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Tributyltin disrupts fin development in *Fundulus heteroclitus* from both PCB-sensitive and resistant populations: Investigations of potential interactions between AHR and PPARγ

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Abstract

Tributyltin (TBT) and dioxin-like polychlorinated biphenyls (PCBs) are environmental contaminants that are highly toxic to fish and co-occur in New Bedford Harbor (NBH), an estuarine Superfund site located in Massachusetts, USA. Atlantic killifish (*Fundulus heteroclitus*) that reside in NBH (and other highly contaminated sites along the east coast of the United States) have developed resistance to activation of the aryl hydrocarbon receptor (AHR) pathway and the toxicity of dioxin-like chemicals, such as 3,3',4,4',5-pentachlorobiphenyl, PCB126. In many biological systems, TBT disregulates adipose and bone development via the PPARγ-RXR pathway; AHR activation also disrupts adipose and bone homeostasis, potentially through molecular crosstalk between AHR and PPARγ. However, little is known about how co-exposure and the interaction of these pathways modulate the toxicological effects of these contaminants. Here, we tested the hypotheses that TBT would induce teratogenesis in killifish via activation of PPARγ and that PCB126 co-exposure would suppress PPARγ pathway activation in PCB-sensitive killifish from a reference site (Scorton Creek, SC, PCB-sensitive) but not in PCB-tolerant NBH killifish. Killifish embryos from both populations exposed to TBT (50 and 100 nM) displayed caudal fin deformities. TBT did not change the expression of *pparg* or its target genes related to adipogenesis (*fabp11a* and *fabp1b*) in either population. However, expression of *osx/sp7*, an osteoblast marker gene, and *col2a1b*, a chondroblast marker gene, was significantly suppressed

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by TBT only in SC killifish. An RXR-specific agonist, but not a PPARγ-specific agonist, induced caudal fin deformities like those observed in TBT-treated embryos. PCB126 did not induce caudal fin deformities and did not exacerbate TBT-induced fin deformities. Further, PCB126 increased expression of *pparg* in SC embryos and not NBH embryos, but did not change the expression of *fabp1b*. Taken together, these results suggest that in killifish embryos the PPARγ pathway is regulated in part by AHR, but is minimally active at least in this early life stage. In killifish, RXR activation, rather than PPARγ activation, appears to be the mechanism by which TBT induces caudal fin teratogenicity, which is not modulated by AHR responsiveness.

1. Introduction

New Bedford Harbor (NBH) is a marine Superfund site located in southeastern Massachusetts (See map in (Crawford et al., 2019)). NBH is well known for its contamination by polychlorinated biphenyls (PCBs), which were used in electronics manufacturing and discharged to the environment, leading to widespread, severe contamination throughout the harbor. Organotins were used as an antifouling agent in marine paints, potentially contaminating major fishing and shipping ports such as NBH (Pesch and Garber, 2001). We recently showed that elemental tin, a marker of organotin contamination, also is elevated in NBH (Crawford et al., 2019).

NBH is home to a number of wildlife populations, including the Atlantic killifish ("killifish," *Fundulus heteroclitus*). Killifish are found in estuarine environments along the east coast of North America and have been studied extensively for their response to contaminants, most notably PCBs and polycyclic aromatic hydrocarbons (PAHs) acting through the aryl hydrocarbon receptor (AHR)(e.g., (Nacci et al., 2002; Nacci et al., 2010; Oleksiak et al., 2011; Osterberg et al., 2018; Proestou et al., 2014)). The ample genetic variation, large population sizes and small home ranges of killifish have led to the evolution of genetically distinct populations that have developed adaptations to the pollutants in the local environment (e.g., (Meyer et al., 2003; Nacci et al., 2002; Reid et al., 2016)) and have emerged as an important example of convergent evolution (Reid et al., 2016; Whitehead et al., 2012). Killifish living in PAH- or PCB-contaminated sites, including NBH, demonstrate dramatic heritable resistance, or evolved tolerance, to AHR pathway activation and to toxicity of dioxin-like PCBs (Arzuaga and Elskus, 2010; Bello et al., 2001; Nacci et al., 2002; Reid et al., 2016).

TBT is a ligand for both peroxisome proliferator activated receptor γ (PPARγ) and its DNA binding partners, the RXRs (Baker et al., 2015a; Cui et al., 2010; Grun et al., 2006; Kanayama et al., 2005; le Maire et al., 2009). In mammals, PPARγ is essential for adipogenesis, mature adipocyte function and systemic metabolic homeostasis (Gumbilai et al., 2016; He et al., 2003; Jiang et al., 2014; O'Donnell et al., 2016; Tontonoz et al., 1994; Zhang et al., 2004). Rosiglitazone, a PPARγ-activating therapeutic, and TBT induce adipogenesis in humans and rodents, *in vitro* and *in vivo* (Adams et al., 1997; Bertuloso et al., 2015; Grun et al., 2006; Kirchner et al., 2010; Miyazaki et al., 2001). Killifish, along with other fish species characterized to date, express a single form of PPARγ. Killifish express at least one form of RXRα and multiple forms of both RXRβ and RXRγ (Karchner

and Hahn, unpublished analysis of XP_012719647; (Baldwin et al., 2017). Rosiglitazone and TBT induce adiposity in zebrafish larvae (Ouadah-Boussouf and Babin, 2016; Tingaud-Sequeira et al., 2011).

In mammalian models, activation of PPARγ by rosiglitazone both induces adipogenesis and suppresses bone formation (Akune et al., 2004; Lecka-Czernik et al., 1999). *In vitro,* TBT promotes commitment of bone marrow mesenchymal multipotent stromal cells (BM-MSCs) towards an adipocyte lineage at the expense of the osteoblast lineage (Baker et al., 2015a; Carfi et al., 2008; Kirchner et al., 2010; Koskela et al., 2012; Watt and Schlezinger, 2015; Yanik et al., 2011). *In utero* exposure to TBT in rodent models delays ossification, induces vertebral malformations and predisposes multipotent stromal cells to favor adipogenesis over osteogenesis (Adeeko et al., 2003; Ema et al., 1995; Kirchner et al., 2010; Tsukamoto et al., 2004). Adult TBT exposure *in vivo* also reduces bone formation in the cortical compartment (Watt et al., 2018).

Early life exposure to organotins (e.g. tributyltin (TBT), triphenyltin) is teratogenic in diverse fish and amphibian species. Organotins cause axial skeletal deformities, which are characterized by craniofacial deformities, bent or shortened spines and tails and failure to form fins (medaka, *Oryzias latipes* (Hano et al., 2007); rockfish, *Sebastiscus marmoratus* (Zhang et al., 2012); mahi, *Coryphaena hippurus* (Adema-Hannes and Shenker, 2008); zebrafish, *Danio rerio* (Huang et al., 2015);West African clawed frog, *Xenopus tropicalis* (Guo et al., 2010; Yu et al., 2011)). Further, TBT reduces osteoblast activity in scales of goldfish (*Carassius auratus*), nibbler (*Girella punctata*) and wrasse (*Pseudolabrus sieboldi*) (Satone et al., 2011; Suzuki et al., 2006).

Studies suggest that the AHR also regulates adipose and bone homeostasis, potentially through molecular crosstalk between AHR and PPARγ. In mammals, AHR knockout increases adiposity (Baker et al., 2015b) and AHR activation suppresses adipogenesis by downregulating PPARγ expression (Alexander et al., 1998; Arsenescu et al., 2008; Brodie et al., 1996; Gadupudi et al., 2015; Ishihara et al., 2018; van den Dungen et al., 2017). AHR has been shown to regulate bone development in a ligand-, species- and age-dependent manner (Fader et al., 2018). Following embryonic exposure, dioxin disrupts craniofacial development and suppresses ossification in zebrafish and medaka (Bums et al., 2015; Watson et al., 2017). Further, AHR2 is required for craniofacial and fin development in zebrafish (Garcia et al., 2018; Souder and Gorelick, 2019).

Collectively, there is strong evidence that TBT and PCB126 (a representative dioxin-like PCB) co-occur in urban waterways and that both target receptors that regulate adipose and bone. However, little is known about how co-exposure to common aquatic pollutants may modulate the toxicological effects of compounds acting through PPARγ-RXR and AHR pathways. Here, we tested the hypotheses that TBT would induce teratogenesis in killifish via activation of PPARγ and that differential AHR sensitivity in SC (PCB-sensitive) and NBH (PCB-tolerant) killifish would modulate PPARγ pathway responsiveness following TBT exposure. Further, we investigated the impact of co-exposure to TBT and PCB126 to interrogate how these responses change following simultaneous activation of both transcriptional pathways.

2. Methods

2.1 Chemicals and reagents

Dimethyl sulfoxide (DMSO) was purchased from American Bioanalytical (Natick, MA, USA). 3,3′,4,4′,5-pentachlorobiphenyl (PCB126, purity: ≥ 97%) was purchased from Ultra Scientific (North Kingstown, RI, USA). Tributyltin chloride (TBT, purity: 96%) and LG100268 (purity: ≥ 98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). S26948 (Purity: ≥ 99%) was purchased from Tocris Bioscience (Minneapolis, MN, USA). All other reagents were from Thermo Fisher Scientific (Suwanee, GA, USA) unless noted.

2.2 Animal Care and Breeding

Reproductively active, adult killifish (F0) were collected from New Bedford Harbor (NBH, PCB-contaminated) and Scorton Creek (SC, reference) using baited traps and held in common garden laboratory conditions as described in Nacci et al. (2002). Killifish were maintained in flow-through aquaria receiving filtered ambient seawater from Narragansett Bay, Rhode Island, and fed *ad libidum*. F0 killifish and their offspring (F1) spawned with semi-lunar periodicity throughout the summer months (late May through August in New England) and were reared to reproductive maturity (1-2 years per generation) under the conditions described above. In order to obtain a sufficient number of embryos, a combination of non-invasive techniques (breeding baskets and manual spawning) were used to collect F2 eggs from F1 females during seasonal, semi-lunar spawning cycles between May 2016 and August 2017. Basket eggs were fertilized *in situ* by male killifish. Manually-spawned eggs were fertilized *in vitro* by expressing milt from males directly into a glass dish containing the eggs. Fertilized embryos were held at 23°C for 24 hours and were then screened microscopically to remove unfertilized or improperly developing embryos.

2.3 Chemical Exposures

Exposures were conducted at 23°C under 14:10 hour light:dark regimen. All exposures occurred in 20-mL glass scintillation vials beginning at 24 hours post fertilization (i.e., 1 day post fertilization (dpf)). Each vial contained one embryo in 10 mL of treatment solution. Solutions were made in 5 μM filtered ambient seawater. Embryos remained in treatment solution until 7 dpf, at which point they were transferred to individual wells of a 12-well plate containing filter paper moistened with filtered seawater. Embryo phenotype was screened microscopically at 10 dpf (further detail provided in Section 2.4).

2.3.1 Individual Chemical Exposures—Individual chemical exposures were conducted over two replicate experiments, each containing 20 embryos per treatment (total N = 40 per chemical treatment per population). Treatment groups were as follows: naïve (untreated), vehicle control (0.01% DMSO), TBT (Low: 5 nM, Medium: 50 nM and High: 100 nM), S26948 (a synthetic PPARγ agonist (Carmona et al., 2007), 1 μM), and LG100268 (a synthetic RXR agonist (Boehm et al., 1995), 200 nM). TBT concentrations were selected to establish a dose-response relationship around the EC_{50} in fish and mammals (~50 nM). To the best of our knowledge, S26948 and LG100268 have not been studied in teleost fish so *in vitro* EC_{50} concentrations for each therapeutic served as the basis for our dose selection (S26948: 8.83 nM in COS-7 cells transfected with human PPARγ, (Carmona et al., 2007);

LG100268: 2 nM in COS-7 cells transfected with human RXRα, (Grun et al., 2006)). Since both compounds are therapeutics designed to have a short biological half-life, concentrations one to two orders of magnitude higher than the published EC_{50} were selected for S26948 and LG100268 given the six-day static exposure. Due to limited F2 SC embryo availability during the 2017 breeding season, LG100268 exposures were only conducted on NBH embryos.

2.3.2 Chemical Co-Exposures—Chemical co-exposures were conducted over three replicate experiments containing at least 20 embryos per treatment (total N ≥ 60 per chemical treatment per population). Chemical treatments consisted of naïve (untreated), a vehicle control (0.02% DMSO), TBT (Medium: 50 nM and High: 100 nM), PCB126 (61 pM), and TBT + PCB126 (Medium TBT + PCB126 and High TBT + PCB126). Medium and High TBT doses were selected for use in co-exposures because of their ability to consistently induce caudal fin malformation visible during 10 dpf phenotype screening. The PCB126 dose was selected based on prior studies showing that this concentration will activate the AHR pathway in SC, but not NBH, killifish embryos and produce low prevalence of cardiac teratogenicity (Nacci et al., 2002). This concentration was selected to minimize the possibility that the AHR-mediated response to PCB126 would overwhelm the effects of TBT exposure in co-exposure treatment groups.

2.4 Phenotypic Screening and Embryo Pooling

At 10 dpf, embryos were examined using a Nikon SMZ1500 stereomicroscope to identify TBT- and PCB126-mediated teratogenesis. TBT-mediated teratogenesis presented as caudal fin malformation, specifically stunted fin ray development and reduced overall fin length. The severity of caudal fin deformity was scored as 0 (normal), 1 (mild deformity – slightly shortened caudal fin), 2 (moderate deformity – shortened caudal fin, less discernable fin rays), and 3 (severe deformity – extremely short caudal fin, poorly defined or indiscernible fin rays) (Figure 1A). Some individuals scored as 3 appeared to have overall shorter tails; however, this was difficult to quantify in an intact embryo, and therefore was not considered during phenotypic scoring.

2.5 RNA Extraction and qPCR

Following phenotypic screening, two pools of embryos (N=5 embryos per pool) from each treatment within each experiment were flash frozen for subsequent gene expression analysis. Embryos with phenotypes representative of the rest of the treatment group were selected for pools. Individual chemical exposure experiments consisted of N=4 pools per chemical treatment, whereas chemical co-exposures experiments consisted of N=6 pools per chemical treatment.

Total RNA was extracted from pooled embryos using RNA STAT-60™ (Tel-Test Inc. Friendswood, TX, USA). Genomic DNA was removed using the Aurum™ Total RNA Mini Kit (BioRad, Hercules, CA, USA). cDNA was prepared from total RNA using the GoScript™ Reverse Transcription System (Promega, Madison, WI, USA), using equal parts random and Oligo (dT)15 primers. qPCR reactions were performed using iTaq Universal SYBR® Green Supermix (BioRad) with 400 nM forward and reverse primers. Previously

published primers were used for *actb* (Oleksiak et al., 2011), *rn18s* (Patel et al., 2006), *ef1α* (Bears et al., 2006), and *cyp1a* (Oleksiak et al., 2011). All new primers were designed to overlap exon junctions to prevent potential amplification of contaminating genomic DNA. Primer sequences are shown in Table 1. Thermocycling parameters 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 critical threshold (CT) values to standardize across multiple plates.

All gene expression data were normalized with the Pfaffl method (Pfaffl, 2001), using primer-specific amplification efficiency and the expression of constitutively expressed (housekeeping) genes and naïve (untreated) pooled embryo samples. Three housekeeping genes were evaluated for stability across populations and experimental replicates. Variability was greatest in *actb* (overall mean (standard deviation): 1.33 (0.996)), with differences between populations (SC > NBH) and experimental replicates. Expression of *ef1α* was less variable (0.982 (0.498)), again with SC > NBH. *rn18s* was most stably expressed (1.06 (0.248)), with no discernable pattern of variability between populations, experimental replicates or chemical treatments. Thus, all gene expression data presented herein are normalized to *rn18S* and reported as a relative expression ratio.

2.6 Statistical Analysis

All exposures were repeated over the course of at least two experimental replicates. Fifteen to thirty individually exposed embryos, depending on availability in a particular spawning cycle, were included in each treatment group for each experimental replicate. For gene expression analyses, 5 embryos were pooled to produce a single biological replicate, two independent biological replicates were generated per experiment, and each experiment was performed 2 to 3 times. In the figures, data points/biological replicates generated from the same experiment are the same color. Embryo mortality never exceeded 20% in control treatments. Power calculations were conducted using G*Power (Faul et al., 2007). All other statistical analyses were performed using Microsoft R Open 3.3.2. Gene expression and phenotypic data were analyzed separately for individual and chemical co-exposures. Gene expression was evaluated using one and two factor ANOVA and t-tests to evaluate differences in mean relative expression between populations and treatment groups. All data points, including possible outliers (points falling more than 1.5 times the interquartile range below or above the first or third interquartile range, respectively), were used in analyses due to the small sample size ($N \leq 6$). Mean caudal fin deformity score and standard error were calculated for each treatment group and chemical exposure groups were compared to DMSO-exposed controls (ANOVA, Dunnett's post-hoc comparison). Statistical significance was evaluated at $\alpha = 0.05$ for all analyses.

3. Results

3.1 TBT-Induce Caudal Fin Deformity

Exposure of killifish to TBT during early embryonic development and organogenesis impaired caudal fin development (Figure 1a). The severity and prevalence of caudal fin

deformities increased in a dose-dependent manner (Figure 1b). Embryos exposed to TBT-Low (5 nM) did not exhibit caudal fin teratogenesis above the background level found in control embryos (> 95% normal caudal fin phenotype). Mild to moderate caudal fin deformities were observed with exposure to TBT-Medium (50 nM) and moderate to severe deformities were observed with TBT-High (100 nM); both concentrations significantly increased the mean deformity score relative to controls. The mean deformity score did not differ significantly between SC and NBH embryos within a treatment group (ANOVA: $p = 0.435$; Figure 1b).

In an effort to understand the molecular targets involved in TBT-mediated caudal fin deformities, we measured the relative expression of genes known to be markers of osteoblasts (*osx/sp7*) and chondroblasts (*col2a1a* and *col2a1b*). No differences in basal expression of these genes were found between populations (Figure 2a-c). Following TBT exposure, *osx/sp7* was suppressed in a dose-dependent manner in SC embryos and significantly suppressed in the highest TBT treatment (Figure 2d). Similar expression patterns were seen in SC embryos for *col2a1a* and *col2a1b* (Figures 2e-f), with *col2a1b* being significantly suppressed at the highest TBT concentration. While a trend toward suppression of *osx/sp7* expression by TBT was observed in NBH embryos, there was no discernable treatment-related pattern for *col2a1a* and *col2a1b* (Figures 2d-f).

3.2 PPARγ-RXR/AHR Crosstalk

Next, we investigated whether PCB126 could modify the ability of TBT to induce fin deformities. PCB126 is well-known to induce AHR-dependent *cyp1a* expression and teratogenic effects (e.g., cardiac malformations in sensitive but not tolerant killifish populations (Arzuaga and Elskus, 2010; Clark et al., 2014). Basal *cyp1a* expression was not different between SC and NBH embryos ($p = 0.672$, t-test; Figure 3a). As expected, PCB126 significantly induced *cyp1a* in SC embryos (Figure 3b). PCB126 + TBT also induced *cyp1a* in NBH embryos (Figure 3c); however, “population” was a significant term in the two factor ANOVA suggesting that the induction was less in the NBH embryos. In contrast, PCB126 alone appeared to increase caudal fin deformities in SC embryos—although the increase was not statistically significant—and did not induce caudal fin deformities in NBH embryos (Figure 4). The same extent of caudal fin deformity was observed when SC and NBH embryos were exposed to TBT + PCB126 compared to TBT alone (Figure 4).

To determine whether chemical exposures caused changes in the PPARγ pathway and whether they differed between populations, we characterized the expression of *pparg,* itself, as well as two target genes previously studied in teleost *fish, fabp11a* and *fabp1b. Fabp11a* is the recognized ortholog to mammalian FABP4, a commonly used marker of the PPARγ-RXR pathway and mature adipocytes (Flynn et al., 2009; Imrie and Sadler, 2010). *Fabp1b* also has been shown to be a selective target of *pparg* in zebrafish (Laprairie et al., 2016). Basal gene expression of *pparg, fabp11a*, and *fabp1b* did not vary between SC and NBH embryos (Figure 5). Further, TBT did not induce significant changes in expression of these genes (Figure 5). In co-exposure experiments, *pparg* expression showed an increasing trend with PCB126 alone and significantly increased with TBT + PCB126 in SC embryos (Figure 6a). A similar trend was seen in NBH embryos although the extent of induction was less

than in SC embryos, *fabp1b* expression was highly variable, with PCB126 potentially increasing *fabp1b* expression in SC embryos and decreasing *fabp1b* expression in NBH embryos (Figure 6b).

3.2 RXR-induced caudal fin deformity

Gene expression analyses did not support a role for PPARγ activation in TBT-induced effects in killifish embryos. Furthermore, a synthetic PPARγ ligand (S26948 (Carmona et al., 2007) also did not induce PPARγ target gene expression (data not shown). Since TBT is known to bind and activate both PPARγ and RXR, we also evaluated caudal fin development in F2 killifish embryos exposed to selective therapeutic ligands for each receptor (PPARγ: S26948 and RXR: LG100268 (Boehm et al., 1995)) to determine which receptor is involved in TBT-induced caudal fin deformities. S26948 exposure did not produce caudal fin deformities in killifish embryos (Figure 7). However, the RXR ligand LG100268 induced deformities in embryos similar to those observed following TBT exposure (Figure 7).

4. Discussion

Killifish is a robust species, multiple populations of which have adapted to living in sites highly contaminated with PCBs and/or PAHs by developing resistance to AHR activation and AHR-mediated toxicity. Few studies have investigated how this adaptation influences the response to other chemical stressors; one has shown that there is cross-resistance to pesticides in a PAH-adapted population (Clark and Di Giulio, 2012) while another found enhanced sensitivity to the pro-oxidant tert-butylhydroquinone in a PCB-adapted population (Harbeitner et al., 2013). We sought to explore how PCB-resistance modifies the biological responses to TBT, a toxicant that is found commonly in urban waterways and likely co-occurs with PCBs in NBH (Crawford et al., 2019). Because TBT is a PPARγ ligand and AHR-PPARγ crosstalk is known in mammals, we investigated the hypothesis that TBT-induced effects on PPARγ-related endpoints would be distinct in PCB-sensitive and PCB-resistant populations. TBT induced caudal fin teratogenicity with equal potency and efficacy in SC and NBH killifish, but activation of PPARγ was not the mechanism. The PPARγ pathway is regulated, at least in part, by AHR in killifish embryos but is minimally active at this early life stage. Rather, the data support the conclusion that RXR activation is the mechanism by which TBT induces caudal fin teratogenicity.

4.1 Lack of Response of the PPARγ Pathway

Overall, the PPARγ pathway was largely unresponsive to TBT exposure in killifish embryos, regardless of population. This was surprising because TBT is widely known to stimulate adipogenesis and/or lipid accumulation in a variety of vertebrate models, including amphibians and fishes (Grun et al., 2006; Kirchner et al., 2010; Lutfi et al., 2017; Ouadah-Boussouf and Babin, 2016). However, others have shown that PPARγ activation by TBT occurs in cartilaginous fish and early diverging ray finned fishes, which split from the teleost lineage prior to the teleost genome duplication, and that PPARγ responsiveness to TBT has been lost in teleost species (Capitao et al., 2018). Cysteine 285 in the PPARγ ligand binding pocket appears to be critical for TBT to activate PPARγ, and this residue has been replaced

by either tyrosine or leucine in the teleosts studied so far (Capitao et al., 2018). Even if TBT were able to activate PPARγ, it is unclear whether PPARγ is biologically active in the fish embryo.

We chose a non-thiazolidinedione synthetic PPARγ ligand (S26948) to independently test the biological activity of PPARγ in killfish embryos. S26948 also did not induce expression of a PPARγ target gene in killifish embryos. Lack of responsiveness of killifish to S26948 can be attributed any of several factors: (1) S26948 did not cross the chorion, (2) it was metabolized during the course of the six-day static exposure and three-day depuration prior to embryo preservation (i.e., S26948 may have a short biological half-life), (3) it is not a ligand for killifish *pparγ*, and/or (4) PPARγ signaling is minimal in the fish embryo. Adipose tissue development, which requires PPARγ activity, has not been chronicled in embryonic killifish and 10 dpf embryos may not have many, if any, differentiated adipocytes. Zebrafish begin to develop adipose tissue around 10-15 dpf (Imrie and Sadler, 2010; Minchin and Rawls, 2011), and the developmental timeframe for killifish is appreciably slower. To the best of our knowledge, S26948 has not been tested as a PPARγ ligand in teleost fish. We selected S26948 rather than the classic PPARγ positive control, rosiglitazone, given that reports do not agree as to whether rosiglitazone is an agonist of fish PPARγ (Capitao et al., 2018; Laprairie et al., 2016; Ouadah-Boussouf and Babin, 2016; Riu et al., 2011; Sanchez-Gurmaches et al., 2010; Tingaud-Sequeira et al., 2011). While timing of initiation of PPARγ activity during development is uncertain, we have also shown that S26948 did not activate the PPARγ pathway in liver of adult killifish following a three-day exposure by intraperitoneal injection (Crawford et al., 2019). Thus, it is likely that S26948 is not an agonist of killifish PPARγ.

4.2 Fin Teratogenicity

The caudal fin and caudal bone complex are recognized as skeletal regions particularly sensitive to disruption during teleost development (Fernández et al., 2008; Fernández et al., 2009; Haga et al., 2002). The skeleton and fin formation/regeneration are targets of dioxin toxicity in zebrafish (Baker et al., 2013; Zodrow and Tanguay, 2003). Further, AHR2 is required for craniofacial and fin development (Garcia et al., 2018; Souder and Gorelick, 2019). Therefore, it was surprising that PCB126, at most, modestly induced fin teratogenicity. The likely explanation is that the concentration of PCB126 we used was relatively low, approximating the EC_{50} for *cyp1a* induction, a highly sensitive AHR response in killifish embryos (Arzuaga and Elskus, 2010; Wills et al., 2010). Higher concentrations may be required to elicit fin deformities.

Our observation of TBT-induced caudal fin deformities in killifish is in line with previously published studies showing TBT-induced axial skeleton and fin abnormalities in other fish and amphibian species (Adema-Hannes and Shenker, 2008; Guo et al., 2010; Hano et al., 2007; Huang et al., 2015; Yu et al., 2011; Zhang et al., 2012). A limited number of killifish exposed to TBT *in ovo* were raised for approximately 12 months after hatching. Deformity of the caudal peduncle remained in one year-old fish, indicating the persistence of the phenotype (data not shown). While PPARγ activation is well known to interfere with osteogenesis (Lecka-Czernik et al., 1999), TBT did not activate PPARγ in developing

killifish. However, TBT also binds and activates RXRs (le Maire et al., 2009). While PPARγ expression in zebrafish is minimal through the larval stage (Ibabe et al., 2005), RXRs are ubiquitously expressed during gastrulation and become more strongly expressed and spatially restricted by 1 dpf (Tallafuss et al., 2006; Waxman and Yelon, 2007), suggesting that RXR signaling is likely to precede PPARγ signaling in killifish embryos, as well. Guo et al. (2010) proposed that TBT-activation of RXR interferes with retinoic acid or thyroid signaling to cause skeletal and ocular deformities in *Xenopus tropicalis*, respectively, and TBT has been shown to interfere with thyroid signaling in *Xenopus laevis* (Mengeling et al., 2018). Our results show that an RXR ligand (LG100268) induced a caudal fin teratogenesis similar to that caused by TBT. Previous studies have shown a reduction in *osx/sp7* expression and bone nodule formation in BM-MSCs following TBT and LG100268 exposure (Baker et al., 2015a; Watt and Schlezinger, 2015). This finding aligns with our observation of caudal fin deformities, characterized by poor cartilage and bone development. Activation of RXR, rather than PPARγ, is a likely mechanism by which TBT induces fin deformity.

Both TBT and LG100268 efficaciously induced fin deformity in SC and NBH killifish. However, expression of marker genes for osteoblast and chondroblast activity were only significantly suppressed in the SC killifish. Why the altered gene expression occurs in SC but not NBH embryos is an intriguing question. In contrast, PCB126 only modestly induced fin deformity in SC killifish alone. The difference in efficacy is likely explained by the exposure to maximally efficacious concentrations of TBT and LG100268 (100x the *in vitro* EC_{50}'s) and sub-maximally efficacious concentrations of PCB126 (≈ 1x the *in vivo* EC_{50}). The similar sensitivity of SC and NBH killifish to TBT- and LG100268-induced fin teratogenicity suggests that RXR signaling is not affected by adaptation to the contaminated environment. On the other hand, the likely difference in sensitivity of SC and NBH killifish to PCB126-induced fin teratogenicity are in line with previous observations of AHR's role in skeletal development in zebrafish (Garcia et al., 2018; Souder and Gorelick, 2019).

4.3 Evidence of Crosstalk Between PPARγ and AHR Pathways

NBH killifish have evolved heritable resistance to environmental AHR agonists, such as dioxin-like PCBs, which is often measured by recalcitrance to the induction of AHR target genes, notably CYP1A (Bello et al., 2001; Nacci et al., 2002; Reid et al., 2016). As expected, PCB126 most efficaciously induced *cyp1a* expression in SC embryos (23-fold); however, it also significantly induced *cyp1a* expression in NBH embryos (12-fold). Although the reduced sensitivity of NBH fish to *cyp1a* induction is typically heritable through several generations (Nacci et al 2010), NBH fish are not completely insensitive; *cyp1a* induction of lower magnitude or requiring higher doses of inducer has been observed previously (Bello et al 2001; Oleksiak et al 2011).

In mammals, AHR activation downregulates PPARγ expression (Alexander et al., 1998; Arsenescu et al., 2008; Brodie et al., 1996; Gadupudi et al., 2015; Ishihara et al., 2018; van den Dungen et al., 2017). Thus, in fish with dysregulated AHR (i.e., NBH killifish), we hypothesized that the PPARγ pathway would be more efficaciously activated. As noted above, neither TBT nor S26948 induced expression of PPARγ target genes in SC or NBH

killifish embryos. It is difficult to interpret the significance of a lack of differential responsiveness to PPARγ ligands, because this may simply result from TBT and S26948 not being ligands for killifish PPARγ. The phthalate metabolite mono-2-ethylhexyl phthalate has been shown to be an efficacious agonist of zebrafish PPARγ (Grimaldi et al., 2015) and may be better suited for testing differences in PPARγ pathway responsiveness in the two populations.

A much more intriguing result was observed in killifish treated with PCB126. *Pparγ* expression was significantly increased in SC embryos exposed to TBT High + PCB126 but not in NBH embryos. We also found that PCB126 induces *pparg* expression in liver of SC, but not NBH, killifish following adult exposure in males (Crawford et al., 2019). These results are the opposite of what has been shown in mammals, in which PCB126 reduces *pparg* expression and, subsequently, expression of its target genes (e.g., (Gadupudi et al., 2015)). Given the unexpected induction of *pparg* expression by PCB126 in SC killifish, we wondered whether this induction also occurs in other sensitive populations. We compared the PCB126-induced expression of *pparg* in a previously generated dataset comparing four sensitive/tolerant population pairs (Reid et al., 2016). First, *pparg* was identified as a gene with significant exposure-by-population interaction (Reid et al., 2016). Second, in each population pair, PCB126 increased expression of *pparg* to a significantly greater extent in the sensitive populations than the tolerant populations (Figure 8). The physiological significance of this observation is unknown, but it may suggest that lipid homeostatic pathways are altered in NBH killifish and potentially other tolerant killifish populations under study (Reid et al., 2016).

4.4 Variability

On the whole, gene expression in pooled embryo samples across experimental replicates was highly variable. Given our small sample sizes (N=4 or 6 per chemical treatment per population), it was extremely difficult to detect significant differences and identify true trends in the expression of the PPARγ-pathway, osteogenic and chondrogenic genes between chemical treatments. Killifish are well known to have high genetic diversity (Reid et al., 2017). Even individuals from the F2 generation are effectively an outbred, wild population, which naturally implies greater variability among individuals than in populations of inbred or genetically identical animals, such as zebrafish or mice.

Intriguingly, the relative expression of each gene in replicate embryo pools from a given replicate experiment (two pools of N=5 embryos per pool, per experiment) tended to be clustered together compared with other embryo pools in the same treatment group from other experiments. This suggests that the specific characteristics of embryos from a given spawning cycle are more similar to one another than they are to embryos from other cycles. This kind of batch-to-batch variability has been characterized in other teleost fish species, including a meta-analysis of fathead minnow (*Pimephales promelas*) and zebrafish (*Danio rerio*), where temporally proximate batches were found to have greater transcriptomic similarity (Wang et al., 2014). Going forward, the ability to detect subtle changes in gene expression would be improved by generating at least 6 biological replicates per treatment

comprised of pools of embryos from multiple independent clutches all generated in the same spawning cycle.

5. Conclusions

These studies show that TBT is a teratogen in killifish. While we hypothesized that TBT would impair bone development through activation of PPARγ, this pathway was unresponsive to TBT. Rather, the data suggest that TBT is likely acting through RXR in killifish. Thus, it is not surprising that TBT efficaciously induced caudal fin deformities in both SC and NBH killifish embryos, as the potential for PPARγ-AHR crosstalk was not at play. Interestingly, PCB126 induced *pparg* expression in a population dependent-manner, supporting the conclusion that PPARγ-AHR crosstalk is biologically relevant in teleosts, although not in the same manner as in mammals. Future studies are needed to identify killifish PPARγ agonists, to define how RXR activation impairs skeletal development in killifish, and to investigate the physiological and ecological significance of the effect of evolved AHR resistance on PPARγ function.

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Highlights

- TBT induced caudal fin deformity in killifish embryos.
- Susceptibility was the same in PCB-sensitive and PCB-tolerant killifish.
- An RXR-ligand recapitulated the TBT-induced caudal fin deformity.
- Co-treatment with an AHR ligand, PCB126, did not affect the caudal fin deformity.
- AHR regulates PPARγ in killifish but PPARγ activity is minimal in embryos.

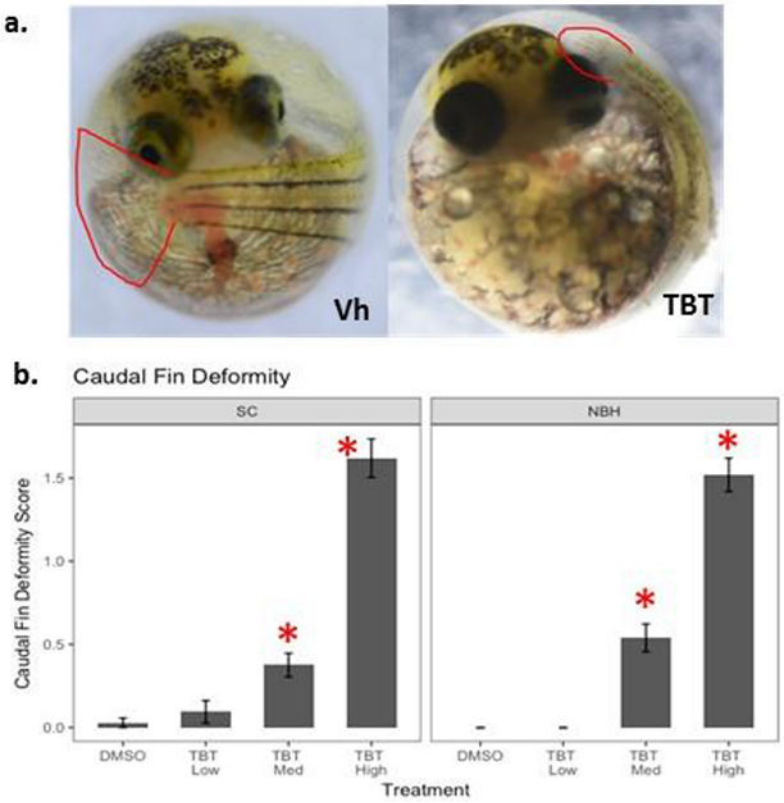

Figure 1. TBT-induced caudal fin teratogenesis.

F. heteroclitus embryos were treated from 1 to 7 dpf with DMSO (0.01%, vehicle), TBT (Low: 5 nM, Medium: 50 nM and High: 100 nM) in seawater and then moved to a filter paper moistened with clean water until 10 dpf. **(a)** Representative images of caudal fin development. **(b)** Caudal fin deformity score for 10 dpf embryos. Deformity score ranged from 0 to 3, with 0 = normal and 3 = severe deformity. Data are presented as mean ± SEM. Total N≥50, screened from two replicate experiments. Two Factor ANOVA: Population p = 0.435, Treatment p <0.0001, Interaction p = 0.483. * Significantly different from DMSO in the same population (p<0.05, ANOVA, Dunnett's)

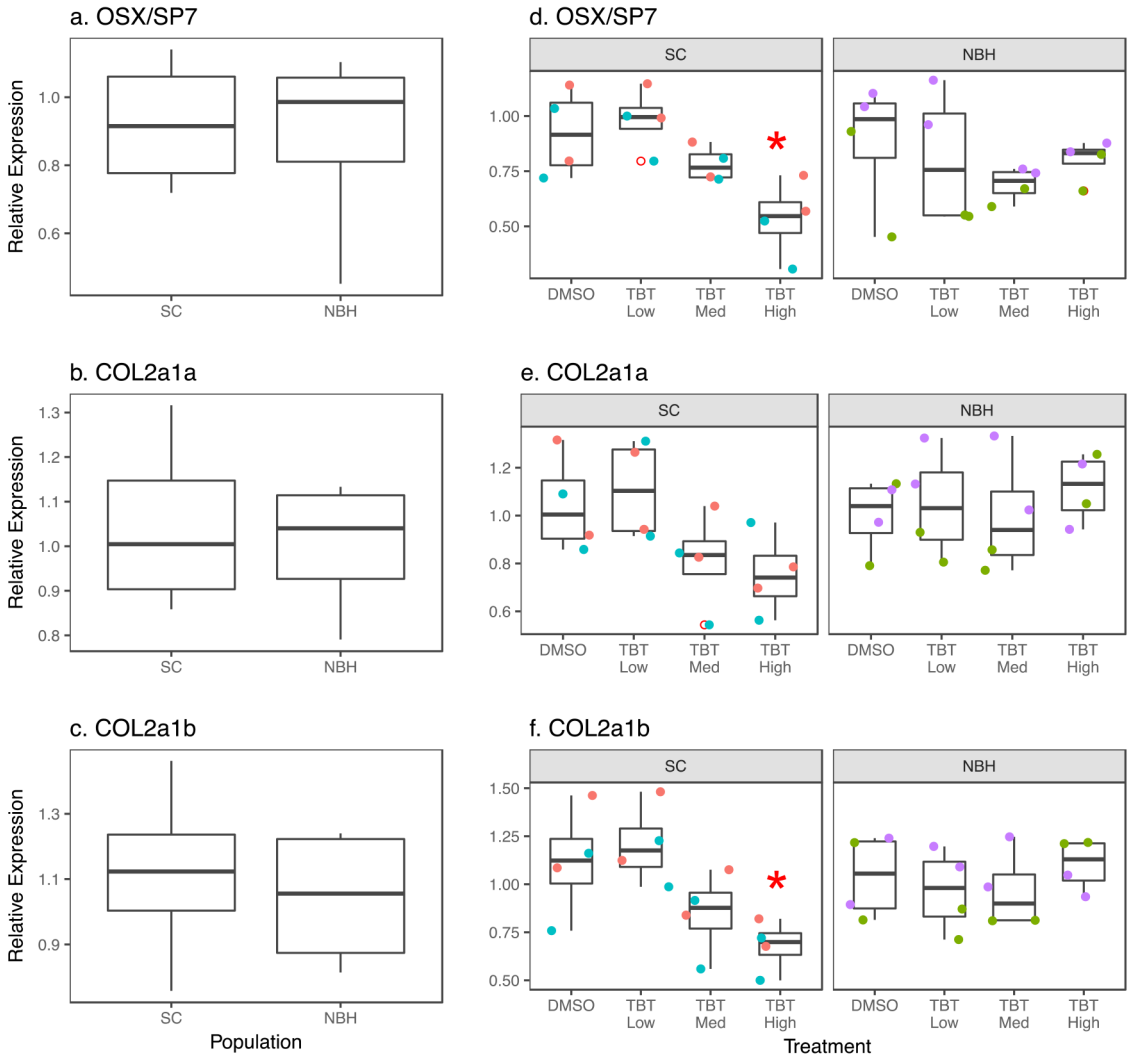

Figure 2. TBT reduces osteoblast and chondroblast gene expression in SC embryos.

F. heteroclitus embryos were treated as described in Figure 1. mRNA expression was measured by RT-qPCR in pooled embryonic killifish. Osteoblast marker gene: **(a)** Basal *osx/sp7* expression. **(d)** Chemical induced *osx/sp7* expression. Two Factor ANOVA: Population p = 0.876, Treatment p = 0.051, Interaction: 0.133. Chondroblast marker genes: **(b)** Basal *col2a1a* expression, **(e)** Chemical-induced *col2a1a* expression. Two Factor ANOVA: Population p = 0.131, Treatment p = 0.301, Interaction p = 0.1330. **(c)** Basal *col2a1b* expression, **(f)** Chemical-induced *col2a1b* expression. Two Factor ANOVA: Population p = 0.448, Treatment p = 0.128, Interaction: p = 0.025. Each pool consisted of 5 embryos. N=4 pools per chemical treatment, per population from two experimental replicates from SC and NBH. Data points of the same color are from biological replicates produced in the same experiment. * Significantly different from DMSO in the same population (p<0.05, ANOVA, Dunnett's)

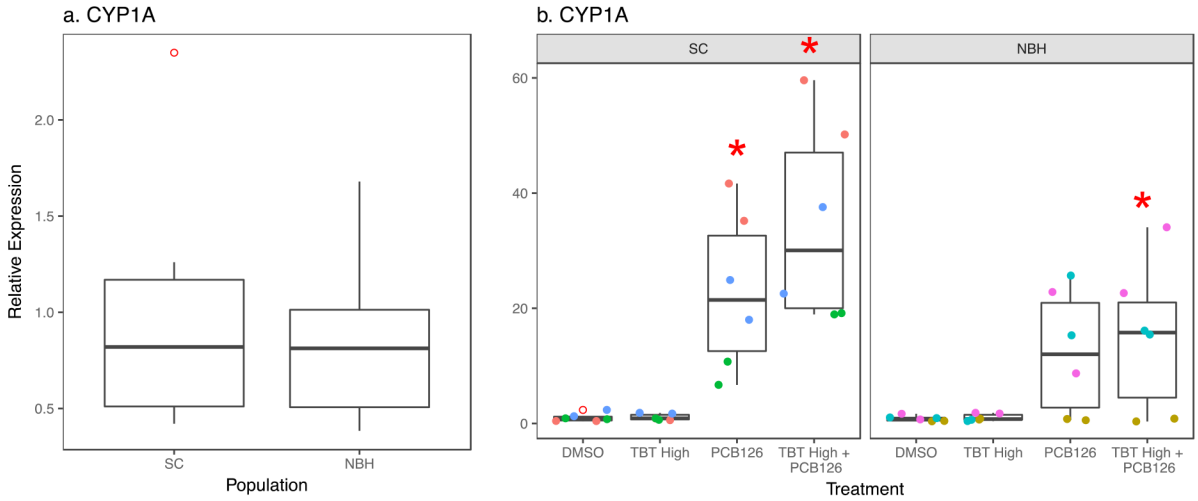

Figure 3. TBT does not alter *cyp1a* expression induced by PCB126.

F. heteroclitus embryos were treated from 1 to 7 dpf with DMSO (0.01%, vehicle), TBT (Medium: 50 nM and High: 100 nM) and/or PCB126 (61 pM) in seawater and then moved to a filter paper moistened with clean water until 10 dpf. mRNA expression was measured by RT-qPCR in pooled embryonic killifish. **(a)** Basal *cyp1a* expression in SC and NBH embryos. **(b)** Chemical-induced *cyp1a* expression. Two Factor ANOVA: Population p = 0.015, Treatment p < 0.001. Interaction: p = 0.190. Each pool consisted of 5 embryos. N=6 pools per chemical treatment, per population from three experimental replicates from SC and NBH. Data points of the same color are from biological replicates produced in the same experiment. * Significantly different from DMSO in the same population (p<0.05, ANOVA, Dunnett's)

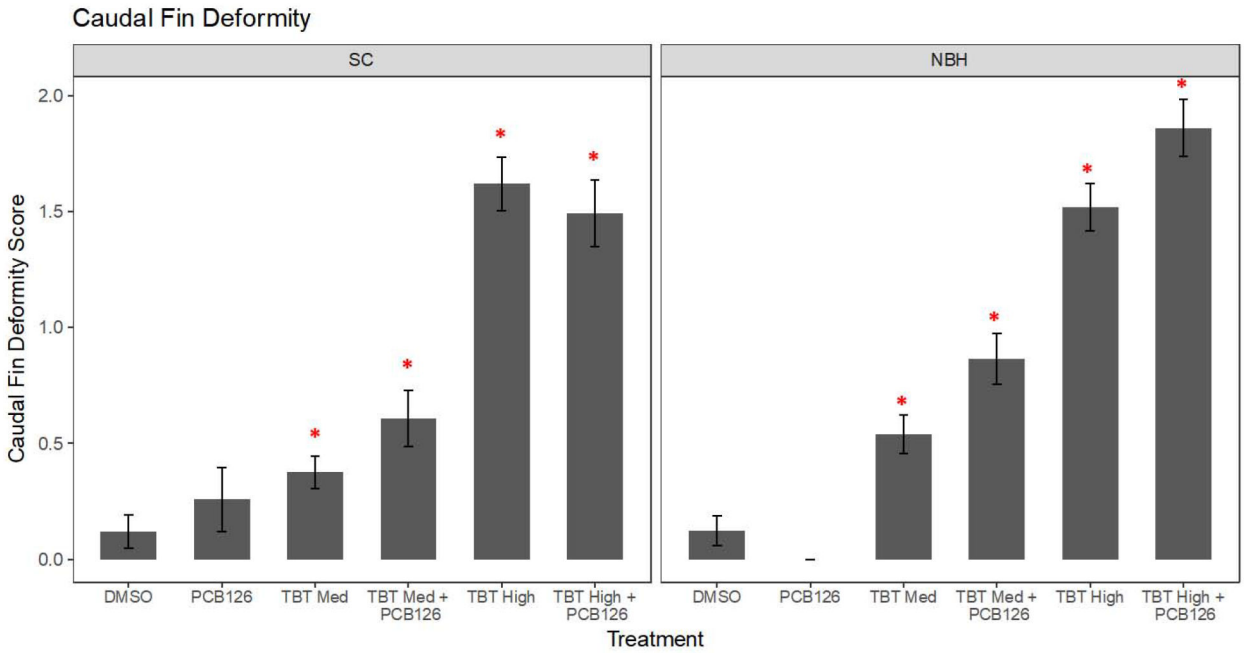

Figure 4. Low level PCB126 exposure does not induce fin teratogenesis and does not alter TBT-induced fin teratogenesis.

F. heteroclitus embryos were treated as described in Figure 3. Caudal fin deformity score for 10 dpf embryos was determined from 0 to 3, with 0 = normal and 3 = severe deformity. Data are presented as mean ± SEM. Total N≥50, screened from three replicate experiments. Two Factor ANOVA: Population $p = 0.161$, Treatment $p < 0.001$, Interaction $p = 0.098$. * Significantly different from vehicle ($p<0.05$, ANOVA, Tukey's HSD)

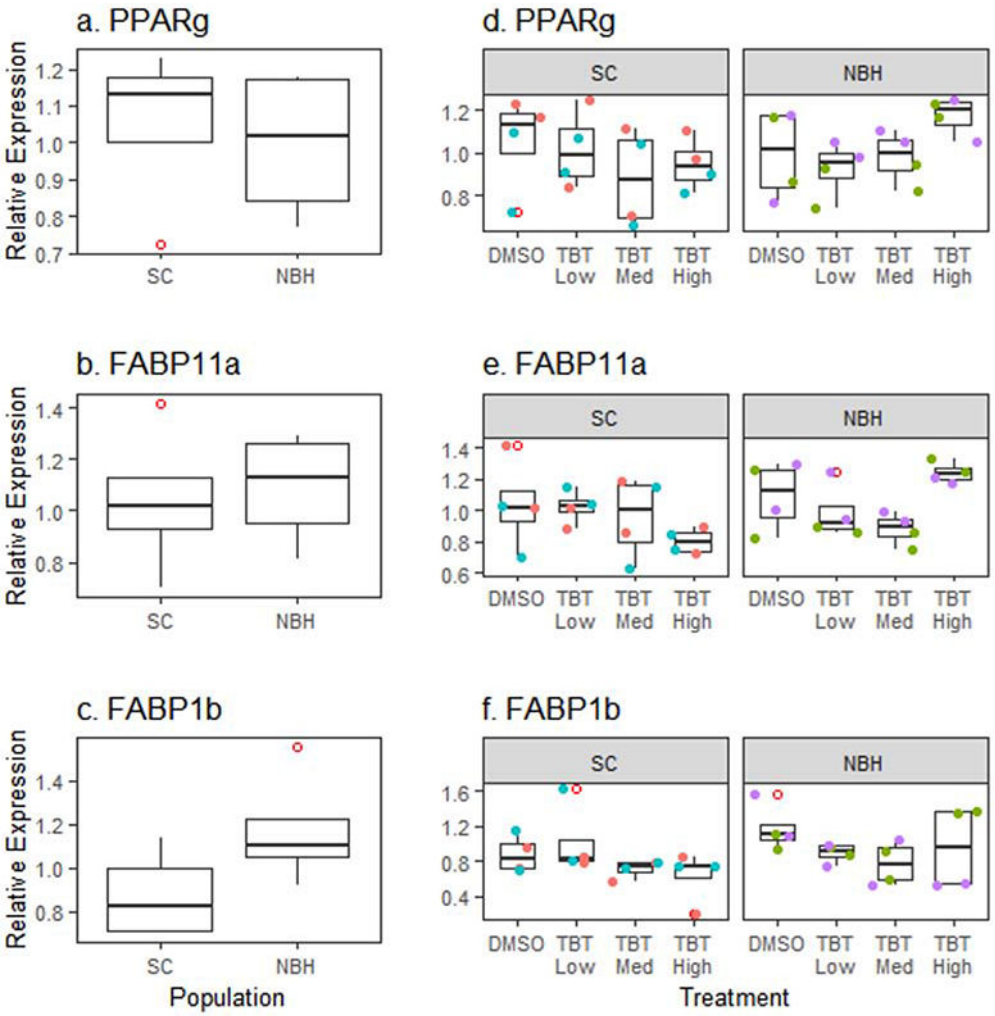

Figure 5. Lack of responsiveness of PPARγ pathway in killifish embryos.

F. heteroclitus embryos were treated as described in Figure 1. mRNA expression of *pparg* and its target genes *fabp11a* and *fabp1b* were measured by RT-qPCR in pooled embryonic killifish. **(a-c)** Basal expression in SC and NBH embryos. **(d-f)** Expression in TBT exposed embryos. Each pool consisted of 5 embryos. N=4 pools per chemical treatment, per population from two experimental replicates from SC and NBH. Data points of the same color are from biological replicates produced in the same experiment. No statistically significant differences were detected (ANOVA).

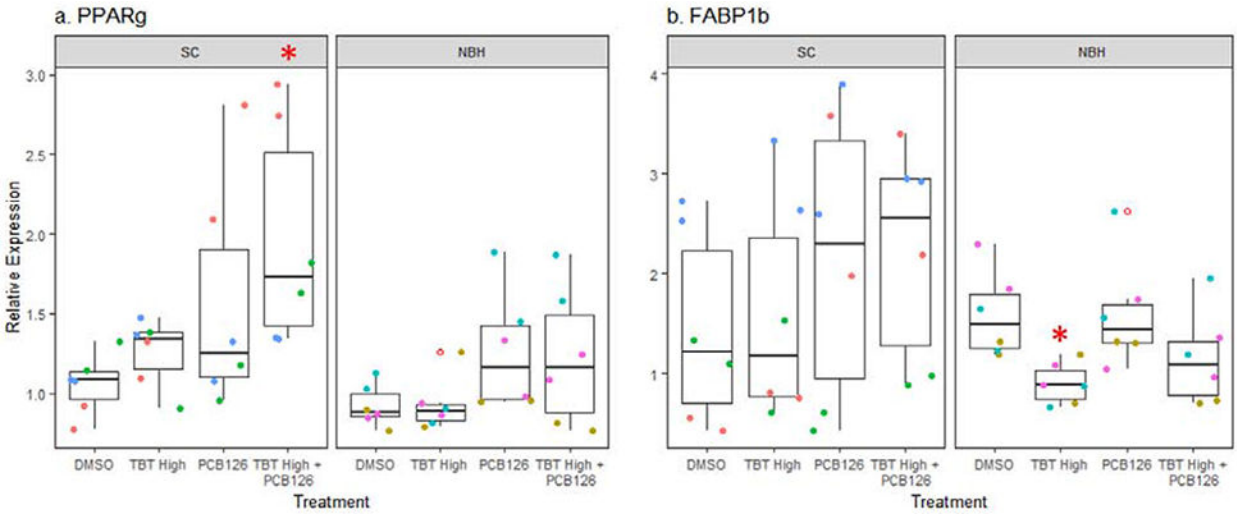

Figure 6. PCB126 induces *pparg* expression only in SC embryos.

F. heteroclitus embryos were treated as described in Figure 3. mRNA expression was measured by RT-qPCR in pooled embryonic killifish. **(a)** Expression of *pparg*. Two Factor ANOVA: Population, p = 0.004. Treatment p = 0.004. Interaction p = 0.364. **(b)** Expression of *fabp1b*. Two Factor ANOVA: Population p = 0.036, Treatment p = 0.363, Interaction: p = 0.407. Each pool consisted of 5 embryos. N=6 pools per chemical treatment, per population from three experimental replicates from SC and NBH. Data points of the same color are from biological replicates produced in the same experiment. * Significantly different from DMSO in the same population ($p<0.05$, ANOVA, Dunnett's)

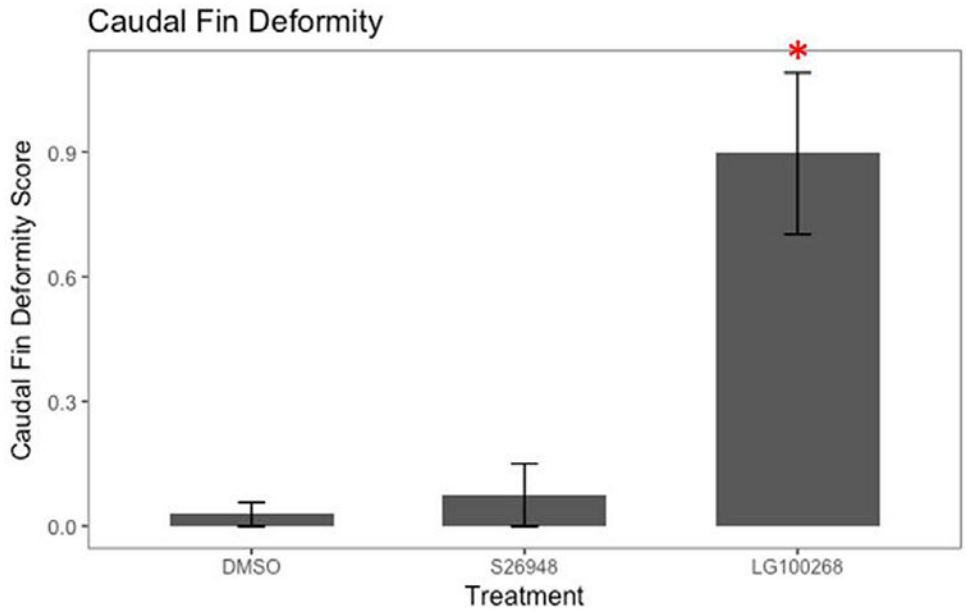

Figure 7. An RXR ligand, but not a PPARγ ligand, induces caudal fin teratogenesis.

F. heteroclitus embryos were treated from 1 to 7 dpf with DMSO (0.01%, vehicle), S26948 (1 μM), and LG100268 (200 nM) in seawater and then moved to a filter paper moistened with clean water until 10 dpf. Caudal fin deformity score for 10 dpf embryos was determined from 0 to 3, with 0 = normal and 3 = severe deformity. Data are presented as mean ± SEM pooled across SC and NBH embryos. Total N≥30, screened from two replicate experiments. * Significantly different from vehicle ($p<0.05$, ANOVA, Dunnett's)

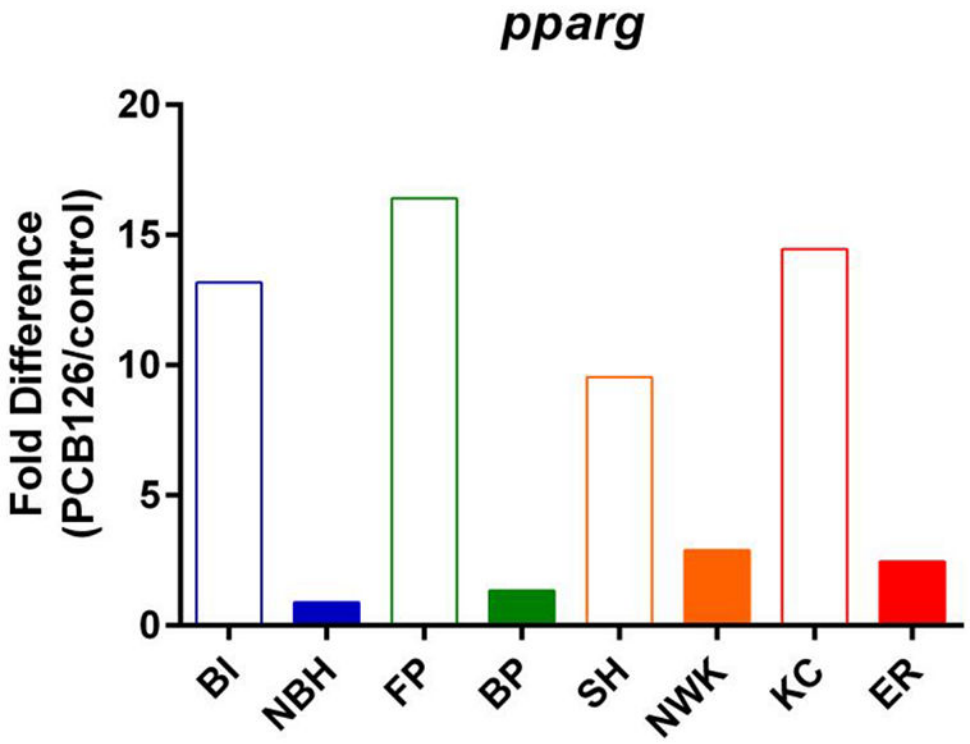

Figure 8. PCB126 induces expression of *pparg* across multiple sensitive killifish populations but not in resistant populations.

Pparg expression data are from (Reid et al., 2016). Killifish from sensitive and resistant population pairs were treated with DMSO or PCB126 (610 pM) in seawater from 1 dpf to post-organogenesis (stage 35, ~10 dpf). mRNA expression was determined by RNA-Seq. A map of the locations can be found in (Reid et al., 2016). Sensitive populations (open bars): BI = Block Island, RI. FP = Flax Pond, NY. SH = Sandy Hook, NJ. KC = Kings Creek, VA. Resistant populations (closed bars): NBH = New Bedford Harbor, MA. BP = Bridgeport, CT. NWK = Newark, NJ. ER = Elizabeth River, VA. *Pparg* expression following PCB126 treatment was determined by dividing the average, normalized expression level (N=3-5) in PCB126 treated embryos by that in vehicle treated embryos. Overall, there was 13.4 ± 1.5 fold induction in sensitive populations versus 1.9 ± 0.9 fold induction in resistant populations ($p<0.003$, Student's t-test).

Table 1.

Primer information.*

Gene	Forward (5' --> 3')	Reverse (5' --> 3')	nt	Tm (°C)
actb	TGGAGAAGAGCTACGAGCTCC	CCGCAGGACTCCATTCCGAG	114	66[a]
rn18S	TGGTTAATTCCGATAACGAACGA	CGCCACTTGTCCCTCTAAGAA	97	65
ef1a	GGGAAAGGGCTCCTTCAAGT	ACGCTCGGCCTTCAGCTT	55	60
cyp1a	CTTTCACAATCCCACACTGCTC	GGTCTTTCCAGAGCTCTGGG	125	66[a]
pparg	GGAAAGAGATGGAGACGCACAACC	CCGACGCATCGTCTGGGAACTTGG	102	66
fabp11a	GGCAAGCTTATTCAGAAACAGAGC	CCACGTCGCCCATTACGCATTTCGC	103	66
fabp1b	AGCCTTTCATGAAGGCCCTTGGTC	GTGGTGACCGTCACCTTGAAGTCG	115	66
col2a1a	GGTGAGACCTGCGTCTACCCAAGC	CCATCCTGAGCATAGCTGAAGTGG	137	66
col2a1b	GGGAGTCCTGCGTGAACCCCAAGC	CTGTCGTCACCATAACTGAAGTGG	136	66
osx/sp7	CATCTGTTCTGGAGCTAGGGAATGC	TTACTGGAGAGCTGCCGGTTTTGC	150	66

*For citations, see section 2.5.