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ESTROGEN RESPONSES IN KILLIFISH (*FUNDULUS HETEROCLITUS*) FROM POLLUTED AND UNPOLLUTED ENVIRONMENTS ARE SITE- AND GENE-SPECIFIC

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Abstract

Epidemiological, ecological, and laboratory-based studies support the hypothesis that endocrine disrupting chemicals (EDCs) in the environment are responsible for developmental and reproductive abnormalities. We have previously described a killifish population resident in a highly polluted Superfund site (New Bedford Harbor, NBH) that shows evidence of exposure to an estrogenic environment and endocrine disruption. Here, we compare NBH with a local reference population (Scorton Creek, SC) for developmental patterns and direct effects of exogenous estradiol on the estrogenic markers, brain cytochrome P450 aromatase (CYP19A2 or AroB), hepatic vitellogenin (Vtg), and hepatic estrogen receptor alpha (ERα). In contrast to our previous observation of elevated ERα in NBH embryos, developmental levels of AroB and Vtg mRNAs did not differ between the two sites, demonstrating that not all estrogen-responsive genes are upregulated in NBH embryos. A dose-response experiment showed that NBH larvae are less responsive (lower maximum induction, as measured by ERα) and less sensitive (higher EC50 for induction, as measured by AroB) to estradiol than SC larvae, changes that would be adaptive in an estrogenic environment. In contrast, induction of Vtg mRNA is similar in the two populations, indicating that the adaptive mechanism is target gene-specific. Based on the lower basal levels of ERα mRNA in several tissues from adult NBH fish vs SC fish (Greytak and Callard, 2007), we predicted estrogen hyporesponsiveness; however, induction of ERα by estradiol exposure in reproductively inactive males did not differ between the two sites. Moreover, AroB was more responsive and Vtg induction was greater (2d) or similar (5d) in NBH as compared to SC males. Worth noting is the high inter-individual variability in estrogen responses of gene targets, especially in NBH killifish, which may indicate evolving preadaptive or adaptive mechanisms. In conclusion, although multi-generational exposure to a highly polluted environment is associated with changes in basal levels of ERα mRNA, this is not a simple predictor of estrogen responsiveness. We hypothesize that adaptation of killifish to the estrogenic and polluted environment may be occurring through diverse mechanisms that are gene-, tissue type- and life-stage specific.

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Keywords

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1. INTRODUCTION

Compounds released into the environment by anthropogenic activity have been shown to disrupt critical hormonally regulated processes of reproduction and development (Phillips and Harrison, 1999). Endocrine disruption has generated increasing public concern because reproductive and physiological effects have been observed in both humans and wildlife, including lowered fertility (Foote, 1999; Gray et al., 1997a; Gray et al., 1997b; Wolf et al., 1999), precocious (Honma et al., 2002) or delayed puberty (Faqi et al., 1998), skewed sex ratios (Bergeron et al., 1994; Saidapur et al., 2001; Willingham and Crews, 1999), gonadal abnormalities (Cook et al., 2003; Roos et al., 2001), and population decline (Cook et al., 2003; Roos et al., 2001). Laboratory studies indicate that long-term exposure to endocrine disrupting chemicals (EDCs) results in an overall desensitization through several different mechanisms, such as transcriptional or post-translational downregulation of receptors (Saceda et al., 1988), a change in steroid clearance rates (Edmunds et al., 1990; Loukovaara et al., 1995; Toscano et al., 1992), and/or an increase or decrease in co-activators and repressors (reviewed in (Shibata et al., 1997)). Further, at least some effects of EDC can be transmitted to successive generations by epigenetic mechanisms (Anway and Skinner, 2008; Chang et al., 2006). It remains unclear whether adaptations that confer desensitization, resistance or tolerance are acquired after EDC exposure in the natural environment and, if so, what mechanisms are involved.

To address these questions, we are comparing two populations of Atlantic killifish (*Fundulus heteroclitus*): one inhabiting a highly polluted Superfund site (New Bedford Harbor, MA: NBH), and another from a reference site (Scorton Creek, MA; SC). From the 1940's until 1978, two capacitor manufacturing facilities in New Bedford discharged PCBs and heavy metals both directly into the harbor and through the city's sewer system (Weaver, 1983). An EPA investigation of the harbor in 1976 found 18,000 acres of PCB contamination with concentrations in sediment in some areas exceeding 100,000 ppm (Weaver, 1983). Indeed, NBH is one of the most extensively PCB contaminated sites in the country (Weaver, 1983). Because of this, the NBH killifish population has been exposed to high levels of polychlorinated biphenyls (PCBs) for >50 yr (~20 generations) (Weaver, 1983). PCBs are a diverse class of environmentally persistent pollutants that have been linked to endocrine disruption by several different mechanisms. Through their interaction with the ligand binding pocket of estrogen receptors (ER), certain PCB congeners or their in vivo metabolites have the potential to directly activate or block transcription of estrogen responsive genes required for normal reproduction and development (Layton et al., 2002; Lind et al., 1999; Rankouhi et al., 2004). Consistent with this, a recent study reported estrogenicity of PCBs found in air, soil, dust and human tissues from the PCB- contaminated NBH environment (DeCastro et al., 2006). Other PCB congeners are strong ligands for the aryl hydrocarbon receptor (AHR). Activation of AHR not only mediates the toxic and biochemical effects of PCBs but also can disrupt estrogen signaling by altering levels of estrogen receptor (ER) mRNA (Dasmahapatra et al., 2001; Tian et al., 1998a; Tian et al., 1998b) or protein (Harris et al., 1990; Ohtake et al., 2007; Wang et al., 1993; Wormke et al., 2003); competitively inhibiting ERα binding to estrogen response elements (EREs) (Klinge et al., 1999); interacting with unbound ER protein to potentiate transcription through EREs (Ohtake et al., 2003); or attenuating the function of ligand bound ER (Ohtake et al., 2003).

Despite high liver burdens of PCBs in NBH fish (809 μg/g liver dry weight) relative to SC fish (1.11 μg/g liver dry weight)(Bello, 1999), killifish continue to survive and reproduce in the NBH environment. The survival of the NBH population has been ascribed to adaptations in the AHR signaling pathway leading to resistance and/or tolerance to dioxins and dioxin-like PCBs, as measured by toxicity and induction of cytochrome P450 1a1 (cyp1a) (Bello et al., 2001; Hahn et al., 2004; Nacci et al., 1999). By analogy to changes in AHR-mediated toxic effects, we hypothesized that long term, multigenerational exposure to NBH pollutants could attenuate the endocrine disrupting effects of environmental pollutants by changes in ER mediated signaling pathways.

Previously, we compared basic reproductive parameters and gene expression in males and females in the NBH and SC killifish populations throughout the annual reproductive cycle (Greytak and Callard, 2007; Greytak et al., 2005). As measured by elevated mRNA levels of two markers of estrogen effect, hepatic vitellogenin (Vtg) (Teo et al., 1998) and the predominant form of aromatase in the brain (CYP19A2, referred to here as AroB) (Kishida et al., 2001; Sawyer et al., 2006) in seasonally inactive fish (when endogenous estrogen is low), NBH is an “estrogenic” environment (Greytak and Callard, 2007; Greytak et al., 2005). However, ERα mRNA, another marker of estrogen exposure and effect in teleosts (Menuet et al., 2004; Menuet et al., 2005; Urushitani et al., 2003), was not similarly elevated in reproductively inactive fish from NBH. Moreover, during the reproductive season, when ERα transcript increases dramatically in the liver, brain, and gonads of SC males and females, ERα transcripts did not increase to the same extent in NBH fish. ERα not only is a critical mediator of hormonal estrogen signaling but also is part of an estrogen-regulated autoregulatory loop that drives ever-increasing expression of ERα and downstream gene targets such as Vtg (Menuet et al., 2004; Urushitani et al., 2003). This estrogen-regulated autoregulatory loop is thought to be especially important at the transition from non-breeding to breeding condition in teleost fish. Based on our observations, we postulated that attenuation of the ERα autoregulatory loop in adult fish is an adaptive response that mitigates the endocrine disrupting effects of environmental estrogen exposure in NBH killifish (Greytak and Callard, 2007).

In contrast to our observations with adult fish, however, we found that ERα was more highly expressed in embryonic and juvenile killifish from NBH relative to those from SC (Greytak and Callard, 2007). A possible interpretation is that high ERα expression in progeny of NBH is due to exposure to estrogenic NBH contaminants accumulated in yolk, as previously observed in embryos derived from fish exposed to steroids (Piferrer and Donaldson, 1994; Yeoh et al., 1996). If so, this implies that the ERα autoregulatory loop is operative, and would be predicted to lead to increased expression of other ERα gene targets. Such activation would be maladaptive in an estrogenic environment, especially during vulnerable early life stages.

In this study, we investigated the functioning of the estrogen signaling system in killifish from NBH and SC. Like other teleosts, killifish have three estrogen receptors (ERα, ERβa, ERβb) but only ERa is estrogen responsive (Greytak and Callard, 2007; Urushitani et al., 2003) To examine whether increased expression of ERα in progeny of NBH fish was associated with widespread alterations in estrogen-dependent signaling, we measured developmental changes in expression of additional markers of estrogen exposure (Vtg, AroB). We then directly tested the effects of estradiol exposure on gene expression in juvenile and adult fish from NBH and SC. Together these studies demonstrate that estrogen sensitivity and responsiveness are altered after multigenerational exposure to EDCs but the changes are complex and differ between genes, tissues and life stages.

2. MATERIALS AND METHODS

2.1 Collection Sites

NBH is located on Buzzard's Bay approximately 55 miles south of Boston. Killifish near our NBH collection site are reported to have total PCB concentrations of 809 μg/g liver dry weight (Bello, 1999). SC is located on Cape Cod Bay in Sandwich, MA approximately 30 miles northeast of NBH and in the same climatic zone. SC is relatively free of PCBs and heavy metal contamination (liver PCB burdens of 1.11 μg/g dry weight) (Bello, 1999), and has previously been used as a reference location for killifish research (Bello et al., 2001; Greytak and Callard, 2007; Greytak et al., 2005; Hahn et al., 2004).

2.2 Collection and care of eggs, embryos and larvae (Expt. 1 – 3)

Eggs, embryos and larvae were obtained as previously described (Greytak and Callard, 2007). Briefly, age- and size-matched adult male and female killifish were collected in May from SC and NBH in collaboration with the US EPA National Health and Environmental Effects Laboratory, Atlantic Ecology Division, Narragansett RI. At this time of year killifish at both sites are reproductively active as indicated by gonadosomatic index, hepatosomatic index, gonadal histology, plasma sex steroids and molecular markers (Greytak et al., 2005). Twelve gravid females from each site were immediately stripped to obtain unfertilized eggs, which were quick-frozen at − 70 °C until analysis. The remainder of the adult fish from each site were kept in separate flow-through tanks at ambient temperature and light cycles at the National Health and Environmental Research Laboratory (Atlantic Ecology Division, US EPA), as previously described (Nacci et al., 1999). The fish were fed three times daily with a diet consisting of Tetramin® fish flakes, and Biodry® 1000 pellet food (Bioproducts, Warrington, Oregon) supplemented with krill and *Artemia* sp. Embryos were obtained by natural spawning at the time of the new or full moon (within 2 weeks (Expt. 1) or 6 weeks (Expts. 2 and 3) of capture). This time frame is significantly less than the reported half-life of PCBs in teleosts (79–182 days) (Buckman et al., 2004). Although PCB levels likely varied between embryos from adults that were freshly collected or held in the lab for 6 weeks, this difference did not affect the endpoints of interest (Greytak and Callard, 2007). Fertilized eggs were maintained in Petri dishes in 35 ppt seawater 22–23° C on a rotary shaker until hatch (~10–12 days post fertilization (dpf) under these conditions). Post-hatch larvae were transferred to glass beakers containing 30 ml of 35 ppt seawater and fed *Artemia* sp. daily. Experimental treatments are described in Results and Figure and Table legends. Water and estradiol were replaced daily, and any dead or deformed embryos were removed. Mortality was no greater than one per group. Embryos and larvae were killed by quick freezing on dry ice and stored at −70°C until processing.

To determine developmental expression profiles of estrogen-responsive (AroB, Vtg) and nonresponsive (CYP19A1, referred to here as AroA) mRNAs at each site (Expt. 1), fertilized eggs from SC and NBH killifish were allowed to develop in beakers (25 per 50 ml beaker) and collected at timed intervals between 0 and 18 dpf (3 replicate pools per site and time point). These were banked samples used previously to measure ERα (Greytak and Callard, 2007). To determine the ontogeny of estrogen responsiveness of AroB and Vtg, (Expt. 2), we used fertilized eggs from SC killifish. Three replicate pools, each with 8 – 10 individuals per beaker, were exposed to vehicle alone (0.001 % DMSO) or to low or high doses of 17-β estradiol (E2, Sigma St Louis, MO; 0.1 or 1.0 μM, final concentration) between 0 and 10 dpf or between 10 and 18 dpf, and sampled at 10 dpf or 18 dpf, respectively. The doses chosen reflect standard doses used in embryonic fish (Menuet et al., 2005; Sawyer et al., 2006). To determine site-related differences in dose-response characteristics of three different estrogen-responsive genes (Expt. 3), larvae from SC and NBH killifish were housed individually in beakers and exposed to vehicle alone or to one of

six different concentrations of E2 (0.001 – 0. 3 μM) (n = 6 per treatment group and site) from 15 – 18 dpf, and then sampled for isolation of RNA (section 2.4).

2.3 Collection and care of adult males (Expt. 4)

Twelve (SC) or 24 (NBH) adult male killifish were collected in November 2003 using minnow traps. Killifish are reproductively regressed at these sites in November, as indicated by molecular and physiological parameters (Greytak et al., 2005). Fish from each site were maintained separately at ambient temperature and light cycles in recirculating seawater tanks at the Woods Hole Oceanographic Institution (Woods Hole, MA) and fed pellet food daily. After 7 d acclimation, fish from each group were injected intraperitoneally with E2 (5 mg/kg body weight; 1 μg/μl sesame oil) or vehicle alone. The dose of E2 was chosen based on previously published studies in killifish (Pait and Nelson, 2003; Urushitani et al., 2003) including our previous work (Tarrant et al., 2006). The concentration and method of exposure are predicted to produce a high spike in plasma estradiol concentration that is cleared rapidly (Pankhurst et al., 1986), causes a robust induction of vitellogenesis in male killifish, and is just below the EC_{50} of E2 (Pait and Nelson, 2003). Two or five days post-injection, fish were anesthetized with 0.06% ethyl 3-aminobenzoate methanesulfonate salt (MS-222), and then decapitated. Brains, livers, eyes and testes were immediately flash-frozen and stored at −70°C.

2.4 RNA Extraction and Reverse Transcription

Processing and analysis of mRNA has been described previously (Greytak and Callard, 2007; Greytak et al., 2005). In brief, RNA was extracted from frozen pooled embryos, individual larvae, or adult tissues using Trizol (Sigma Aldrich, St. Louis, MO) and treated with DNAseI (Roche Diagnostics, Indianapolis, IN). RNA quality was visualized using gel electrophoresis, and yield was determined spectrophotometrically. cDNA was synthesized from 3 μg of total RNA using oligo(dT) primers (Promega) and SuperScriptII reverse transcriptase (Invitrogen Corp.), according to the manufacturer's instructions, and diluted 5-fold before analysis.

2.5 Real-time quantitative (Q)PCR analysis

Real-time PCR was performed on an ABI PRISM 7900 (Applied Biosystems) instrument using real time PCR primers and conditions described previously (Greytak and Callard, 2007; Greytak et al., 2005). Primer sets were designed to amplify all alternative transcripts identified in our previous studies (Greytak and Callard, 2007; Greytak et al., 2005). Reactions were set up such that all samples for analysis of target and control mRNAs from both sites were on the same plate. The final reaction contained 50% SYBR Green Master Mix (Applied Biosystems), 1 μM of each primer (0.5 μM for β-actin, 0.25 nM for Vtg), and separate aliquots (3%) of cDNA. Amplification was performed under default conditions with the addition of a denaturing step to confirm the presence of a single amplicon. Data were analyzed using the Q-Gene package, a publicly available Excel script package (Simon, 2003). Cycle threshold (Ct) values were adjusted for differences in the amplification efficiency (E) of each primer set/gene target where $E = 10^{(-1/\text{slope})}$ as determined by linear regression analysis of a dilution series of reactions (expressed as percent input cDNA), and normalized to β-actin as an internal reference ($NE = (E_{\text{ref}})^{Ctref} / (E_{\text{target}})^{Cttarget}$).

2.6 Statistics and data analysis

Statistical analysis was performed using the SigmaStat 2.0 package (Jandell Scientific). See figure legends for details. Significance was set at $p = <0.05$. EC_{50} values were obtained using CurveExpert v.1.3 (Hyams, 2005).

3. RESULTS

3.1 Developmental patterns of expression of estrogen responsive (Vtg, AroB) and nonresponsive (AroA) genes in NBH and SC killifish (Expt. 1; Fig. 1)

To test the possibility that high expression of ERα (an estrogen inducible gene in teleosts) is due to the presence of maternally-transmitted estrogenic contaminants in yolk of NBH fish, we utilized the same samples as in our previous study (Greytak and Callard, 2007) and real-time PCR to compare the developmental profiles of two additional estrogen responsive genes (AroB and Vtg) and an estrogen unresponsive gene (AroA) as control (Fig. 1). All three mRNAs were detected in unfertilized eggs from both sites. Although steady-state levels at 0 hpf were gene-specific (Vtg > AroA ⋙ AroB) ($p = <0.001$; $F = 596.76$), no site–related differences were observed ($p = 0.700$; $F = 0.156$) (Fig. 1). During the first 24 hr of development, all mRNA levels decreased markedly, signifying degradation of maternally transcribed mRNA; however, the degradation rate of AroA was greater (2500-fold) than that of AroB and Vtg (10- and 60-fold, respectively).

Over the course of development, expression of AroA, AroB and Vtg mRNAs was significantly stage-dependent ($p = <0.001$; $F = 49.223$; $p = <0.001$; $F = 11.280$; $p = <0.001$; $F = 20.640$ respectively) but not site-related ($p = 0.338$; $F = 0.941$; $p = 0.909$; $F = 0.0132$; and $p = 0.444$; $F = 0.600$ respectively)(Fig. 1). Indicative of embryonic transcription, AroB increased between 1 and 2 dpf, but Vtg accumulation did not begin until 4 dpf, possibly due to the later development of liver (~ 4 dpf) as compared to brain (~ 2 dpf). Between 2 and 12 dpf (time of hatching), AroB and Vtg levels increased progressively (300- and 40-fold respectively) and then reached a plateau through the larval stages. By contrast, the developmental increase in AroA mRNA was modest (4-fold between 2 and 18 dpf).

3.2 Ontogeny of the estrogen response system in SC killifish (Expt. 2; Fig. 2)

To rule out the possibility that AroB and Vtg do not normally acquire estrogen regulation before 18 dpf, we tested effects of E2 at two concentrations (0.1 and 1.0 μM) in SC embryos (0 – 10 dpf) and late embryos/ early larvae (10 – 18 dpf). As shown in Fig. 2, one-way ANOVA revealed a significant effect of high but not low dose E2 on AroB and Vtg ($p = 0.009$; $F = 11.243$; $p = P = 0.029$; $F = 6.797$ respectively) in embryos, but the inductive response was weak (~5 and 4 fold, respectively). Later in development (10 – 18 dpf), both low and high dose E2 treatment robustly increased AroB ($p = 0.013$; $F = 6.412$) and Vtg ($p = <0.001$; $F = 21.206$) levels (>50- and ~1000-fold, respectively). AroA was not estrogen responsive early or late in development ($p = 0.873$; $F = 0.139$; $p = 0.722$; $F = 0.335$, respectively.

3.3 Gene- and site-specific dose-response characteristics of estrogen inducible genes (Expt.3; Fig. 3; Table S1)

Real-time PCR was used to measure mRNA levels of three estrogen-responsive genes (Vtg, AroB and ERα) and three estrogen non-responsive genes (AroA, ERβa and ERβb) in posthatch larvae (18 dpf) exposed from 15 – 18 dpf to a range of E2 concentrations (0.001 – 0. 3 μM). Test parameters were based on results of Expt. 2. As shown in Fig. 3A, two-way ANOVA revealed that Vtg induction was dose-dependent ($p = <0.001$; $F = 31.816$) but not site- related ($p = 0.978$; $F = 0.000768$). Maximal Vtg induction was obtained with 0.01μM E2 and then reached a plateau at a level that was 1000-fold greater than vehicle treated controls. When expressed levels of AroB and ERα were rank transformed and analyzed by two-way ANOVA, significant dose-related effects ($p = <0.001$; $F = 17.940$ and $p = <0.001$; $F = 11.386$ respectively) and site-related effects ($p = 0.006$; $F = 8.01$; and $p = < 0.001$; $F = 28.646$ respectively) were observed (Fig. 3B and C). Compared to the Vtg response of 1000-fold, the maximal response to E2 was lower for AroB (~100-fold) and ERα (<15-fold). Whereas

the maximal AroB response was the same in NBH and SC larvae, the dose-response curve was right-shifted in NBH fish, suggesting reduced E2 sensitivity. By contrast, the ERα gene was significantly less responsive to E2 in NBH than SC larvae at all E2 concentrations, but the sensitivity of the response, as measured by calculated EC_{50} values, did not differ in larvae from the two populations. Mean levels of ERα mRNA in vehicle treated controls were ~5-fold higher in NBH as compared to SC larvae, but significance was not achieved in the present study due to high variance.

As expected, expression of mRNAs of estrogen non-responsive genes (AroA, ERβa, and ERβb) were found by two-way ANOVA on rank transformed data to be unaffected by treatment (p= 0.953; F= 0.261; p=0.603; F= 0.761; p=0.272; F=1.295; and P= 0.643; F= 0.709, respectively) and collection site (p= 0.877; F= 0.0241; p= 0.538; F= 0.384; p= 0. 272; F= 1.225; and p= 0.654; F= 0.203, respectively) (Table S1).

3.4 Estrogen responses in reproductively inactive males from SC and NBH (Expt. 4; Fig. 4)

To assess possible site-related differences in the estrogen response system of adult killifish, we collected reproductively inactive males from the two sites and measured mRNA levels of estrogen responsive and nonresponsive mRNAs in brain, liver, testis, and eye at two and five days after administration of a single dose of E2 (5 mg/kg) or vehicle alone. Results of real time PCR confirmed our previous findings with newly collected males (Greytak et al., 2005) by showing that hepatic Vtg mRNA is higher in vehicle-injected NBH fish as compared to SC fish, even after 9 days (2 days post-injection) in the laboratory. However, after 12 days (5 days post-injection), Vtg did not differ significantly in vehicle-injected NBH and SC fish, suggesting depuration of estrogenic compounds. In contrast to Vtg, hepatic ERα and AroB levels did not differ in vehicle controls from the two sites at either 2 or 5 d postinjection.

As determined by two-way ANOVA, hepatic Vtg levels 2 days post injection were significantly affected by E2 treatment (p= <0.001; F= 52.578) and site (p= 0.025; F= 6.3) (Fig. 4A). Although Vtg was induced by E2 in both populations, induced levels were 2-fold higher in NBH fish as compared to SC fish, although the fold-increase was greater in SC fish than in NBH fish (respectively, >100 vs ~13-fold), due mainly to much lower baseline levels in vehicle-injected SC controls. As determined by two-way ANOVA, hepatic Vtg levels 5 days post-injection were significantly affected by treatment (p= <0.001; F= 26.001) but not site (p= 0.279; F=1.249) (Fig. 4B). By 5 days post-injection baseline Vtg mRNA levels did not differ between control SC and NBH fish, nor was there a difference in the fold-response (~ 2,500 fold) to E2. There was a high degree of variability in the estrogen response of the NBH fish as measured by each of the three marker genes, which resulted in low statistical power.

AroB expression was significantly higher in the brains of estrogen-treated fish relative to vehicle control, but site had no effect (Figure 4C, two way ANOVA on rank-transformed data, treatment p= <0.001; F= 49.77, site p = 0.086; F= 3.462). Two days after E2 injection, brain AroB expression increased 2 – 3-fold in both populations. Five days post-injection AroB expression in brain was significantly affected both by treatment (p= <0.001; F=17.844) and site (p= 0.021; F= 6.599) (Figure 4D, two-way ANOVA on rank-transformed data). Whereas the SC killifish showed only a 1.5 fold increase in AroB, the NBH killifish showed a mean 6-fold increase.

Two-way ANOVA of ERα levels in liver two days post-injection revealed treatment (p=0.007; F=10.15) but not site-related (p=0.912; F= 0.0126) differences (Fig. 4E). When the data were further analyzed by Tukey test, it was found that E2 induced a small but significant increase in ERα levels in SC fish (p=<0.05) but effects of E2 in NBH fish were not significant. By five day post-injection, significant effects of treatment (p=0.006;

F=9.823) but not site (p=0.606; F= 0.279) were found by two-way ANOVA. In contrast to results two-day post injection, however, E2 had a significant effect in fish from both sites.

Further, comparison of Fig. 3 and 4 shows that adult fish did not respond as robustly as larvae to the E2 treatment. As expected, levels of the estrogen non-responsive markers, ERβa, and ERβb in brain, liver, testis, and eye were not significantly affected by treatment (p=>0.05), nor were there site-dependent differences (p=>0.05) (Table 1).

4. DISCUSSION

Despite exposure to dioxin- and estrogen-like chemicals in the NBH environment, and evidence of an impacted hypothalamic-pituitary-gonadal axis (e.g., decreased gonadosomatic index, hepatosomatic index, plasma sex steroids), the killifish resident in NBH continue to display normal seasonal and semilunar cyclicity, spawning behavior and production of viable embryos (Black et al., 1998; Greytak and Callard, 2007; Greytak et al., 2005). Survival of individual killifish in NBH has been ascribed to an evolved resistance to the toxic effects of chemicals that activate AhR-mediated signal transduction (Bello et al., 2001; Nacci et al., 1999). By analogy to adaptations in AhR signaling, we hypothesized that adaptations in reproductively relevant ER-mediated signaling pathways can explain the continued reproductive success of the population. In an earlier study, we described site-related differences in expression of ERα (but not ERβa or ERβb) in embryos and in different tissues of reproductively active and inactive males and females (Greytak and Callard, 2007). Here we report differences in the functionality of the estrogen response system in the two populations, but the differences are complex. Estrogen responses in the two populations differed between larvae and adults, and the underlying mechanism cannot be explained by differences in expressed levels of ERα alone.

Based on dramatically elevated expression of ERα mRNA throughout the development of embryos and larvae from NBH, we initially predicted estrogen hyperresponsiveness, which would be maladaptive in an estrogenic environment. However, detailed dose-response experiments with estradiol show that NBH larvae are less responsive (as measured by ERα), less sensitive (as measured by AroB) or not different (as measured by Vtg) than SC larvae. One possible explanation for decreased ERα responsiveness in the face of increased ERα mRNA is that a substantial fraction of the ERα transcripts may be untranslatable, have low translation efficiency, or encode non-functional proteins. Killifish ER antibodies are not currently available to test this idea directly; however, we previously identified a number of alternatively spliced transcripts and polymorphisms among the three ER subtypes (Greytak and Callard, 2007). Similar ER variants in humans are produced in a tissue-specific manner (Herynk and Fuqua, 2004; Ogawa et al., 1998) and, when coexpressed with functional ERα, heterodimerize and act as dominant negatives (Ogawa et al., 1998). The killifish variant forms, if differentially expressed between populations, may act similarly to those in human and attenuate (ERα, AroB) or neutralize (Vtg) responses to administered or environmental estrogen. The role of environmental chemicals in regulating splicing decisions is an important but unexplored question.

Although alternative exon splicing is one plausible mechanism to explain discordance between ERα expression and estrogen responsiveness in killifish larvae, possible causal factors are still unclear. Initially, we ascribed elevated levels of ERα in NBH embryos/larvae to ER activation caused by estrogenic contaminants present in yolk, but measurement of additional estrogen markers (AroB and Vtg) did not confirm this hypothesis. As both AroB and Vtg are estrogen-responsive in killifish beginning as early as 10 dpf, the lack of a difference in mRNAs of these genes when the two populations are compared indicates that the elevated level of ERα mRNA observed in NBH embryos might not be the direct result of

estrogenic contaminants. An alternative explanation is that the elevated levels of ERα could reflect a loss of inhibition. In mice, AHR transcriptionally represses ERα (Tian et al., 1998b). As previous studies have established that there are changes in the AHR signaling pathway in the NBH killifish, measured by resistance to toxicity and induction of cyp1a (Bello et al., 2001; Nacci et al., 1999; Powell et al., 2000), perhaps the change in basal ERα levels in larval killifish reflects de-repression resulting from defective AHR signaling.

Similar to observations in embryos/larvae, expressed levels of ERα mRNAs in tissues of adult fish do not reliably predict differences in estrogen responsiveness between populations. Based on low levels of ERα mRNA in several different tissue types of NBH males or females and/or times of year when compared to SC controls, we predicted estrogen hyporesponsiveness, which would be adaptive in a high estrogen environment (Greytak and Callard, 2007). Despite a high degree of inter-individual variability for all three marker genes after estradiol treatment, especially in NBH fish, some differences between the populations were found. When adult males were treated with a single submaximal dose of estradiol, responses either did not differ in the two populations (as measured by ERα) or occurred sooner (as measured by Vtg) or were greater in magnitude (as measured by AroB) in NBH fish than in SC fish. To some extent, results in NBH fish could be confounded by prior exposure of these fish to environmental estrogens, as pretreatment with estrogen is known to advance or enhance effects of subsequent exposure, due to either “transcriptional memory” or to stabilization of mRNA (Baker and Shapiro, 1978; Edinger et al., 1997). The environmental estrogens could include estrogenic PCBs or PCB metabolites as well as other established xenoestrogens such as alkyl phenol ethoxylates and their derivatives. Additionally, outflow from wastewater is the presumed source of several natural and synthetic estrogens which have been identified in NBH (Zuo et al., 2006).

In conclusion, these results demonstrate that the functionality of the estrogen response system differs in larvae from polluted and reference environments. The main effect is seen as a loss of the autoregulatory feedback loop that enhances ERα expression, rather than a change in all estrogen responsive genes. Attenuation of a mechanism that normally would advance and amplify estrogenic responses in the ERα -regulated gene network could be adaptive in a high estrogen environment and especially important during critical stages of development. Although we can conclude that the elevated ERα levels in embryos and larvae are unlikely to be due solely to estrogenic contaminants in the yolk, the functional significance of this mRNA pool and the underlying mechanism of ERα overexpression are important unresolved questions. In contrast to the condition of acquired resistance in the AhR signaling pathway in NBH and other polluted environment, which generally affects all tissue types and downstream gene targets and is heritable (Bello, 1999; Bello et al., 2001; Nacci et al., 1999), it is more likely that this change in estrogen signaling is gene-specific and regulated through complex mechanisms such as changes in abundance of alternate transcripts and in transcript turnover, although genetic and epigenetic changes in coding or noncoding regions of the genome due to multigenerational selection pressures cannot be ruled out.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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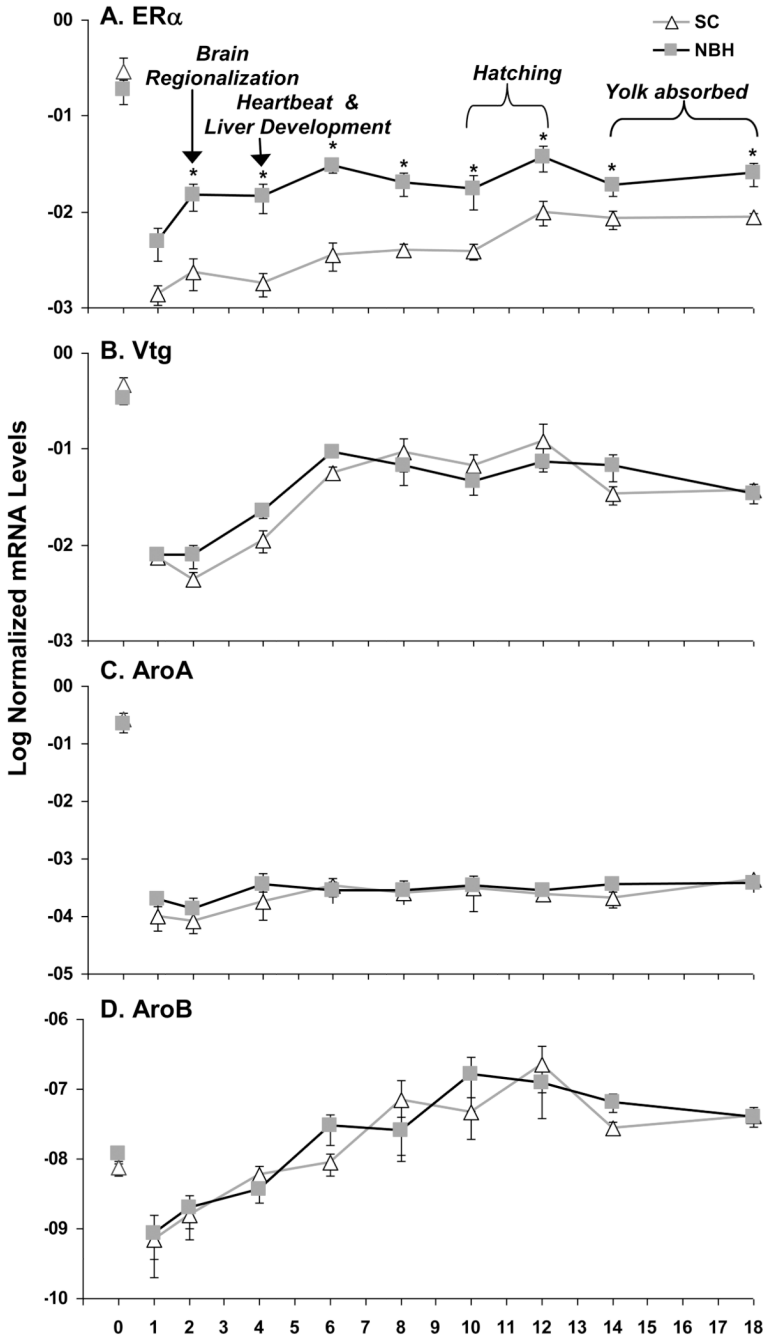

Figure 1.

Developmental patterns of expression of estrogen responsive (ERα1, Vtg, AroB) and non-responsive (AroA) mRNAs in NBH (closed symbols) and SC (open symbols) killifish (Expt. 1). Size-matched reproductively active males and females were collected from both sites (see Methods). Unfertilized eggs (0 dpf) were obtained by stripping gravid females. Fertilized eggs were obtained by group spawning of SC and NBH fish, maintained in

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seawater, and collected at timed intervals during development (1 – 18 dpf). Established developmental landmarks are indicated at the top of the figure (Oppenheimer 1937; Armstrong and Child, 1965). For each RNA extract, 25 eggs, embryos or larvae were pooled and analyzed by real-time PCR. Each data point represents β-actin normalized mRNA levels +/− SEM of three independent biological replicates. For each mRNA species, data were analyzed by two-way ANOVA for stage of development and collection site. Significant effects of site ($p<0.05$) were only observed for ERα For a given mRNA and stage, a significant difference between NBH and SC killifish was determined by Student's paired t-test and is indicated by an asterisk (*). Developmental profiles of ERα, - βa and - βb expression in the same killifish samples have been reported previously (Greytak and Callard, 2007).

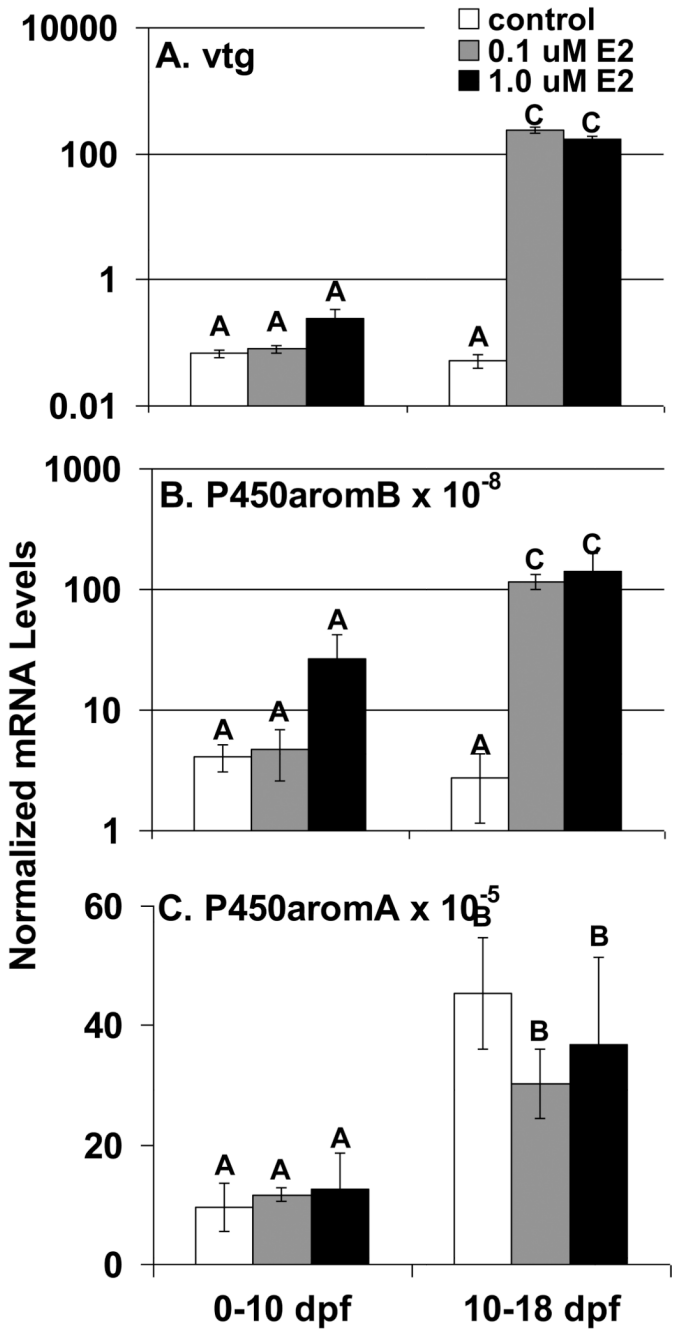

Figure 2.

Ontogeny of estrogen responsiveness as measured by Vtg and AroB induction in SC embryos/larvae (Expt. 2). Groups of 8–10 embryos (0–10 dpf) or late embryos/ early larvae (10–18 dpf) were exposed to vehicle alone (0.001% DMSO; clear bar) or 0.1 or 1.0 μM estradiol (grey and black bars, respectively). Each bar represents β-actin normalized mRNA levels +/− SEM as determined by real-time PCR analysis of three embryo/larval pools, each with of 8–10 individuals. When data were rank transformed and analyzed by two-way ANOVA with the Tukey test for pairwise comparisons, significant effects of treatment and developmental stage on expression of Vtg and P450aroB, but not AroA, were found ($p < 0.05$). Note differences in the scale of the y-axes of panels.

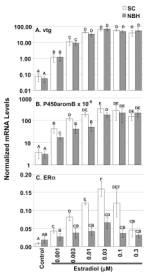

Figure 3.

Dose-related characteristics of the estrogen response of larval killifish from SC and NBH, as determined by real time PCR analysis of (a) Vtg; (b) AroB; and (c) ERα mRNAs. Fertilized eggs were obtained by natural spawning of adult killifish from SC (clear bars) and NBH (shaded bars) and allowed to develop until hatching. Between 15 and 18 dpf, larvae were housed individually and exposed to vehicle alone (0.001% DMSO) or one of a range of E2 concentrations (0.001 – 0.3μM). Each bar represents β-actin normalized mRNA levels +/− SEM of six embryos. Data were analyzed separately for each mRNA by two-way ANOVA for effects of treatment and site, and significance was determined by Tukey test (see Results). Bars with different letters differed significantly ($p < 0.05$). The calculated EC_{50} values for NBH and SC killifish were, respectively: Vtg (~0.0069 μM and ~0.0075 μM), AroB (~0.0072 and ~0.019 μM), and ERα (~0.0044 and ~0.0120 μM). Results are representative of three independent experiments. Note that values in panels A and B, but not C, are shown on a log scale. Estrogen non-responsive mRNAs (AroA; ERβa, and ERβb) were measured as treatment controls (see Table S1).

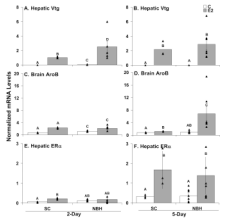

Figure 4.

Estrogen responses of reproductively inactive male killifish from SC and NBH, as determined by real time PCR analysis of (a, b) hepatic Vtg; (c, d) brain AroB; and (e, f) hepatic ERα mRNAs. Male killifish were collected at each site during the period of seasonal reproductive regression (November). After 7 d acclimation, they were given a single intraperitoneal injection of 5 mg/kg estradiol (E2, shaded bars) in sesame oil or vehicle alone (C, clear bars). Two (a, c, e) or 5 (b, d, f) days post-injection, tissues were collected for mRNA analysis. Each bar represents β-actin normalized mRNA levels +/− SEM of 3 (SC) or 6–7 (NBH) individuals. Individual data points are indicated by black triangles to show range and variability of responses. Data were rank transformed and analyzed separately for each mRNA species by two-way ANOVA for effects of treatment and site. As determined by Tukey's test, bars with different letters differed significantly ($p < 0.05$). Note differences in the scale of the y-axes of each panel. See Table 1 for levels of estrogen non-responsive mRNAs.

Table 1

Real time PCR analysis of ERα, -βa and -βb mRNAs in different tissues of reproductively inactive adult male NBH and SC killifish 2 and 5 d after treatment with E2 (5 mg/kg)(Expt. 4). See legend to Fig. 4 for experimental details.

			ERα × 10^2		ERβa × 10^2		ERβb × 10^3	
			2-day	5-day	2-day	5-day	2-day	5-day
Brain	SC	Control	0.25 ± 0.07	1.67 ± 0.93	1.58 ± 0.42	2.50 ± 0.25	3.46 ± 0.57	7.00 ± 2.14
		E2	0.44 ± 0.05	0.91 ± 0.13	1.82 ± 0.66	2.06 ± 0.58	5.41 ± 1.76	4.08 ± 1.78
	NBH	Control	0.45 ± 0.17	1.51 ± 0.49	2.31 ± 1.31	2.25 ± 1.00	4.58 ± 2.04	6.63 ± 2.04
		E2	0.45 ± 0.31	1.75 ± 0.43	1.22 ± 0.70	1.88 ± 0.24	4.42 ± 2.16	3.73 ± 2.40
Liver	SC	Control	6.98 ± 2.11	32.77 ± 8.50	9.04 ± 6.90	10.31 ± 3.99	36.99 ± 20.44	52.44 ± 28.08
		E2	21.75 ± 4.64*	167.54 ± 95.87*	3.16 ± 0.85	13.277 ± 9.48	23.79 ± 9.74	65.28 ± 19.91
	NBH	Control	12.48 ± 5.25	35.09 ± 31.97	4.58 ± 2.89	11.87 ± 7.04	28.29 ± 18.38	48.69 ± 25.28
		E2	17.99 ± 11.79	138.66 ± 95.21*	4.71 ± 5.39	10.96 ± 4.84	23.22 ± 19.24	49.65 ± 20.89
Testis	SC	Control	2.56 ± 0.79	3.65 ± 1.18	7.27 ± 2.94	7.29 ± 2.55	18.34 ± 4.17	22.37 ± 8.96
		E2	2.77 ± 0.77	2.99 ± 0.50	8.21 ± 3.33	6.17 ± 0.81	17.27 ± 3.42	18.29 ± 5.12
	NBH	Control	3.32 ± 0.81	2.75 ± 1.18	9.86 ± 2.31	9.40 ± 1.79	21.41 ± 2.16	19.70 ± 9.53
		E2	3.57 ± 1.71	3.77 ± 0.81	8.05 ± 2.32	6.14 ± 2.60	18.89 ± 7.79	16.62 ± 3.85
Eye	SC	Control	0.07 ± 0.01	0.09 ± 0.03	2.67 ± 0.11	2.11 ± 0.54	0.49 ± 0.10	0.43 ± 0.16
		E2	0.11 ± 0.03	0.07 ± 0.01	1.74 ± 0.39	2.11 ± 0.81	0.47 ± 0.12	0.40 ± 0.09
	NBH	Control	0.08 ± 0.07	0.09 ± 0.05	2.18 ± 0.95	1.76 ± 0.37	0.43 ± 0.28	0.47 ± 0.18
		E2	0.09 ± 0.04	0.10 ± 0.03	2.74 ± 0.96	2.91 ± 0.89	0.43 ± 0.13	0.41 ± 0.09

Values are given as β-actin normalized mRNA levels +/− SEM of 3 (SC) or 6 (NBH) individuals. With the exception of hepatic ERα (*; see Fig. 4), no estrogen- or site-dependent effects on ERα, -βa and -βb mRNA levels were found by two-way ANOVA on rank transformed data with the Tukey test for pairwise comparisons.