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Resistance to *Cyp3a* induction by polychlorinated biphenyls, including non-dioxin-like PCB153, in gills of killifish (*Fundulus heteroclitus*) from New Bedford Harbor

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Abstract

Previous reports suggested that non-dioxin-like (NDL) PCB153 effects on cytochrome P450 3A *(Cyp3a)* expression in Atlantic killifish *(Fundulus heteroclitus)* gills differed between F0 generation fish from a PCB site (New Bedford Harbor; NBH) and a reference site (Scorton Creek; SC). Here, we examined effects of PCB153, dioxin-like (DL) PCB126, or a mixture, on *Cyp3a56* mRNA in killifish generations removed from the wild, without environmental PCB exposures. PCB126 effects in liver and gills differed between populations, as expected. Gill *Cyp3a56* was not affected by either congener in NBH F2 generation fish, but was induced by PCB153 in SC F1 fish, with females showing a greater response. PCB153 did not affect *Cyp3a56* in liver of either population. Results suggest a heritable resistance to NDL-PCBs in killifish from NBH, in addition to that reported for DL PCBs. Induction of *Cyp3a56* in gills may be a biomarker of exposure to NDL PCBs in fish populations that are not resistant to PCBs.

Keywords

PCB153; Fish; Gills; Cyp3a; Resistance

1. Introduction

Polychlorinated biphenyls (PCBs) are persistent legacy pollutants abundant in many parts of the world. The PCB congeners with *meta*- and *para*- but no *ortho-chlorine* substituents, known as dioxin-like PCBs (DL PCBs), are avid ligands for the aryl hydrocarbon receptor (Ahr) and are acutely toxic, eliciting molecular effects and toxicity through activation of the

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Ahr. Prominent molecular effects of the DL PCBs include induction of drug metabolizing enzymes, especially cytochrome P450 1A (Cyp1a) and other members of the *Cyp1* gene family. Induction of Cyp1a has long been used as a biomarker of exposure to DL PCBs and other Ahr agonists in the aquatic environment (Stegeman and Lech, 1991). In contrast to the DL PCBs, the molecular effects of non-dioxin-like PCBs (NDL PCBs) in fish are less well known, despite the fact that they are vastly more abundant in the environment. This study addresses the molecular effects of the NDL PCBs including an exposure biomarker, and a possible molecular adaptation to high levels of NDL PCBs, in the estuarine fish model, killifish (*Fundulus heteroclitus)*.

Some fish populations inhabiting areas heavily contaminated by PCBs show impaired CYP1A biomarker responses, rather than robust induction (Elskus et al., 1999; Förlin and Celander, 1995; Wirgin and Waldman, 2004). A well-studied example is the killifish population inhabiting the New Bedford Harbor (NBH) in Massachusetts (MA; USA), a site severely contaminated by PCBs for more than 70 years (Nacci et al., 2010). Killifish in NBH have evolved a heritable resistance to the effects of DL PCBs, including little or no induction of CYP1A by DL PCBs or other Ahr agonists (Bello et al., 2001; Nacci et al., 1999). In addition to the DL PCBs, killifish in NBH are also exposed to extremely high levels of NDL PCBs (Gräns et al., 2015; Lake et al., 1995), among the highest in the world (Martinez et al., 2017).

Despite severely high body-burdens of PCBs, the NBH killifish population survive and reproduce, which implies that they have developed a resistance to the adverse effects of NDL PCBs in addition to DL PCBs. However, the molecular mechanisms involved in PCB resistance are not known.

In some mammals, NDL PCBs activate the pregnane-X-receptor (Pxr) and/or the constitutive androstane receptor (Car) and induce the expression of *Cyp3a* and *Cyp2* genes (Al-Salman and Plant, 2012; Jacobs et al., 2005; Kopec et al., 2010). Some mammalian Pxr agonists can activate zebrafish Pxr *in vitro* (Bainy et al., 2013), but whether NDL PCBs act through similar pathways in killifish is unclear. In a previous study, *Pxr* and *Cyp3a* mRNA levels in liver of killifish from either NBH or Scorton Creek (SC) in MA (a reference site), showed either slight or no response to laboratory exposures to the NDL congener PCB153 (Gräns et al., 2015). In contrast, gills of killifish from SC treated with PCB153 showed elevated levels of both *Pxr* and *Cyp3a* mRNAs, while *Pxr* and *Cyp3a* mRNA levels in gills of fish from NBH did not show an effect of PCB153 (Gräns et al., 2015). As expected, the DL PCB126 did elicit strong induction of *Cyp1a* mRNA, in both gills and livers of SC fish but not in NBH fish.

In the previous study (Gräns et al. 2015), the fish had been collected from the wild, and while they had been held for a few months in the laboratory before being exposed to PCB153 or PCB126. Previous work indicated that the fish from NBH would still retain extremely high levels of PCBs, including PCB153 (Nacci, D., unpublished). Those PCBs already present in the NBH fish weakened inferences about the effects of added PCB153, and could obscure actual differences in effect between these two populations. In the present study, we examined PCB153 effects on *Pxr* and *Cyp3a* in NBH and SC killifish one or two

generations removed from the wild, with no residual body-burdens of PCBs. The aim of this study was to examine the effects of PCB153 on *Cyp3a56* expression in lab-reared killifish from the NBH and SC populations. We focused on gills based on previous results, where the response in SC fish pointed to a molecular response to PCB153. The absence of such a response in NBH fish suggested an adaptation to NDL PCBs in that population (Gräns et al., 2015). A response to PCB153 in gills also could suggest a biomarker for exposure to NDL PCBs in fish populations that are not resistant to PCBs.

2. Materials and Methods

2.1 Chemicals

PCB126 (3,3',4,4',5-pentachlorobiphenyl) and PCB153 (2,2',4,4',5,5'-hexachlorobiphenyl) were purchased from Ultra Scientific, Kingston, RI, USA.

2.2 Fish collection and breeding

Adult killifish were collected in the summer of 2010 from NBH. These fish (denoted NBH F0 generation) were transported to the Atlantic Coastal Environmental Science Division of the United States Environmental Protection Agency (US-EPA), Narragansett in Rhode Island (RI; USA), and were maintained in the lab until 2012. Embryos (F1 generation) were collected from adult NBH F0 generation fish during free spawning events in the summer of 2012 and raised to maturity. Embryos (F2 generation) were collected from those F1 fish during free spawning events in the summer of 2015 and raised to maturity. In December 2017, a subset of these F2 fish was selected based on size similarity and used in this study. Adult killifish were collected in the summer of 2015 from the reference site SC and maintained in the US-EPA lab (denoted SC F0 generation). Embryos (F1 generation) were collected from those F0 fish during free spawning events in the summer of 2015 and raised to maturity. In December 2017, a subset of these F1 fish was selected based on size similarity and used in this study. All adult fish were held in ambient temperature flowing seawater and fed Tetramin® flakes food *ad libitum.* The average body weights ± standard deviations (SD) were 5.7 ± 0.8 g for the F2 generation from NBH, and 5.3 ± 0.8 g for the F1 generation from SC. All fish were acclimated up to test temperature of 23 °C at the rate of 2 °C twice per week and held at the test temperature for two weeks prior to the initiation of this experiment (December 4, 2017).

2.3. Fish PCB exposure experiment

Fish were treated by intraperitoneal injections of PCB126 (1.38 ng/μL), PCB153 (100 ng/μL) or a mixture of both (1.38 ng/μL PCB126 + 100 ng/μL PCB153) dissolved in corn oil. Corn oil alone was used as vehicle control. The injection volume was 21.20 μl/g fish. In the SC population, there were 8 fish with a male (M) to female (F) ratio of 5:3 in vehicle treated group, 9 fish (M:F-ratio 5:4) in the PCB126 treated group, 11 fish (M:F-ratio 6:5) in the PCB153 group and 9 fish (M:F-ratio 5:4) in the mix PCB126/153 group. In the NBH population, there were 10 fish male (M):female (F) ratio 5:5 in vehicle treated group, 8 fish (M:F-ratio 4:4) in the PCB126 treated group, 10 fish (M:F-ratio 5:5) in the PCB153 group and 8 fish (M:F-ratio 5:3) in the mix PCB126/153 group. The goal of these exposures was to achieve doses of these congeners similar to those measured in livers of wild-caught

fish from NBH (nominal targets of 29 ng PCB126/g fish, 2120 ng PCB153/g fish, and the mixture of both) (Gräns et al., 2015). Fish were injected December 4 and sampled three days later December 7, 2017. Fish were killed by cervical transection. Gills (gill arches and filaments) and livers were immediately dissected out. Livers were cut in three parts, one part for PCB concentration analyses and two parts for RNA analyses. Gills and liver tissues for RNA analyses were snap-frozen in liquid nitrogen and transported to the Woods Hole Oceanographic Institution and stored in −80 °C upon arrival. Procedures used in the studies were conducted under the Woods Hole Oceanographic Institution IACUC permit number BI21981.01 and US-EPA IACUC Eco23–03-002.

2.4. Isolation of total RNA, cDNA synthesis and quantitative PCR (qPCR)

Total RNA was isolated from gills and liver using the TRI Reagent and the Direct-zol RNA Mini Preps Plus kit from Zymo Research, Irvine, CA, USA. Total RNA concentrations were analyzed using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). For cDNA synthesis, 1 μg total RNA was reverse transcribed in a 20 μl reaction volume performed using the iScript Reverse Transcription Supermix for RT-PCR from BioRad, Hercules, CA, USA. The cDNA samples were diluted with nuclease-free water to a final volume of 100 μl and stored in −80 °C prior analysis. For qPCR analysis, 2 μl of the diluted cDNA sample was transferred to a 96-well PCR-plate, and real-time PCR was carried out in a total reaction volume of 10 μl using the SsoFast EvaGreen Supermix from BioRad, and a CFX96 Real-Time System PCR machine from BioRad, Hercules, CA, USA. Two technical replicates were assayed for each fish sample and gene analyzed. Three target genes were analyzed, *Cyp3a56, Pxr* and *Cyp1a.* Three housekeeping genes were analyzed, elongation factor 1α *(ef1α), β-actin* and *Arnt2.* The annealing temperature for *Pxr, Cyp3a56, ef1α,* and *Arnt2* was 58 °C and 55 °C for *Cyp1a* and */β-actin.* The standard qPCR protocol was used followed by melting curve analysis to confirm single PCR products for each primer pair. The pattern of target gene expressions between the different treatment groups appeared independent of the housekeeping gene used. The PCR threshold-levels for *ef1α* were in the same range as that for *Cyp3a56.* The data presented are therefore normalized to ef1α. The primer efficiencies for *Cyp3a56* and *ef1α* in gills were 1.00 and 0.99 respectively. The qPCR data are presented as $2^{-\Delta Ct}$ *(target gene-ef1α)* ± standard deviation (SD). Although there are four different *Cyp3a* genes present in the killifish genome, the principal gene expressed in killifish adults is *Cyp3a56* (Hegelund and Celander,2003; Goldstone, J.V., unpublished) The *Cyp3a56* primers used here are specific, and do not match and will not amplify the other, minor *Cyp3a* genes. The qPCR primer sequences were (5'to 3'):

Cyp3a56 forward: CATGGATGTGGTAACCAGCACGG

Cyp3a56 reverse: GAAAGAAAGCGACGGCGAGGAAG

Cyp1a forward: GCCTTTCACAATCCCACACTGCTC

Cyp1a reverse: TGGGTCGTGGTTTATCTGCCACTG

Pxr forward: AAGCCTTGAGGACTTCCGGTCTC

Pxr reverse: CCCAGTTGCTGGTTGTGGAGTTG

Ef1α forward: CTCAGAGCTTCAACGCTCAGGTC

Ef1α reverse: ATCAGCTCGTTGAACTTGCAGGC

β-actin forward: TGGAGAAGAGCTACGAGCTCC

β-actin reverse: CTCGGAATGGAGTCCTGCGG

Arnt2 forward: TTCCAGGCGCTCCTTTATTTGCC

Arnt2 reverse: TAGACGGTCCCAAGCCATTCCTG

2.5 PCB analyses in livers

Individual livers from control and PCB-treated fish were analyzed for residues of PCB126 and PCB153, using established methods (Gutjahr-Gobell et al., 1999; Jayaraman et al., 2001). Calibration curves ranged from 50 to 500 ng/mL for PCB153 and 1.5 to 48 ng/mL for PCB126. Livers from four vehicle treated fish, one of each sex and population and 61 PCB treated fish of each sex and population were analyzed for PCB126 and PCB153. Two matrix spikes were prepared. The first one was spiked with 500 ng/g PCB153 and 7.50 ng/g PCB126. The recoveries were 101 and 109 for PCB153 and PCB126, respectively. The second one was spiked with 250 ng/g PCB153 and 15 ng/g PCB126. The recoveries were 92.1 and 111 for PCB153 and PCB126, respectively.

2.6 Statistics

All statistical analyses were conducted using Graph Pad Prism 8.4. The data were tested for deviation from the Gaussian ideal using the Shapiro-Wilk normality test. The qPCR data were log-transformed to achieve approximately normal distributed residuals. A three-way ANOVA was conducted to compare the factors of treatment, population, and sex and their interaction effects on gene expression. A two-way ANOVA was conducted to test for statistically significant interactions between treatments and populations. ANOVA comparisons were performed followed by a multiple comparison using Tukey correction to adjust the critical values. Statistical significance was accepted at the $p < 0.05$ level for two-way interactions.

3. Results

Comparisons of levels of *Cyp1a, Pxr* and *Cyp3a56* mRNA between the two killifish populations to various PCB-treatments were performed. We focused on these few genes as they are the ones that the prior study suggested (Gräns et al., 2015), and because of data from other vertebrate species *(e.g.* Kopec et al., 2010). Comparisons between the two populations on *Cyp1a, Pxr,* and *Cyp3a56* mRNA levels in gills from pooled sexes are listed in Table 1. The statistics comparing responses to treatments within each population for each sex and pooled sexes are provided as supplemental material in Table S1. The statistics comparing responses to treatment between the two populations for each sex and pooled sexes are provided as supplemental material in Tables S2.

As expected, when compared to vehicle control, *Cyp1a* mRNA levels were significantly induced in gills in F1 generation fish from SC treated with the DL congener PCB126 *(F(3,* 65) = 6.92; adjusted $p = 0.017$) as well as in fish treated with the PCB126/153 mixture (adjusted $p < 0.001$). These treatments did not affect *Cyp1a* mRNA levels in gills in F2 generation fish from NBH. No changes in *Pxr* or *Cyp3a56* mRNA levels were observed in gills in F2 generation NBH killifish treated with the either NDL PCB153, the DL PCB126, or a mixture of the two congeners compared to vehicle control. In the F1 generation SC killifish, the mean *Cyp3a56* mRNA level was more than three-times higher upon treatment with PCB153 compared to vehicle control fish, yet not significantly different (adjusted $p = 0.475$) due to intragroup variation (Table S1).

Next, mRNA levels in gills from pooled sexes were compared between populations that received the same treatment. The *Cyp1a* mRNA levels differed between SC and NBH ($F(1,65) = 205$; $p < 0.001$) for all exposure groups: Vehicle (adjusted $p = 0.003$), PCB126 (adjusted $p < 0.001$), PCB153 (adjusted $p < 0.001$), Mix PCB126/PCB153 (adjusted $p < 0.001$). Despite a large variation in *Cyp3a56* mRNA levels in each group, the two-way ANOVA revealed a statistically significant difference between populations ($F(1,65) = 16,70$; adjusted $p < 0.001$). In fact, *Cyp3a56* mRNA levels were more than seven times in gills in SC fish treated with PCB153 compared to that in NBH fish treated with PCB153. A post-hoc analysis using Tukey adjusted alpha levels of 0.05 was performed, indicating a significant difference in *Cyp3a56* mRNA levels between PCB 153-treated SC and NBH fish, adjusted $p < 0.001$. In contrast to *Cyp3a56,* levels of *Pxr* mRNA in gills were constant in all treatment groups in both populations (Table 1).

Intrapopulation sex differences in response to PCB153 in gills on *Cyp3a56* mRNA levels were observed. Fish from NBH, treated with PCB153, had eight times lower *Cyp3a56* mRNA levels in males (adjusted $p = 0.724$) and two times lower *Cyp3a56* mRNA levels in females (adjusted $p = 0.479$) compared to vehicle-treated fish of the respective sex. Fish from SC, treated with PCB153, had 1.6 times higher *Cyp3a56* mRNA levels in males ($p > 0.999$) and 5 times higher CYP3A mRNA levels in females ($p = 0.012$) compared to vehicle-treated fish of the respective sex (Table 2).

When comparing the two PCB153 treated populations, SC females had 35 times higher (adjusted $p < 0.001$) and males had two times higher (adjusted $p = 0.979$) *Cyp3a56* mRNA levels compared to that in NBH females and males, respectively (Table S2). Levels of *Cyp1a* and *Pxr* mRNA in gills, separated by sexes, are provided in Table S3. A sex-difference was observed on *Cyp1a* mRNA levels, with males from SC having 6 to 13 times higher *Cyp1a* mRNA levels upon treatment with the DL PCB126 alone or mixed with PCB153. Elevated *Cyp1a* mRNA levels, albeit not statistically significant, were seen in female fish from SC treated with PCB126 alone or mixed with PCB153 (Table S3).

In addition, a three-way ANOVA was conducted to examine the effect of 1) population; 2) sex and 3) treatment on *Cyp3a56* mRNA levels, showing a statistically significant three-way interaction ($F(3, 57) = 6.088$; adjusted $p = 0.001$). The population factor was most significant, adjusted $p < 0.001$. The differences in responses to PCB treatment between the

two populations in *Cyp3a56* mRNA levels and CYP1A mRNA levels in female fish are illustrated in a histobar diagram in Figure 1, and as a scatterplot in Figure S1.

For comparison, *Pxr, Cyp3a56* and *Cyp1a* mRNA levels also were analyzed in livers from these fish. Similar to that in gills, hepatic levels of *Pxr* mRNA were the same in all treatment groups in both populations. In contrast to the response in gills, the *Cyp3a56* were not induced by PCB153 in livers of fish from SC or NBH. As expected, and similar to the effect in gill, CYP1A mRNA levels were induced in livers of F1 SC fish exposed to PCB126, whereas in F2 NBH fish, no increased *Cyp1a* expression was seen in liver (Table 3). The results with PCB126 thus confirm that the F1/F2 generations fish used in these experiments had the expected resistance to effects of DL PCBs in the NBH population.

Levels of PCB153 and PCB126 were measured in 61 of these fish, including both PCB-treated and vehicle-treated fish. A small amount of PCB153 was detected in the liver of one a F1 female fish from SC. The remaining three vehicle-treated liver samples were free of PCB153 and PCB126 (Table 4). The targeted concentration of 29 ng PCB126/g wet weight (ww) was not achieved in most fish; within treatment variation was high and many samples were below detection limit. However, average concentrations were similar between populations, ~20 ng PCB126/g ww. Similarly, within treatment variation was high for PCB153 in most fish, but the average was lower than the targeted concentration of 2120 ng PCB153/g ww in both the single congener and the mixture treatments.

4. Discussion

Ortho-substituted NDL PCB congeners are legacy pollutants ubiquitous in the environment, with incompletely understood effects in humans and animals, especially in non-mammalian animals such as fish. In the present study, we focused on *Cyp3a56* responses in gills in killifish exposed to the NDL PCB153. In a preceding study, F0 generation fish collected directly from the SC reference site showed an increase in expression of *Pxr* and *Cyp3a56* in gills of fish treated with additional PCB153, an effect not seen in F0 fish collected from the PCB-polluted NBH site (Gräns et al., 2015). The environmental levels of NDL PCBs in the SC F0 generation fish are extremely low, and would not be expected to interfere with effects of additional laboratory PCB153 exposures in those fish. In contrast, F0 fish collected from NBH have extremely high levels of environmental PCB153 and other NDL PCB congeners in their tissues (Gräns et al., 2015), which could have interfered with any effect of added PCB153 in the laboratory. Hence, the body burdens of PCBs in the NBH fish reported in the preceding study draw into question interpretations about differences in response to added PCB153 between these two populations.

In the present study, we focused on killifish that were raised in the laboratory and which were one or two generations removed from the wild. The response to PCB153 observed in the F1 SC killifish gills thus confirm that this NDL PCB can indeed induce *Cyp3a56* gene expression in this species. By comparison, the lack an effect of PCB153 on *Cyp3a56* expression in the gills of the F2 NBH fish, without a confounding pre-existing body burden of PCBs in the experimental fish, suggests that the killifish in NBH are resistant to effects of PCB153 and possible other NDL PCBs. As these fish are two generations removed from the

wild, these observations further suggest that this resistance is heritable. This appears to be much like the well-known heritable lack of induction of Cyp1a by the DL PCBs in the NBH killifish population (Bello et al., 2001; Nacci et al., 1999; Gräns et al. 2015).

The mechanism of resistance of killifish even to the DL PCBs is still not completely known, although there are significant genomic differences between resistant and non-resistant populations (Nacci et al., 2016). Laboratory and field studies have identified several genes as probable selective agents for the inherited differences observed in responses to PCBs between NBH and reference killifish. Specifically, genes associated with the Ahr signaling response pathway have been correlated with tolerance to DL PCBs in a Quantitative Trait Locus study (Nacci et al., 2016). Population genetics studies have confirmed genetic variation at these loci when NBH (and other tolerant killifish populations) are compared with reference killifish populations (Hahn et al., 2004: Proestou et al., 2014; Reitzel et al., 2014; Reid et al., 2016). In addition, *Cyp3a* was identified as a variant in a population genetic study that included NBH killifish (Proestou et al., 2014). However, it is currently unknown whether these concurrent genetic variations in the Ahr pathway components and *Cyp3a56* in NBH killifish reflect related, unrelated, or compensatory mechanisms at play in their ability to survive in extremely PCB-contaminated environment.

In another fish species, tomcod *(Microgadus tomcod),* resistance to DL PCB effects involves sequence variation in the Ahr (Wirgin et al., 2011). At some level, DL PCB resistance in killifish is certain to involve the Ahr signaling pathway. Although the exact molecular mechanism is still hidden (Aluru et al., 2011; Reitzel et al., 2014), it appears to be the result of complex interactions between different components of the stress response network (Nacci et al., 2016).

The lack of an effect of PCB153 on *Cyp3a56* expression in liver of F1 SC fish was somewhat surprising, given the *Cyp3a56* induction in gills. However, a lack of effect of PCB153 on *Cyp3a56* mRNA in liver was observed also in the SC reference population fish in the previous study in the F0 generation (Gräns et al., 2015). In addition, immunohistochemical study in killifish showed greater staining of CYP3a proteins in gills compared to that in livers (Hegelund and Celander, 2003). Such a difference could involve different abundances of some responsive cell types in the two organs, or selective expression of a receptor that may be involved. Although intriguing, the difference is not understood, but may be resolved when the mechanism of PCB153 effect on *Cyp3a56* expression is identified. Increased Cyp1a induction in gills relative to liver has been observed for different Ahr ligands, suggesting that gills might be an important bio-indicator tissue (Gao et al., 2011).

In mammals, the effects of NDL PCBs on expression of *Cyp* genes have been linked to the Pxr, and to the related constitutive androstane receptor (Car; NR113) (Gahrs et al., 2013). Teleost fish do not possess a gene for *Car(e.g.* Handschin et al., 2004). In zebrafish Pxr has been implicated in regulation of the expression of *Cyp3a* genes and *Pxr* itself, by a known mammalian Pxr agonist, pregnenolone (Kubota et al., 2015; Salanga et al., 2020). Also in zebrafish, both the Ahr and Pxr are involved in regulation of *Cyp3a65* (Kubota et al., 2015), the ortholog of killifish *Cyp3a56.* The lack of effect of PCB126 on *Cyp3a56* in SC fish gill

suggests that Ahr is not involved in *Cyp3a56* regulation in killifish, at least not in in gills. This hypothesis is supported by the lack of any xenobiotic (dioxin) response elements *(XRE/DRE)* within 2kb of the start site of *Cyp3a56* gene, and only 2 *DRE* sequences in the 5kb *cis* regulatory region, in distinct contrast to zebrafish (Zeruth and Pollenz, 2007). The lack of a clear-cut effect of PCB153 on *Pxr* expression in gills further suggests that the regulation is different from the action of pregnenolone in zebrafish. Additional unpublished data also suggest that *Pxr* is not the primary regulator of transcriptional responses to PCB153 in zebrafish (Goldstone et al., 2019).

As with the organ differences in response to PCB153, the basis for the sex difference showing a stronger PCB effect in females in the F1 SC fish is unknown. However, both PCB153 and PCB126 have been shown to affect hormonal function in other systems *(e.g.* Sechman et al., 2016), and the NBH environment itself has been reported to be estrogenic (Graytak and Callard, 2007) Thus, some hormonal pathway also may be involved in the effect of PCB153 on *Cyp3a56* expression.

There may be other molecular adaptions to the NDL PCBs in NBH fish. Unlike the DL PCBs, the NDL PCBs are not acutely toxic. However, the levels of NDL PCBs in F0 NBH fish (up to 118 000 μg/g dry weight (Fritsch et al., 2015) are as high as those that can activate and severely disrupt ryanodine receptor (RyR) (intracellular calcium channel) function (Holland et al., 2017a) causing toxicity in animals. The fact that the NBH fish are a reproducing population implies that there is adaptation in a variety of other pathways, including some involving the RyRs. Although there are some NBH-SC population differences in RyR pathways (Fritsch et al., 2015; Holland et al., 2017b), we do not expect these to be related to the response in *Cyp3a56* seen in the present study. Identifying the receptors involved in *ortho*-PCB-induced effects and the roles of target genes is essential to a full understanding of *ortho*-PCB toxicity, and the diversity of systems that may be involved.

Biomarker implications

For many years the induction of *Cyp1a* gene expression has been used to indicate exposure to biologically significant levels of Ahr agonists, including the DL PCB congeners. Simple gene expression biomarkers of exposure to NDL PCBs have been lacking. The expression of *Cyp3a56* in gills may be such a biomarker in fish populations that have not developed resistance to PCBs exposures on Ahr-Cyp1a signaling. Induction of *Cyp1a* in gills has long been known (Miller et al., 1989), and the gill tissue has been suggested as a relevant organ for biomarker analysis (Husøy et al., 1994; Jönsson et al., 2004). The gills are good targets for direct exposures in the water as well as exposure by the diet, or even experimentally via injection, as in this study. There could be different cell types involved, although pillar cells (endothelial cells) are likely to be involved, as is the case with Cyp1a (Jönsson et al., 2004; Miller et al., 1989).

There are still unanswered questions about the results here, regarding the sex difference in the responses to PCBs, and the difference between gills and liver. Until the mechanism of *Cyp3a56* induction by the NDL PCB153 is identified, the mechanism of resistance to that effect in killifish from NBH will remain unknown. Further specifics are needed on the regulation of *Cyp3a56* in killifish, addressable in part through genomic comparisons of

the wild-type and PCB-resistant populations. Such questions are to be addressed in further studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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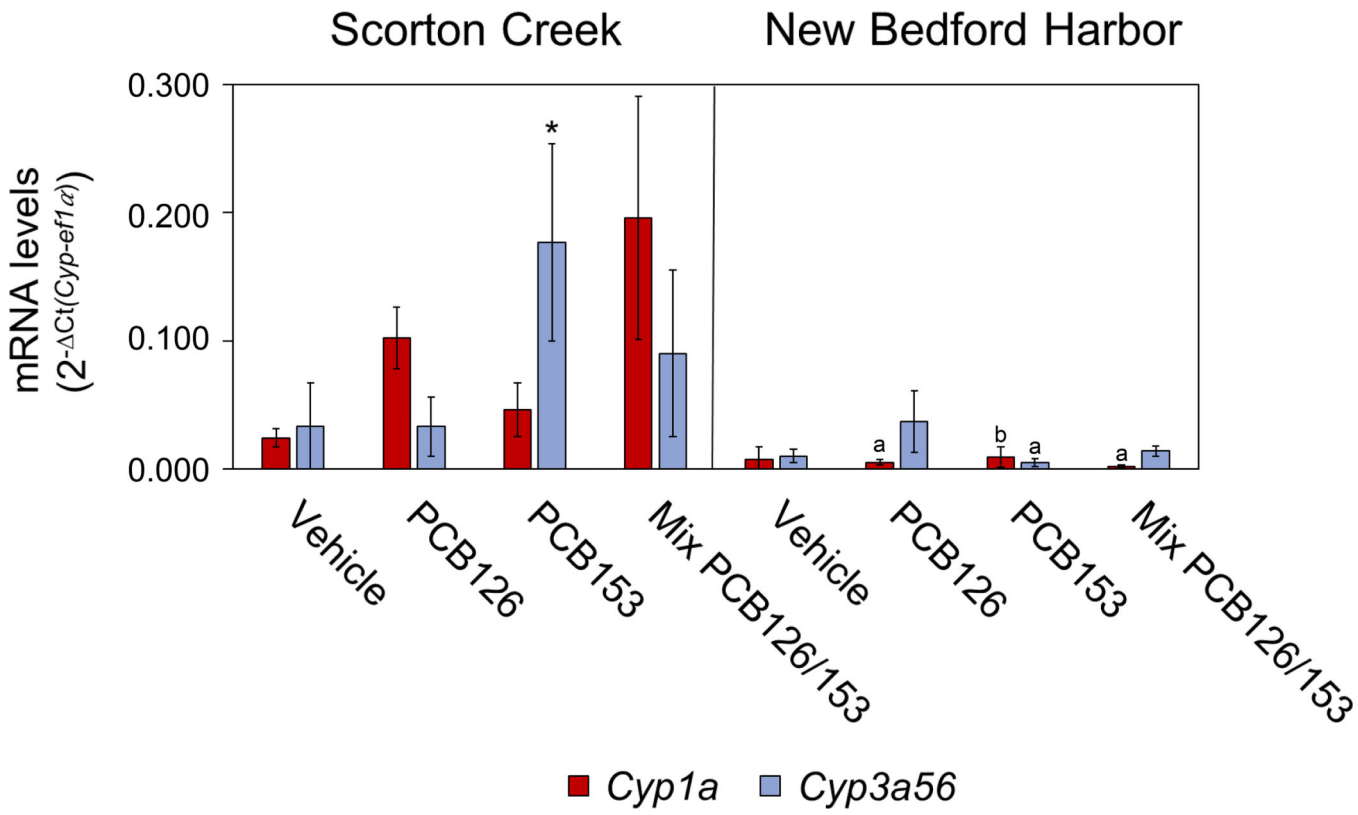

Figure 1.

Cyp1a and *Cyp3a56* mRNA levels in gills in F1 generation female killifish from Scorton Creek population and in F2 generation female fish from the New Bedford Harbor population raised and exposed in the laboratory with PCB126, PCB153 or a mixture of both congeners. Each bar represents the mean of 3 to 5 fish ± standard deviation; * $p < 0.05$ compared to vehicle control in the Scorton Creek population; [a] $p < 0.001$ and [b] $p < 0.05$ population comparisons to the same treatment. The statistics for exposure comparisons within each population are provided in Table S1 and for population comparisons in Table S2. A scatterplot showing *Cyp3a56* mRNA *versus Cyp1a* mRNA in female fish from both populations and the different treatments is provided in Figure S1.

Table 1

Gill mRNA levels from pooled sexes of F1/F2 generation killifish.

Treatment	***Cyp1a*** **Mean** **adj. *p*-value**	**±SD**	***Pxr*** **Mean** **adj. *p*-value**	**±SD**	***Cyp3a56*** **Mean** **adj. *p*-value**	**±SD**
Scorton Creek (SC) population – F1 Generation						
Vehicle (n=8; M:F ratio 5:3)	0.050	0.033	0.003	0.001	0.030	0.019
PCB126 (n=9; M:F-ratio 5:4)	0.275	0.219	0.002	0.001	0.052	0.044
PCB153 (n=11; M:F-ratio 6:5)	0.057	0.029	0.004	0.001	0.105	0.089
Mix PCB126/153 (n=9; M:F-ratio 5:4)	0.571	0.479	0.002	0.001	0.069	0.053
New Bedford Harbor (NBH) population – F2 Generation						
Vehicle (n=10; M:F-ratio 5:5)	0.014 adj. $p = 0.003$ **	0.016	0.006 adj. $p = 0.580$	0.004	0.099 adj. $p > 0.999$	0.197
PCB126 (n=8; M:F-ratio 4:4)	0.005 adj. $p < 0.001$ ***	0.003	0.002 adj. $p = 0.997$	0.001	0.025 adj. $p = 0.850$	0.021
PCB153 (n=10; M:F-ratio 5:5)	0.006 adj. $p < 0.001$ ***	0.006	0.003 adj. $p = 0.843$	0.002	0.014 adj. $p < 0.001$ ***	0.016
Mix PCB126/153 (n=8; M:F-ratio 5:3)	0.011 adj. $p < 0.001$ ***	0.016	0.003 adj. $p = 0.708$	0.001	0.026 adj. $p = 0.473$	0.021

Each value represents the mean ±standard deviation (SD). The number of fish (n) are presented in parenthesis. The male (M) and female (F) ratios are provided for each treatment group. The mRNA data are presented as $2^{-\Delta Ct(target\ gene - ef1\alpha)}$ ±SD, followed by gene-specific two-way ANOVA Tukey adjusted (adj.) *p*-value comparing the two populations of each treatment.

** $p < 0.01$

*** $p < 0.001$.

One female SC vehicle sample, one female SC PCB126 sample and one male NBH PCB126 sample were classified as outliers based on house keeping gene correlation analyses (*ef1α* vs. *Amt2* and *ef1α* vs. *β-actin*, respectively). These three fish are excluded.

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Table 2

Cyp3a56 mRNA levels in gills in F1/F2 generation killifish, separated by sexes.

Treatment	***Cyp3a56* – Males Mean (n) adj. *p*-value**	**±SD**	***Cyp3a56* – Females Mean (n) adj. *p*-value**	**±SD**
Scorton Creek population – F1 Generation				
Vehicle	0.028 (n=5)	0.005	0.033 (n=3)	0.034
PCB126	0.068 (n=5) adj. $p = 0.955$	0.053	0.033 (n=4) adj. $p > 0.999$	0.023
PCB153	0.045 (n=6) adj. $p > 0.999$	0.040	0.177 (n=5) adj. $p = 0.012$ *	0.077
Mix PCB126/153	0.052 (n=5) adj. $p = 0.999$	0.040	0.090 (n=4) adj. $p > 0.435$	0.065
New Bedford Harbor population – F2 Generation				
Vehicle	0.188 (n=5)	0.259	0.010 (n=5)	0.005
PCB126	0.013 (n=4) adj. $p = 0.381$	0.006	0.037 (n=4) adj. $p = 0.143$	0.024
PCB153	0.023 (n=5) adj. $p = 0.724$	0.019	0.005 (n=5) adj. $p = 0.479$	0.003
Mix PCB126/153	0.033 (n=5) adj. $p = 0.857$	0.024	0.014 (n=3) adj. $p > 0.990$	0.002

Each value represents the mean and the mRNA data are presented as $2^{-\Delta Ct(Cyp3a56\text{–}ef1\alpha)}$ ±standard deviation (SD). The number of fish (n) are presented in parenthesis followed by sex-specific two-way ANOVA Tukey adjusted (adj.) *p*-value comparing PCB treatment to vehicle control.

* $p < 0.05$.

Table 3

Liver mRNA levels from pooled sexes of F1/F2 generation killifish.

Treatment	***Cyp1a*** **Mean** **adj. *p*-value**	**±SD**	***Pxr*** **Mean** **adj. *p*-value**	**±SD**	***Cyp3a56*** **Mean** **adj. *p*-value**	**±SD**
Scorton Creek population – F1 Generation						
Vehicle (n=8; M:F-ratio 4:4)	0.047	0.026	0.003	0.001	0.142	0.105
PCB126 (n=8; M:F-ratio 4:4)	0.660 adj. $p = 0.046$ *	0.370	0.004 adj. $p > 0.999$	0.002	0.113 adj. $p = 0.993$	0.108
PCB153 (n=7; M:F-ratio 4:3)	0.060 adj. $p > 0.999$	0.081	0.003 adj. $p > 0.999$	0.002	0.120 adj. $p > 0.999$	0.060
Mix PCB126/153 (n=8; M:F-ratio 5:3)	0.123 adj. $p = 0.837$	0.131	0.005 adj. $p > 0.999$	0.008	0.097 adj. $p = 0.988$	0.060
New Bedford Harbor population – F2 Generation						
Vehicle (n=7; M:F-ratio 3:4)	0.170	0.155	0.014	0.026	0.239	0.192
PCB126 (n=7; M:F-ratio 4:3)	0.074 adj. $p = 0.842$	0.059	0.003 adj. $p = 0.924$	0.002	0.193 adj. $p = 0.908$	0.128
PCB153 (n=8; M:F-ratio 4:4)	0.082 adj. $p = 0.834$	0.090	0.004 adj. $p > 0.999$	0.003	0.196 adj. $p = 0.992$	0.113
Mix PCB126/153 (n=8; M:F-ratio 5:3)	0.071 adj. $p = 0.631$	0.065	0.004 adj. $p = 0.956$	0.005	0.200 adj. $p > 0.999$	0.088

Each value represents the mean ±standard deviation (SD). The number of fish (n) are presented in parenthesis. The male (M) and female (F) ratios are provided for each treatment group. Fish with non-detectable mRNA levels are excluded. The mRNA data are presented as $2^{-\Delta Ct(\textit{target gene}-\textit{ef1a})}$ ±SD, followed by gene-specific two-way ANOVA Tukey adjusted (adj.) *p*-value comparing PCB treatments with vehicle control.

* $p < 0.05$.

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Table 4

Liver PCB126 and PCB153 concentrations in F1/F2 generation killifish.

Treatment	PCB126 Mean	±SD	PCB153 Mean	±SD
Scorton Creek population – F1 Generation				
Vehicle – Males (n=1*)	2.5		2.5	
Vehicle – Females (n=1)	2.5		52.0	
PCB126 – Males (n=5; 3*)	19.1	23.5		
PCB126 – Females (n=5; 2*)	15.4	12.6		
PCB153 – Males (n=6)			1384.4	749.5
PCB153 – Females (n=5)			939.7	344.3
Mix PCB126/153 – Males (n=5; 2*)	17.6	15.8	881.3	318.3
Mix PCB126/153 – Females (n=4)	15.6	3.5	539.4	201.7
New Bedford Harbor population – F2 Generation				
Vehicle – Males (n=1*)	2.5		2.5	
Vehicle – Females (n=1*)	2.5		2.5	
PCB126 – Males (n=5; 2*)	21.9	23.6		
PCB126 – Females (n=4; 3*)	8.3	11.6		
PCB153 – Males (n=5)			653.8	77.6
PCB153 – Females (n=5)			688.0	252.7
Mix PCB126/153 – Males (n=5; 2*)	12.2	9.8	615.4	202.8
Mix PCB126/153 – Females (n=3)	30.7	19.8	1188.4	775.7

Each value represents the mean ±standard deviation (SD) for the number of samples measured (n). The PCB concentrations are presented as ng PCB/g wet liver weight ±SD. For the sake of this calculation, samples below detection limit (n*) were assigned the values of ½ detection limit equal to 2.5 ng/g.