multiple Linear Regression	Adjusted Mixed Effects Linear Regression	2-Way ANOVA	Adjusted Multiple Linear Regression	Adjusted Mixed Effects Linear Regression	2-Way ANOVA	Adjusted Multiple Linear Regression	2-Way ANOVA	Adjusted Multiple Linear Regression	Adjusted Mixed Effects Linear Regression
	β (SE)										
Population (NBH)	39.30 (4.66)	32.05 (4.64)	32.08 (4.64)	-2.80 (0.73)	-2.79 (0.53)	-2.79 (0.53)	1.67 (0.29)	1.67 (0.28)	1.04 (0.23)	0.85 (0.21)	0.85 (0.21)
Treatment (Vh)											
PCB126	15.10 (7.12)	-0.45 (7.28)	-0.44 (7.28)	0.32 (1.25)	1.42 (0.91)	1.42 (0.91)	-0.03 (0.49)	-0.03 (0.48)	0.33 (0.39)	0.19 (0.33)	0.19 (0.33)
PCB153	1.20 (6.67)	0.80 (6.43)	0.80 (6.43)	-0.25 (1.12)	-0.27 (0.80)	-0.27 (0.80)	-0.07 (0.44)	-0.07 (0.43)	0.40 (0.35)	0.49 (0.30)	0.49 (0.30)
TBT	-2.55 (7.50)	5.01 (6.45)	5.03 (6.45)	-0.27 (1.12)	-0.33 (0.80)	-0.34 (0.80)	0.05 (0.44)	0.45 (0.43)	0.41 (0.35)	0.34 (0.29)	0.34 (0.29)
S26948	1.68 (7.50)	-0.01 (6.44)	-0.05 (6.44)	0.02 (1.12)	0.21 (0.81)	0.22 (0.81)	0.45 (0.44)	0.05 (0.43)	-0.05 (0.35)	0.01 (0.29)	0.03 (0.29)
Season (Fall)		40.86 (5.37)	mixed effect		-3.12 (0.67)	mixed effect				1.83 (0.24)	mixed effect
Weight		-4.29 (1.32)	-4.39 (1.32)		0.55 (0.17)	0.57 (0.17)				0.15 (0.06)	0.15 (0.06)
Sex (M)					-4.43 (0.54)	-4.41 (0.54)		-0.66 (0.28)		-0.50 (0.20)	-0.50 (0.20)
ASI		3.12 (1.17)	3.10 (1.17)							0.12 (0.04)	0.11 (0.05)