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Chronic exposure of killifish to a highly polluted environment desensitizes estrogen-responsive reproductive and biomarker genes

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ABSTRACT

Reproductive and endocrine disruption is commonly reported in aquatic species exposed to complex contaminant mixtures. We previously reported that Atlantic killifish (*Fundulus heteroclitus*) from the chronically contaminated Newark Bay, NJ, exhibit multiple endocrine disrupting effects, including inhibition of vitellogenesis (yolk protein synthesis) in females and false negative vitellogenin biomarker responses in males. Here, we characterized the effects on estrogen signaling and the transcriptional regulation of estrogen-responsive genes in this model population. First, a dose–response study tested the hypothesis that reproductive biomarkers (*vtg1*, *vtg2*, *chg H*, *chg Hm*, *chg L*) in Newark Bay killifish are relatively less sensitive to 17β-estradiol at the transcriptional level, relative to a reference (Tuckerton, NJ) population. The second study assessed expression for various metabolism (*cyp1a*, *cyp3a30*, *mdr*) and estrogen receptor (*ER α*, *ER βa*, *ER βb*) genes under basal and estrogen treatment conditions in both populations. Hepatic metabolism of 17β-estradiol was also evaluated *in vitro* as an integrated endpoint for adverse effects on metabolism. In the third study, gene methylation was evaluated for promoters of *vtg1* (8 CpGs) and *vtg2* (10 CpGs) in both populations, and *vtg1* promoter sequences were examined for single nucleotide polymorphism (SNPs). Overall, these studies show that multi-chemical exposures at Newark Bay have desensitized all reproductive biomarkers tested to estrogen. For example, at 10 ng/g 17β-estradiol, inhibition of gene induction ranged from 62% to 97% for all genes tested in the Newark Bay population, relative to induction levels in the reference population. The basis for this recalcitrant phenotype could not be explained by a change in 17β-estradiol metabolism, nuclear estrogen receptor expression, promoter methylation (gene silencing) or SNPs, all of which were unaltered and normal in the Newark Bay population. The decreased transcriptional sensitivity of estrogen-responsive genes is suggestive of a broad effect on estrogen receptor pathway signaling, and provides insight into the mechanisms of the endocrine disrupting effects in the Newark Bay population.

1. Introduction

Adverse reproductive effects are frequently reported in aquatic species living within contaminated environments (Tyler et al., 1998). Chemicals that disrupt hormonal pathways and modulate gene expression can result in deleterious effects throughout the hypothalamus–pituitary–gonad–liver axis (Rempel and Schlenk, 2008). We have previously characterized a population of Atlantic killifish (*Fundulus heteroclitus*) from the historically polluted Newark Bay, NJ (USA), which exhibit reproductive dysfunction and abnormal biomarker responses indicative of complex endocrine disruption (Bugel et al., 2010, 2011). Integrated biomarkers are commonly used for ecological risk assessments (Amiard-triquet et al., 2013). Most biomarkers are physiologically relevant, apical endpoints that respond predictably to single chemicals or simple mixtures (*e.g.* cytochrome P4501A, metallothionein, vitellogenin). However, environmental exposures typically involve complex

Abbreviations: AHR, aryl hydrocarbon receptor; *chg*, choriogenin; *cyp*, cytochrome P450; E2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; mdr, multidrug resistance; SNP, single nucleotide polymorphism; *vtg*, vitellogenin.

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mixtures with diverse mechanisms that may result in atypical biomarker responses (Celander, 2011). Atlantic killifish are a model teleost widely used for comparative ecotoxicological studies pertaining to exposures and effects, adaptations and tolerance, endocrine disruption, and population genetics (Burnett et al., 2007). In the present study, we used the Newark Bay killifish population as a model to study mechanisms of endocrine disruption associated with chronic exposure to complex mixtures.

Newark Bay and the interconnected greater New York–New Jersey Harbor Estuary have a long history of contamination by PAHs, PCBs, heavy metals, polychlorinated dibenzo-*p*-dioxins and furans, and other emerging chemicals of concern (Panero et al., 2005; Muñoz et al., 2006; Valle et al., 2007). Reproductively active female killifish from Newark Bay exhibit reduced expression levels of vitellogenin, correlated to reduced fecundity and inhibited vitellogenin-dependent follicular development (Bugel et al., 2010, 2011). In most oviparous vertebrates (*i.e.* birds, amphibians, fish, *etc.*), vitellogenins are hepatically derived phosphoglycolipoprotein precursors to egg yolk proteins that serve as growth substrate during embryogenesis (*e.g.* amino acids, lipids, sugars) (Arukwe and Goksøyr, 2010). Vitellogenins are highly expressed during oogenesis, and expression directly correlates with fecundity (Miller et al., 2007; Thorpe et al., 2007). In Newark Bay killifish, vitellogenin protein levels are much less responsive to induction by 17β-estradiol (E2), relative to a reference population (Bugel et al., 2011). Thus, a functional desensitization of the vitellogenin pathway likely contributes to the adverse reproductive effects observed in the female population and undermines the use of vitellogenin as a biomarker in males.

Expression of vitellogenin and other estrogen-responsive genes (*e.g.* choriogenin egg envelope genes) are regulated by 17β-estradiol activation of estrogen receptors (ER), which dimerize and bind to *cis*-regulatory estrogen-responsive elements to induce transcription (Menuet et al., 2005). In killifish, three estrogen receptors have been identified (*ER α*, *ER βa*, *ER βb*), although the exact transcriptional role of each for different estrogen-responsive genes is not yet known (Greytak and Callard, 2007). In zebrafish, vitellogenin transcription is regulated primarily by ER α, and secondarily by ER βb, with no clear role of ER βa (Griffin et al., 2013). Epigenetic mechanisms are also important to vitellogenin gene regulation. When transcriptionally active, during spawning or when challenged with 17β-estradiol, CpG sites in promoters of vitellogenin genes are demethylated to facilitate high levels of induction (Saluz et al., 1988; Strömqvist et al., 2010). When transcriptionally inactive (*i.e.* in males or non-spawning females), CpG sites in promoters are methylated to maintain suppressed basal expression (gene silencing). In males, estrogen receptors are functionally expressed and sensitive to low levels of xeno-estrogens. Estrogen-responsive genes (*e.g.* vitellogenin, choriogenin) in males are therefore commonly used as universal biomarkers for exposures to endocrine disrupting compounds because of relatively low basal expression and high induction levels that can be achieved across a broad dosage range (Sumpter and Jobling, 1995; Lee et al., 2002; Pait and Nelson, 2003).

Three studies are presented here with the purpose of characterizing the endocrine disrupting effects influencing the transcriptional regulation of estrogen-responsive genes in the chemically impacted Newark Bay population, relative to a reference population from Tuckerton (Fig. 1). The first was a challenge study conducted using adult male killifish from Newark Bay and Tuckerton to compare the transcriptional sensitivity of various hepatic reproductive biomarker genes (*chg H*, *chg Hm*, *chg L*, *vtg1*, *vtg2*) to 17β-estradiol. This dose–response challenge study was used as an integrated endpoint that may be influenced by many potential effects on ER signaling that could result in adverse effects on ER-mediated gene expression. The second study evaluated

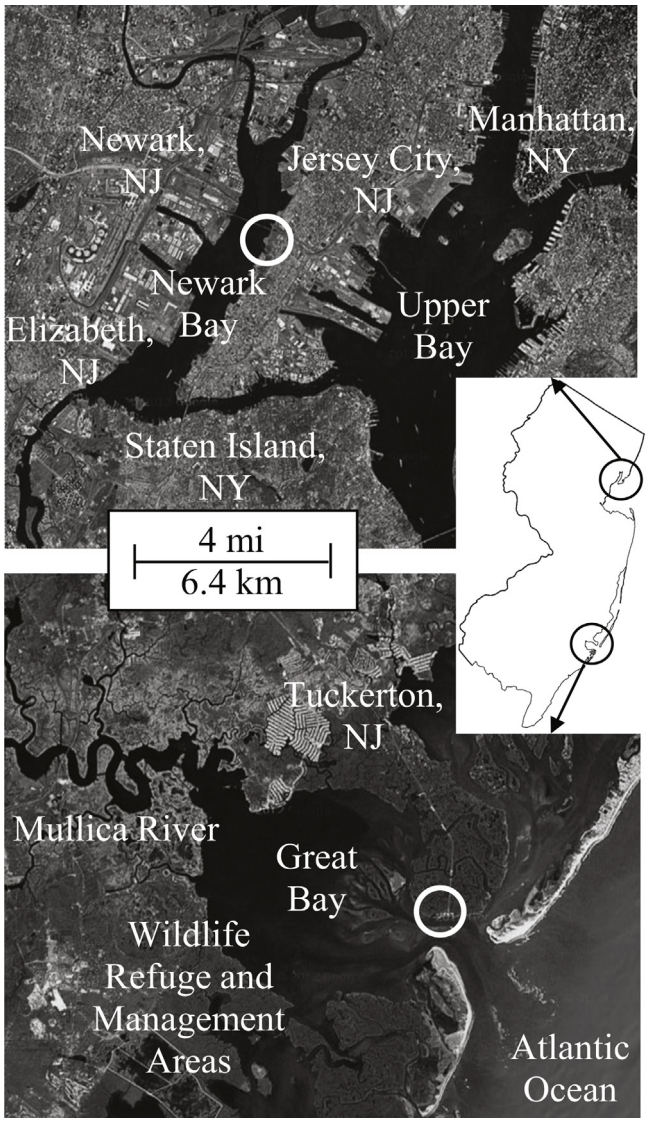

Fig. 1. *F. heteroclitus* collection locations are indicated by circles at a reference site in Tuckerton, and the chemically impacted Newark Bay, NJ (USA) within the interconnected NY–NJ Harbor Estuary.

17β-estradiol metabolism (hepatic elimination activity) and expression levels for genes involved in nuclear estrogen receptor signaling (*ER α*, *ER βa*, *ER βb*) and E2 metabolism (*cyp1a*, *cyp3a30*, *mdr*). In the third study, we investigated differential methylation of CpG sites (gene silencing) in *vtg* promoters and single nucleotide polymorphisms (SNPs) in the promoter of *vtg1* as possible explanations for the refractory sensitivity of various genes in Newark Bay killifish. Our overall hypothesis was that Newark Bay killifish are transcriptionally less sensitive to E2, which may correlate with changes in metabolism, receptor expression, or gene promoter methylation and sequence.

2. Materials and methods

2.1. Site selection, animal necropsy and husbandry protocols

All animal husbandry and handling methods were approved by the Rutgers University Animal Rights Committee in accordance with AALAC accreditation and NIH guidelines. Adult killifish (3–10 g, 5–9 cm) were collected and transported to the laboratory to be either sacrificed, or acclimated to laboratory conditions for 1