FINAL STUDY REPORT

INVESTIGATION OF THE IMPACTS OF COMMON ENDOCRINE DISRUPTING COMPOUNDS ON MULTIPLE EARLY LIFE STAGES OF ENDANGERED ATLANTIC SALMON AND SHORTNOSE STURGEON AND THREATENED ATLANTIC STURGEON

PREPARED FOR

NATIONAL MARINE FISHERIES SERVICE

NORTHEAST REGIONAL OFFICE

BY

KIER ASSOCIATES

TURNERS FALLS, MASSACHUSETTS

AND

SAN RAFAEL, CALIFORNIA

www.kierassociates.net

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TARA DUFFY, PHD, USGS CONTE LAB, PRINCIPAL CO-INVESTIGATOR
STEPHEN MCCORMICK, PHD, USGS CONTE LAB, CO-INVESTIGATOR
MEGHAN NICHOLS, PROJECT TECHNICIAN
BARBARA ARTER, PROJECT FIELD LIAISON AND TECHNICAL ADVISOR
BILL KIER, PROJECT ADMINISTRATOR

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1 INTRODUCTION

1.1 Origins of this Investigation

Under Section 7 of the federal Endangered Species Act, (ESA, 7 U.S.C. § 136, 16 U.S.C. § 1531 et seq), the National Marine Fisheries Service (NMFS) conducts consultations, as needed, with agencies and parties engaged in actions within the designated habitat of ESA-listed species to identify, and negotiate the reduction of, activities capable of further jeopardizing the existence of federally listed species. Such consultations include those of agencies and parties permitted to discharge wastewater into 'the waters of the United States' under the terms of the federal Clean Water Act's National Pollution Discharge Elimination System (NPDES).

Questions have arisen among the responsible agencies concerning the efficacy of the standard 'whole effluent toxicity' ('WET') test, a biological assessment required of NPDES permittees, to detect harm to ESA-listed Atlantic salmon (*Salmo salar*), shortnose sturgeon (*Acipenser brevirostrum*), and Atlantic sturgeon (*Acipenser oxyrinchus*). The questions concern both the fish species normally used in these WET tests (whether they are true biological surrogates for the ESA-listed species) and the efficacy of the testing to identify wastewater components like endocrine disrupting compounds (EDCs) which may be contributing further to the jeopardy of the ESA-listed species.

1.1.2 Project Synopsis

This study was undertaken to determine whether current U.S EPA-recommended whole effluent testing (WET) is effective for predicting negative wastewater discharge impacts to ESA-listed endangered Atlantic salmon (*Salmo salar*), endangered shortnose sturgeon (*Acipenser brevirostrum*), and endangered Atlantic sturgeon (*Acipenser oxyrinchus*). The scope of the investigation has been to determine the appropriateness of the fish species normally used in WET testing - whether they are true biological surrogates for the ESA-listed species - and the efficacy of the testing, itself, to identify wastewater components like endocrine disrupting compounds (EDCs) which may be contributing further to the jeopardy of the ESA-listed species.

This investigation had three specific objectives: 1) to determine if EDCs commonly discharged into waters of the United States under National Pollution Discharge Elimination System permits, identified by NMFS as biologically relevant and which contain Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon, can negatively affect the species and, if possible, at what concentrations and exposure durations such negative effects occur, 2) to examine differences in sensitivity to EDCs at various life stages of shortnose sturgeon, Atlantic salmon, and Atlantic sturgeon and 3) to provide information as to whether the test species commonly used for WET

testing [e.g., fathead minnow (*Pimephales promelas*) and brook trout (*Salvelinus fontinalis*)] are appropriate surrogates for shortnose sturgeon, Atlantic sturgeon, and Atlantic salmon.

To accomplish these tasks, the team exposed these three species to acute (96 hour) and/or chronic (21-day) exposures to three common EDCs - α-ethinylestradiol, 17 β-estradiol, and nonylphenol - using vitellogenin (Vtg) as a primary biomarker of responsiveness.

The results presented here demonstrate that routine WET test methods are insufficient for determining impacts from EDCs to Atlantic salmon, shortnose sturgeon and Atlantic sturgeon. Measures of mortality and growth are not appropriate indicators of potential impacts from three common EDCs, as mortality was not compound- or dose-dependent and impacts to growth were only detected after 21 days of exposure.

Vitellogenin was determined to be an appropriate and sensitive biomarker of EDC exposure in most life stages of Atlantic salmon, but Vtg mRNA was not responsive to EDC exposure in early life stages of shortnose and Atlantic sturgeon. Vtg may be a good biomarker of EDC exposure in older sturgeon, but the lack of inducible Vtg response in shortnose and Atlantic sturgeon suggests that younger sturgeon (<1 year old) may not be as sensitive to EDCs as older juveniles, Atlantic salmon and other fish species. Brook trout were more responsive to EDCs than Atlantic sturgeon, but show comparable responses to Atlantic salmon. Therefore, brook trout may be a useful surrogate species *if the precautionary principle is to be applied* and the measure of response is an EDC-responsive biomarker such as Vtg.

This research underscores the need to understand potential responses to EDC exposure in both salmonids and sturgeon, and suggests that short-term assessments of mortality and growth in surrogate species—where those are used as end-points of WET testing—are insufficient to determine potential impacts of EDC exposure in endangered species. The results presented here are intended to be published in peer-reviewed primary literature. Please contact Kier Associates for publication details as they become available.

1.1.3 EDCs have been shown to impair the fitness of fish

Substances referred to as EDCs include a large number of organic compounds that may act as estrogen mimics, or act to block normal hormonal control, thus causing imbalanced hormonal regulation in fish through a number of different pathways. EDCs have been shown to impair the fitness of freshwater, estuarine, and marine fish by causing reproductive failure, skewed sex ratios, and developmental abnormalities (Sumpter 2005).

These EDC compounds are most often associated with municipal and industrial wastewater effluent and they can feminize male fish in environments receiving large volumes of effluent

(Sumpter 2005, Desbrow et al. 1998, Blazer et al. 2007), resulting in local extinction (Kidd et al. 2007). Two of the most prevalent estrogenic compounds in rivers and estuaries include the natural steroid, 17 β-estradiol (E2), and a synthetic estrogen, 17 α-ethinylestradiol (EE2) (Desbrow et al. 1998). Less potent but often more concentrated estrogenic compounds such as nonylphenol (NP) are also present in wastewater effluent (Servos et al. 2003).

Routine wastewater treatment does not completely remove these compounds and in some cases it actually increases their effect (Johnson and Sumpter 2001). Any fish in the receiving waters of wastewater effluent may be subject to endocrine disruption (Oberdorster and Cheek 2001, Matthiessen 2003).

Under certain conditions, exposure to endocrine disruptors can elicit severe and long-lasting impacts, primarily when fish are exposed early in their development (Strussmann and Nakamura 2002, Mills and Chichester 2005). Several studies have indicated that particular developmental stages may be more vulnerable to EDCs than others. For example, Liney et al. (2005) exposed the common roach, *Rutilus rutilus*, to a gradient of wastewater effluent and found little response in adults, but 100% feminization in subjects exposed during the embryonic through juvenile stages of this teleost fish. Similar life stage-dependent results have been demonstrated in zebrafish (Brion et al. 2004), medaka (Koger et al. 2000), and fathead minnows (Van Aerle et al. 2002). Thorpe et al. (2007) found adverse impacts to reproduction and male survival due to exposure to EE2 and estrone (E1) in fathead minnows.

One factor that may undermine current efforts to conserve and restore populations of Atlantic and shortnose sturgeon and Atlantic salmon is degraded water quality (Collins et al. 2000, Thorstad et al. 2008). Over-wintering and spawning of these species within the Gulf of Maine and other Northeast watersheds often occur near urban centers (Kynard et al. 2000, Fernandes et al. 2010). For example, shortnose sturgeon were found to be present year-round in the Penobscot River estuary downstream from urban areas and several pulp and paper processing mills (Fernandes et al. 2010). The same study found that Atlantic sturgeon also spent several months in the Penobscot River estuary and that both of the sturgeon species of interest here migrated between the Penobscot River estuary and the Kennebec River, another urbanized estuary. Therefore, both spawning adults and developing offspring may be exposed to effluent with high concentrations of EDCs, potentially leading to recruitment failure (Kidd et al. 2007).

Relatively little is known of the impacts of EDCs on Atlantic salmon or any species of sturgeon, including the life-stage sensitivity to EDCs of these species. Such knowledge, were it available, could prove critical to the conservation of these species.

1.2 Purpose and Scope of this Investigation[1]

The purpose of this investigation is to gather data on the impacts of impaired water quality on Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon to determine whether the surrogate species commonly used in WET tests provide results that are truly applicable to Atlantic salmon and Atlantic and shortnose sturgeon sensitivity. The investigation will:

1. provide information as to whether EDCs commonly discharged into the waters of the United States that contain Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon, and identified by NMFS as biologically relevant, can negatively affect the species and, if possible, at what concentrations and exposure durations such negative effects occur;
2. investigate the most vulnerable life stages of Atlantic and shortnose sturgeon; and
3. provide information as to whether test species commonly used for WET testing [e.g., fathead minnow (*Pimephales promelas*), brook trout (*Salvelinus fontinalis*), and rainbow trout (*Oncorhynchus mykiss*)] are suitable surrogates for Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon.

1.2.2 Year One of the Investigation (September 2010-September 2011)

The goals set for the project's base year were to 1) determine the fishes' baseline responses to three common endocrine disrupting compounds when exposed for four days (96 hours), 2) further determine differential responsiveness to these compounds across different life stages, and 3) develop and validate the assays to be used as biomarkers for endocrine disruption in these species. Achievement of these goals would establish which contaminants appear to have the greatest influence on Atlantic salmon and shortnose sturgeon, which life stages are most sensitive to these contaminants, and how these species respond (i.e. reduced growth, altered gonad development, etc.) to the contaminants.

Atlantic sturgeon were unavailable during the base year. Study of this species was, therefore, delayed to the second project year.

1.2.3 Year Two (2011-2012)

The goals of the project for year two were to 1) continue to determine short-term responses to endocrine disruptors in shortnose sturgeon life stages (thereby completing all short-term response testing), 2) expose Atlantic sturgeon to environmentally relevant levels of EDCs and determine long-term effects of these exposures (see note below regarding shortnose sturgeon), and 3) determine the impacts of EDCs on a commonly used WET test species (brook trout) for comparison.

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[1] From NMFS' 17 August 2010 Request for Quotes #495852 **'**Sturgeon and Salmon Water Quality Study'

Notes regarding items 2 and 3: The federal permit for shortnose sturgeon research was not granted to the Conte Lab in time to obtain embryos from spawning fish, therefore long-term exposures on shortnose sturgeon could not be carried out. Atlantic sturgeon embryos became available during the last quarter of year two, and the fish were reared until they reached the stage where gonad development was expected to occur and long-term exposures were initiated at the start of year three. The team attempted to obtain brook trout used in municipal wastewater WET tests conducted by Maine-based consultants Lotic, Inc. but, for whatever reasons, Lotic failed to respond to any of our several phone and email requests for assistance.

1.2.4 Year Three (2012-2013)

The primary goal of year three was to conduct 21-day exposures on three-month old Atlantic sturgeon juveniles (an age at which we anticipated the animals would be capable of displaying a feminizing response as measured by levels of vitellogenin (Vtg) - see Section 2.1) and to determine potential impacts to gonad development. The team developed a Vtg assay to determine if Atlantic sturgeon are responsive to these compounds at this life stage. Sampling occurred at four- and 21 days in order to obtain information about short-term and long-term responses. The goal of this exposure was to determine the stage at which these compounds may induce Vtg transcription and protein production, and to determine if exposure impacts normal gonad differentiation.

The team also carried out 21-day exposures in Atlantic salmon to determine impacts to gonad differentiation and development. The team assessed Vtg mRNA transcription to compare this to the other species tested in the lab. Sampling occurred at both four and 21 days to assess short-term and long-term effects on growth, gonad development, and Vtg transcription. (Note: gonadal tissue is currently being processed by colleague Kristy Forsgren of California State University, Fullerton, to determine long-term impacts to gonad development in Atlantic sturgeon and Atlantic salmon. These experiments got underway late in year three of the project and the histological processing is underway at the time of this writing. Gonadal differentiation and development results will be provided to the National Marine Fisheries Service in an addendum when these data become available.)

The team also worked in Dr. Luke Iwanowicz's lab (USGS) to run yeast E-screen (estrogen-screen) assays to determine the estrogenicity of water samples used in the exposures and also to determine the estrogenicity of six water samples collected from wastewater treatment plants in Massachusetts and Maine. Additionally, the team worked to assess changes in the gene expression of genes potentially impacted by EDC exposure. The genes assessed were those involved in growth and salinity tolerance.

2. EXPERIMENTAL DESIGN

Throughout the three-year project the team carried out both acute (four-day) and chronic (21-day) exposures using the three species of concern (Atlantic salmon, shortnose sturgeon, and Atlantic sturgeon) and a fourth species commonly used in WET tests, brook trout. Acute exposures were carried out in Atlantic salmon and shortnose sturgeon. Chronic exposures were carried out in Atlantic salmon, Atlantic sturgeon, and brook trout. All chronic exposures included an acute sampling time point for comparison. In these exposures we utilized two common endocrine disrupting compounds for long-term exposures (EE2 and NP), or three common EDCs for acute exposures (EE2, NP plus 17 β-estradiol) (Table 1). These compounds have known estrogenic impacts on both wild and lab-reared fish populations (Desbrow et al. 1998, Servos et al. 2003).

Table 1. Nominal concentrations of compounds used in the acute (96-hour) exposures of Atlantic salmon and shortnose sturgeon, with the range of concentrations of each compound reported for multiple measures in surface water.

		Nominal Concentrations				
		Acute exposures (96-hour)		**Chronic exposures (21 days)**		
		nM	ng l^{-1}	nM	ng l^{-1}	Range of conc. in surface waters
α-ethinylestradiol (EE2)	*low*	0.004	1.2	0.008	2.3712	0.04-28.6 ng l^{-1} [a]
	medium	0.04	11.9	0.04	11.9	
	high	0.4	118.6	--	--	
17 β-estradiol (E2)	*low*	0.04	10.9	--	--	0.11-84.3 ng l^{-1} [a]
	medium	0.4	109.0	--	--	
	high	4	1089.6	--	--	
Nonylphenol (NP)	*low*	4	881.4	--	--	15-30,000 ng l^{-1} [b]
	medium	40	8814.0	40	8814.0	
	high	400	88140.0	200	44070	

[a] Range of mean concentrations of compounds in surface waters, compiled by Leet et al. (2011).

[b] Approximate range of concentrations of NP compiled by Mao et al. (2012).

The doses examined in this study represent the range of doses initially proposed by the project team, and for each compound there is 1) a representative dose of contaminant that is expected to elicit a positive response, 2) a dose similar to that found in sewage effluent, and 3) a dose that would normally be found in an urbanized estuary (Dwyer et al. 2005). The long-term exposure concentrations were decreased because nearly identical effects were seen between E2 and EE2, and because previous work in the lab suggested that the highest positive control doses used in the short-term exposures would result in excessive mortality.

In addition, the highest doses used as positive controls would not be representative of what fish experience in the wild and were therefore removed from the long-term exposure protocol.

The principal biomarker of comparison in this study is Vitellogenin, (Vtg), a precursor egg yolk protein that is often used as a biomarker of feminizing EDC exposure (Bernanke and Kohler 2009, Leet et al. 2011). The two most common methods of Vtg measurement are measuring either the Vtg protein itself (by immunoassay) or by quantifying Vtg mRNA (messenger RNA) which is a measure of gene transcription or activity. Higher levels of the protein or mRNA can indicate a stronger response by the individual to the stimuli - in this case, EDC exposure (Leet et al. 2011).

Elevated Vtg is normally associated with spawning females (Tyler and Sumpter 1996) and is often a more sensitive endpoint to contaminant exposure than phenotype or population-level responses (Leet et al. 2011). Vitellogenin production in juvenile or, especially in male fish, can be a very sensitive indicator of exposure to feminizing compounds at environmentally relevant concentrations (Leet et al. 2011). Elevated plasma concentrations of vitellogenin have been found in juvenile and male fish from sewage-impacted aquatic ecosystems around the world (Bernanke and Kohler 2009), which suggests Vtg may be a useful biomarker for estrogenic EDC exposure (Tyler and Routledge 1998). Elevated plasma Vtg in fish has been associated with enlarged livers (Kaptaner et al. 2009), abnormal gonad development (Rodgers-Gray et al. 2001, Lye et al. 1997), and reduced growth (Woltering 1984). It has also been used to compare sensitivities of multiple life stages to estrogenic exposure (Leet et al. 2011).

One goal for this project was to evaluate responses to endocrine disruptors in a common WET test species, brook trout, for comparison to the responses in the ESA-listed species. The team chose to use brook trout rather than another common WET test fish, the fathead minnow, for two reasons. First, brook trout may be more sensitive to environmental contaminants than fathead minnows (Dwyer et al. 2005) and second, brook trout are the species of preference in WET tests in Maine where all three species of concern are known to spawn.

This three-year project then, consisted of three major components: 1) assay development, 2) animal exposure, and 3) analysis of results. The details of assay development and the animal exposures may be found in this report's Supplemental Information section. The Results section of this report summarizes the three major objectives of the project set out in Section 1.2 above.

3 RESULTS

3.1 Objective #1 - Do common EDCs negatively impact Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon and, if so, at what concentrations and exposure durations do such impacts occur?

3.1.1 Atlantic salmon Vtg mRNA

Vtg is an appropriate and sensitive biomarker of acute EDC exposure in most life stages of Atlantic salmon.

To determine potential impacts of EDCs to early life stages of salmon, embryos, yolk-sac larvae, feeding fry, and smolts (one-year old fish preparing for migration to seawater) were exposed to three EDCs for four days (96-hours) in a flow-through system with daily renewal to mimic WET testing. Vtg mRNA transcription was analyzed across all four stages, and Vtg plasma protein concentration was assessed in smolts.

All life stages demonstrated upregulation (increases in response) of Vtg mRNA in response to EDCs with maximal transcription in whole body comparisons occurring in feeding fry (one-way ANOVA with Holm-Sidak *post hoc* comparisons, Fig. 1). Embryos increased Vtg transcription in response to 0.4 nM α-ethinylestradiol, but lower concentrations of this compound, as well as 17 β-estradiol and nonylphenol, did not induce a statistically significant vitellogenic response (Fig. 1A). Yolk-sac larvae Vtg transcription increased significantly and in a dose-dependent manner for the two highest concentrations of all three compounds (Fig. 1B).

Feeding fry had a statistically similar response to the compounds compared to yolk-sac larvae, but the magnitude of transcriptional upregulation was greater for feeding fry (~500 times greater than the control) than for yolk-sac larvae (~200 times greater than control) (Fig. 1C). Smolt liver tissue demonstrated similar dose-dependent Vtg mRNA upregulation for both α-ethinylestradiol and 17 β-estradiol, whereas a reduction in Vtg mRNA, i.e., relative to the control, was observed for fish exposed to 40 and 400 nM nonylphenol (Fig. 2). Lowest observable effect concentrations (LOEC) were determined for embryos exposed to EE2, and were identical for all three compounds for yolk-sac larvae and feeding fry (Table 2).

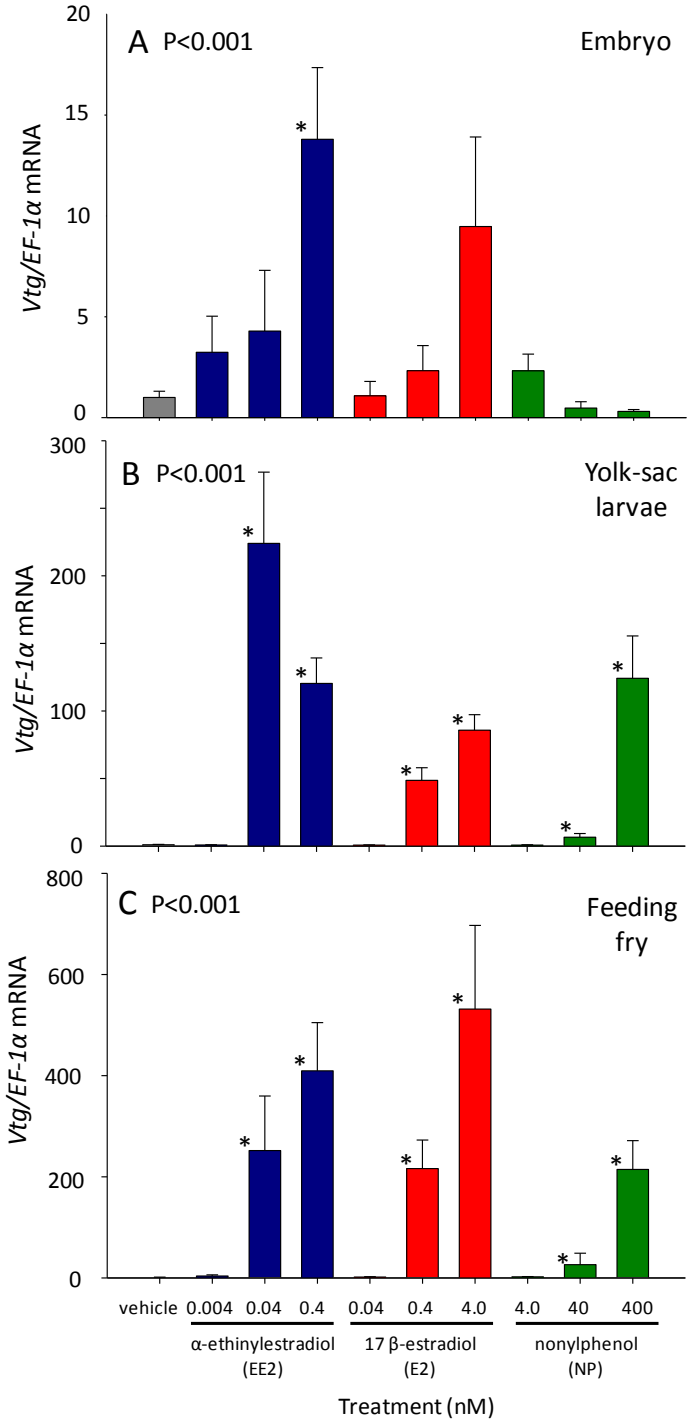

Figure 1. Whole body Vtg mRNA transcription in three life stages of Atlantic salmon exposed to endocrine disrupting compounds: A) embryos, B) yolk-sac larvae, and c) feeding fry. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. For each life stage, the control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. P-values represents overall one-way ANOVA on ranks for each life-stage. Asterisks indicate significant differences between each concentration and the control for each life stage.

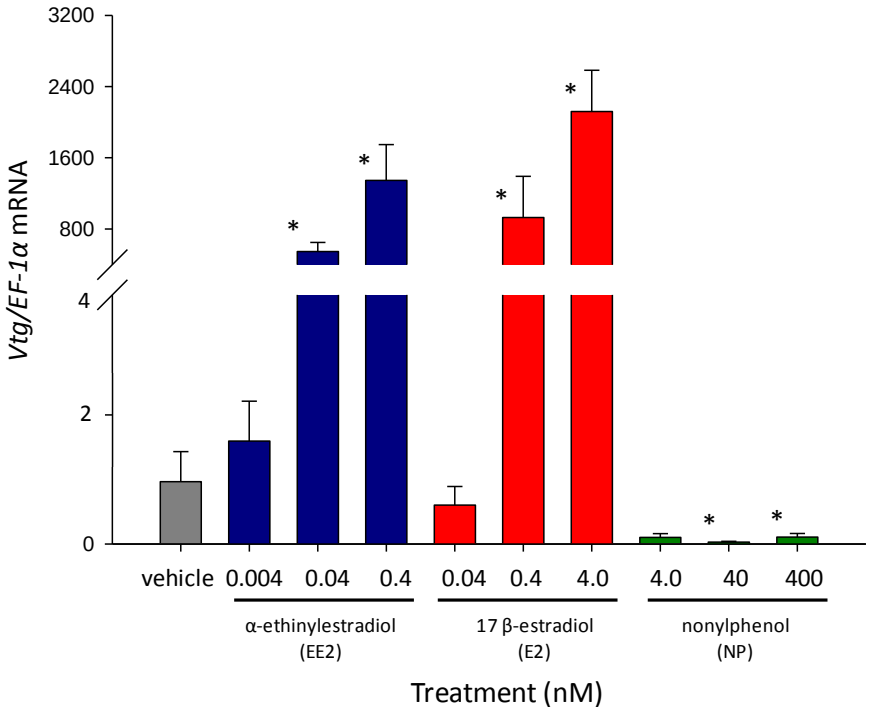

Figure 2. Liver Vtg mRNA levels in Atlantic salmon smolts exposed to endocrine disrupting compounds. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

Table 2. Lowest observable effect concentration (LOEC) for early life stage exposures in Atlantic salmon based on significant difference in Vtg mRNA transcription between the control and dose. ND—not determined.

	Compound (μM)		
	α-ethinylestradiol (EE2)	**17 β-estradiol (E2)**	**Nonylphenol (NP)**
Embryo	0.4	ND	ND
Yolk-sac larvae	0.4	0.04	40.0
Feeding fry	0.4	0.04	40.0

Atlantic salmon demonstrated strong physiological responses to three estrogenic compounds commonly found in wastewater effluent and urbanized river systems and estuaries. All life stages clearly responded to aqueous exposure, but responsiveness appeared to be life stage-, compound-, and dose-dependent. The estrogens, E2 and EE2, elicited the strongest overall Vtg response, while response to NP exposure was muted. Clearly, each life stage was capable of eliciting a vitellogenic response that was partially dose-dependent, but the dose that elicited this response and its magnitude varied among life stages.

3.1.2 Atlantic salmon *Vtg protein*

Vtg is elevated at environmentally relevant concentrations of common EDCs.

Smolt plasma Vtg protein concentration increased in response to all compounds (1-way ANOVA, $p<0.001$), and there was a clear dose-dependent response for both α-ethinylestradiol and 17 β-estradiol wherein all doses elicited elevated Vtg (Fig. 3). Dose-dependency was less clear for nonylphenol, but both the 4.0 and 400 nM doses elicited significant increases in plasma Vtg concentration. No differences for Vtg concentration were found based on sex (2-way ANOVA, $p=0.315$). Further, analysis of Vtg protein indicates that measurement of the protein may be a more sensitive biomarker of acute EDC exposure than Vtg mRNA, potentially due to downregulation of Vtg mRNA at the time of sampling.

One plausible explanation for these life stage differences in Vtg may be due to a developmental increase in estrogen receptor-mediated Vtg induction. The impacts of the endogenously regulated natural estrogen, 17-β estradiol, are mediated by three known estrogen receptors in teleosts, ERα, ERβ, and ERγ (Katsu et al. 2011). Estrogen receptors are important regulators of circulating E2 during ovarian development in fish and are interlinked with growth and development throughout a fish's life (Devlin and Nagahama 2002). Estrogen receptors increase in number during development and, in particular, are likely to increase rapidly prior to and during sexual differentiation (Liao et al. 2009). Gonad differentiation begins early in development in Atlantic salmon, as many of the major genes involved in sex determination and differentiation are upregulated in the embryonic stage (von Schalburg et al. 2011). Gonad development is plastic throughout early development, and exposure to aqueous and dietary EDCs can skew sex ratios (Davidson et al. 1999, Norrgren et al. 1999) by binding to the estrogen receptor(s) (Kloas 2000, Scholz et al. 2013). Exposure to estrogenic compounds early in development could have profound effects on population dynamics and recruitment success of Atlantic salmon in the wild, thereby hindering success of conservation strategies.

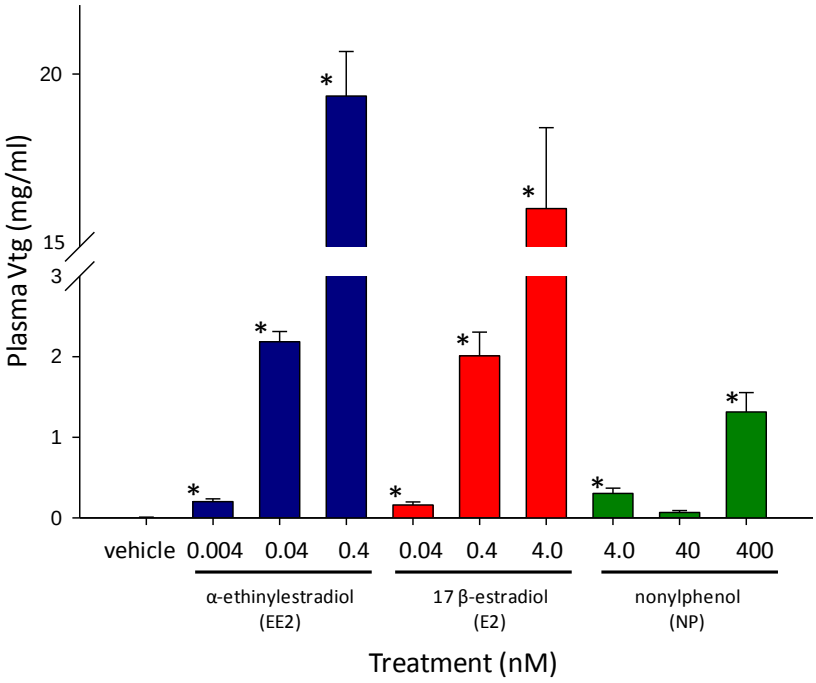

Figure 3. Plasma Vtg protein concentration in Atlantic salmon smolts exposed to endocrine disrupting compounds. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

Additional physiological parameters

Hepatosomatic index (HSI) is the ratio of liver weight to body weight and is an indicator of protein production and metabolite synthesis. It often increases with EDC exposure (Leet et al. 2011). HSI was significantly elevated in response to the highest doses of α-ethinylestradiol and 17 β-estradiol, but no significant differences were noted for nonylphenol (Fig. 4). Additionally significant differences were seen for hematocrit and triiodothyronine (T_3, a thyroid hormone), but T_4 and cortisol did not vary among treatments (Table 3). Nonylphenol exposure (4.0 and 400 nM) significantly reduced hematocrit relative to the control, and T_3 was significantly reduced by exposure to α-ethinylestradiol.

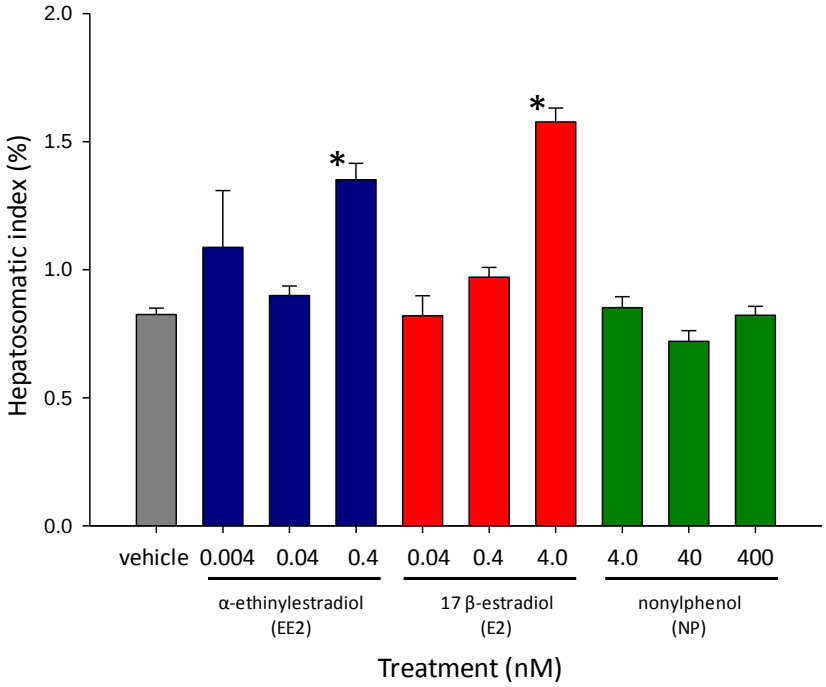

Figure 4. Hepatosomatic index for Atlantic salmon smolts exposed to endocrine disrupting compounds. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control for each compound.

Na^+/K^+ -ATPase (hereinafter, NKA) activity was measured in smolt gill tissue to determine potential impacts to seawater preparedness. Controls from each set of exposures differed significantly, with nonylphenol control NKA activity being significantly lower than control ATPase values for α-ethinylestradiol. Within a given experiment (all doses of a single compound) no significant differences were noted among concentrations. Therefore, ATPase activity did not exhibit compound- or dose-dependent response.

Table 3. Physiological and endocrine changes in smolts in response to four-day EDC exposure. All values are mean ± SE. Significant treatment effects are indicated in the right-hand column. Values in bold indicate significant differences as compared to the control in *post hoc* comparisons.

		Compound			
		α-ethinylestradiol (EE2)	**17 β-estradiol (E2)**	**Nonylphenol (NP)**	**Statistical test and significance level**
T_4 (ng/ml)	*control*	6.2 ± 0.7	4.1 ± 0.1	3.7 ± 0.5	one-way ANOVA
	low	3.6 ± 0.2	4.9 ± 0.2	2.3 ± 0.1	EE2, p=0.44
	medium	3.5 ± 0.2	5.4 ± 0.2	3.1 ± 0.3	E2, p=0.81
	high	3.2 ± 0.2	5.2 ± 0.4	2.5 ± 0.1	NP, p=0.18
T_3 (ng/ml)	*control*	2.4 ± 0.3	2.4 ± 0.1	2.7 ± 0.3	one-way ANOVA
	low	2.1 ± 0.1	2.5 ± 0.3	2.7 ± 0.2	**EE2, p=0.004**
	medium	2.3 ± 0.2	2.6 ± 0.3	2.7 ± 0.3	E2, p=0.35
	high	**1.6 ± 0.1 ***	2.0 ± 0.2	2.8 ± 0.2	NP, p=0.36
Cortisol (ng/ml)	*control*	4.4 ± 1.0	4.2 ± 1.6	24.1 ± 12.5	one-way ANOVA on ranks
	low	31.6 ± 10.6	20.7 ± 7.2	20.3 ± 7.6	EE2, p=0.16
	medium	7.4 ± 2.9	14.3 ± 6.9	22.5 ± 8.6	E2, p=0.43
	high	10.1 ± 1.7	15.8 ± 4.7	14.6 ± 4.5	NP, p=0.89
Na^+/K^+-ATPase (ng/ml)	*control*	4.6 ± 0.4	5. 8 ± 0.4	4.2 ± 0.3	one-way ANOVA
	low	4.3 ± 0.4	5.6 ± 0.4	4.2 ± 0.4	EE2, p=0.73
	medium	4.3 ± 0.4	5.5 ± 0.6	4.7 ± 0.5	E2, p=0.97
	high	4.0 ± 0.3	5. 7 ± 0.5	4.5 ± 0.4	NP, p=0.67

3.1.3 Shortnose sturgeon Vtg mRNA

Vtg mRNA is not responsive to EDC exposure in early life stages, but may be a good biomarker in older animals.

Acute exposure of shortnose sturgeon yielded greatly variable Vtg mRNA transcription among life stages. These data indicate that in the embryos Vtg transcription is not upregulated by the concentrations of EDCs used in this study, and that the responses in both yolk-sac larvae and feeding fry are both variable and ambiguous (Figure 5). No treatment effect was detected in the embryos (one-way ANOVA on ranks, p=0.38, Figure 5A). Yolk-sac larvae display a significant overall treatment effect (p=0.003), but no single treatment was significantly different from the control (Figure 5B). Similarly, newly feeding early juveniles (feeding fry) display a significant overall treatment effect (p=0.009) but no significant differences between treatments and the control (Figure 5C).

Larvae and fry displayed a high degree of variance in Vtg transcription during these life stages, as evidenced by large standard errors. In feeding fry, high concentrations of all three compounds induce a Vtg response in some individuals, but here the variance in response masks any statistical differences between the control and treatments. Estrogen receptor numbers

may increase more slowly in early development of sturgeon as many species of sturgeon do not initiate gonad development until several months of age (Van Eenennaam and Doroshov 1998).

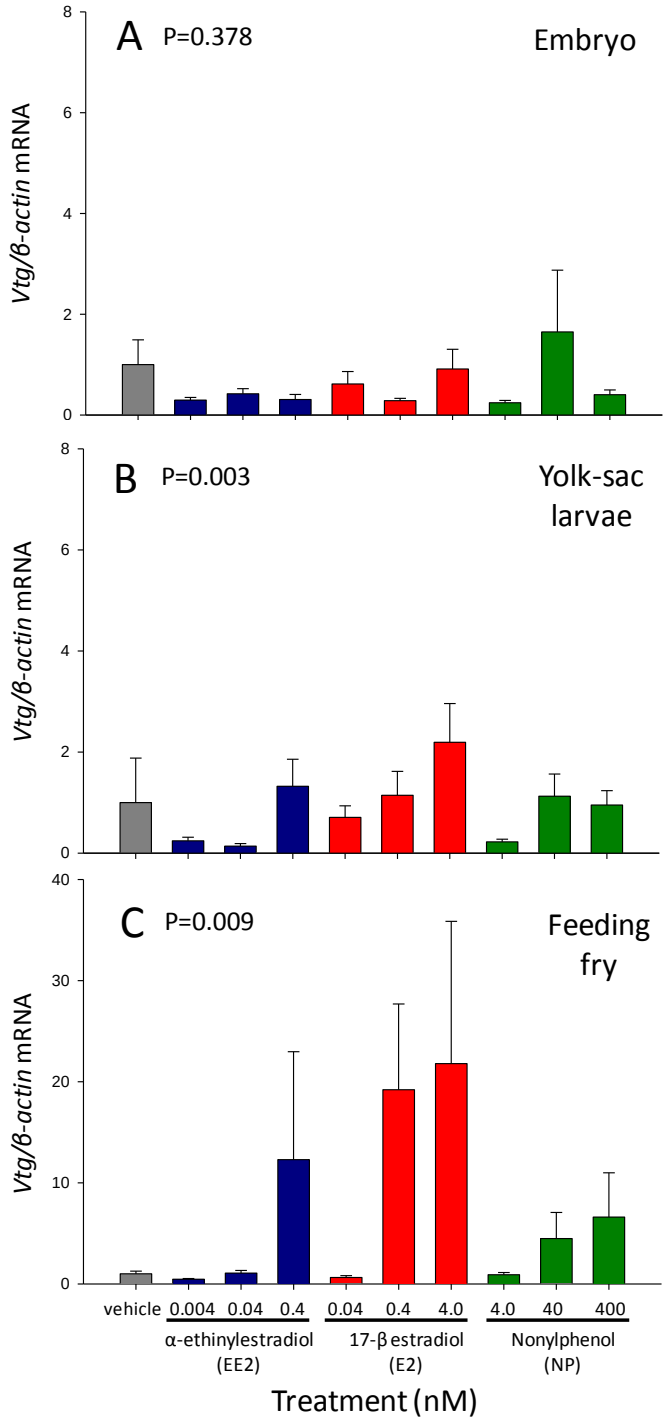

Figure 5. Whole body Vtg mRNA transcription in three life stages of shortnose sturgeon exposed to endocrine disrupting compounds: A) embryos, B) yolk-sac larvae and c) feeding fry. Values are normalized to the housekeeping gene, β-actin, and then normalized to the control. For each life stage, the control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue,

17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. P-values represents overall one-way ANOVA on ranks for each life-stage. No significant differences between the control and individual treatments were detected.

Two-year old juvenile sturgeon displayed a significant overall treatment effect (p=0.001), with significant differences between control (vehicle) and treatments for the highest concentration of α-ethinylestradiol and the two highest concentrations of 17 β-estradiol (Figure 6). In older juveniles, Vtg transcription is highly upregulated in response to the higher doses of estrogens. Nonylphenol does not, however, elicit a statistically significant response despite an increase in the mean transcription at 400 µM.

3.1.4 Shortnose sturgeon Vtg mRNA and Vtg protein

Shortnose sturgeon demonstrate a positive response to Vtg at two years of age, but only to high concentrations of EE2 and E2.

Atlantic salmon smolts (one-year old) showed elevated Vtg mRNA at both intermediate and high concentrations of all compounds (Figure 2) and elevated protein in response to all concentrations of EDC exposure (Figure 3). This indicates that Vtg is sensitive to EDC exposure in this life stage of salmon. In contrast, two-year old shortnose sturgeon have a more muted response to EDCs, with Vtg mRNA transcription in response only to the highest dose of EE2 and the mid to high doses of E2 (Figure 6). Further, no statistically significant response to NP was seen.

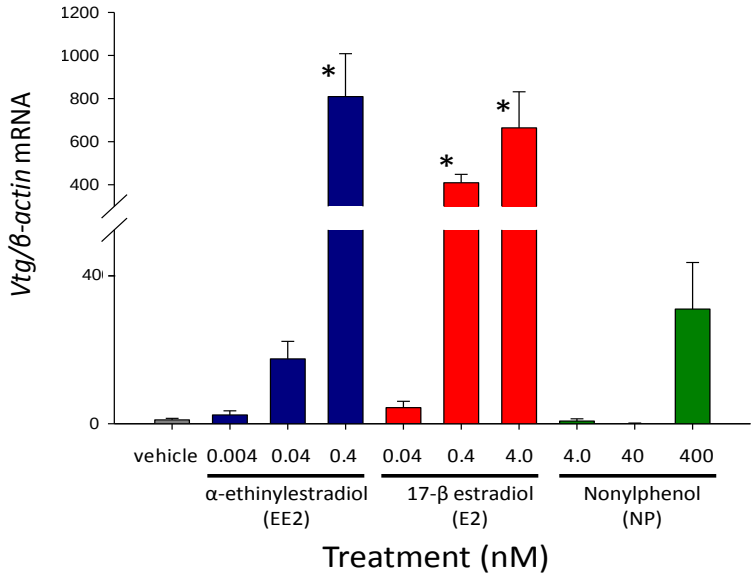

Figure 6. Liver Vtg mRNA levels in shortnose sturgeon juveniles exposed to endocrine disrupting compounds. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

Shortnose sturgeon juvenile plasma Vtg showed a similar pattern to the mRNA with each compound demonstrating a significant treatment effect (E2- $p<0.001$, EE2- $p<0.001$, NP- $p=0.37$) (Figure 7). The highest concentrations of both EE2 and E2 elicited significant increases in Vtg, as did the intermediate dose (0.4 nM) of E2 when compared to the control, but nonylphenol exposure did not result in an increase in Vtg for any concentration.

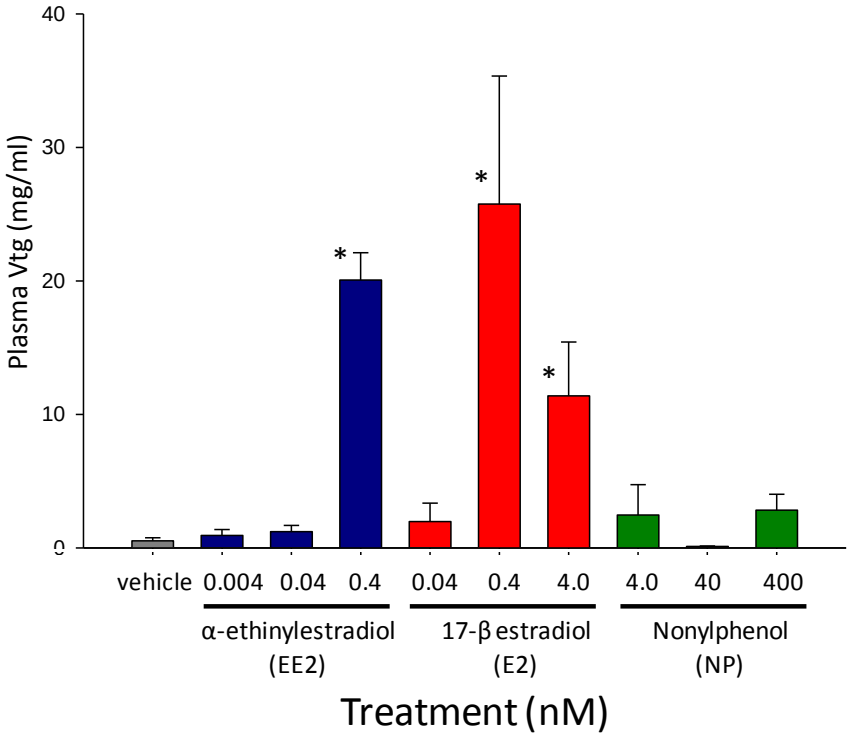

Figure 7. Plasma Vtg protein concentration in shortnose sturgeon juveniles exposed to endocrine disrupting compounds. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

3.1.5 Additional physiological parameters

Short-term exposures to EDCs cause little change to physiological status of shortnose sturgeon.

Plasma hematocrit and cortisol all demonstrated treatment effects for either NP or E2 exposure, but these were limited to a single difference between one dose (in the case of hematocrit following NP exposure), or no *post hoc* differences detected between control and exposed animals (in the case of cortisol). Exposure to E2 did result in an increase in the thyroid hormone, T_4 (thyroxine), in response to all concentrations used in this study. Na^+/K^+ -ATPase did not demonstrate any treatment effects.

Table 4. Physiological and endocrine changes in shortnose sturgeon two-year old juveniles in response to four-day EDC exposure. All values are mean ± SE. Significant treatment effects are indicated in the right-hand column. Values in bold indicate significant differences as compared to the control in *post hoc* comparisons.

		Compound			
		α-ethinylestradiol (EE2)	**17 β-estradiol (E2)**	**Nonylphenol (NP)**	**Statistical test and significance level**
Hematocrit (%)	*control*	24 ± 2	27 ± 2	24 ± 3	one-way ANOVA
	low	12 ± 3	30 ± 1	34 ± 2	EE2, p=0.08
	medium	23 ± 2	34 ± 1	**29 ± 1**	E2, p=0.16
	high	24 ± 2	28 ± 2	24 ± 1	**NP, p=0.03**
T_4 (ng/ml)	*control*	0.8 ± 0.1	0.6 ± 0.1	1.1 ± 0.4	one-way ANOVA
	low	1.4 ± 0.3	**1.3 ± 0.4**	1.1 ± 0.2	EE2, p=0.70
	medium	1.1 ± 0.1	**1.4 ± 0.1**	0.8 ± 0.1	**E2, p=0.01**
	high	1.1 ± 0.2	**1.2 ± 0.1**	1.1 ± 0.1	NP, p=0.50
Cortisol (ng/ml)	*control*	0.8 ± 0.2	0.9 ± 0.5	1.3 ± 0.7	one-way ANOVA
	low	1.0 ± 0.3	0.9 ± 0.4	3.3 ± 1.2	EE2, p=0.64
	medium	2.2 ± 0.8	3.1 ± 0.9	1.2 ± 0.3	**E2, p=0.04**
	high	1.7 ± 0.4	2.4 ± 0.6	1.1 ± 0.3	NP, p=0.54
Na^+/K^+-ATPase (ng/ml)	*control*	5.0 ± 0.3	3.1 ± 0.3	3.9 ± 0.3	one-way ANOVA
	low	5.3 ± 0.4	5.2 ± 0.4	4.2 ± 0.3	EE2, p=0.30
	medium	4.7 ± 0.4	3.4 ± 0.4	7.5 ± 0.5	E2, p=0.21
	high	5.0 ± 0.5	2.7 ± 0.5	3.8 ± 0.7	NP, p=0.19
HSI (%)	*control*	2.6 ± 0.2	2.3 ± 0.4	2.1 ± 0.1	one-way ANOVA
	low	2.2 + 0.2	2.9 ± 0.3	2.3 ± 0.1	EE2, p=0.54
	medium	2.3 ± 0.1	2.4 ± 0.2	2.2 ± 0.2	E2, p=0.31
	high	2.3 ± 0.2	2.6 ± 0.2	2.3 ± 0.1	NP, p=0.77

3.2 Objective #2 - What are the most vulnerable life stages of Atlantic and shortnose sturgeon?

3.2.1 Atlantic sturgeon Vtg mRNA and Vtg protein

Lack of Vtg response in shortnose and Atlantic sturgeon may indicate a lack of sensitivity to three common EDCs during early life-history stages.

The data above (Figures 2 and 3) indicate that Atlantic salmon are highly responsive to the concentrations and types of EDCs used in this study and that this responsiveness (in the form of Vtg mRNA) develops early in the fish's life history. In shortnose sturgeon, this same response in similar age, similar stage fish is highly variable and is not dose-dependent. Shortnose sturgeon

juveniles were clearly able to upregulate the Vtg gene and produce Vtg in the protein in response to EDCs. Because of this, the team chose to expose Atlantic sturgeon at the age of four months, when they may be most responsive to these compounds, and during initial gonad development.

Atlantic sturgeon were exposed for 21 days during what the team hypothesized would be early gonadal differentiation. Vtg response was measured by mRNA transcription (Figure 8) and plasma Vtg protein (Figure 9). Statistical analyses of both assays show no treatment effects. Shortnose sturgeon exposed to EDCs demonstrated detectable levels of Vtg protein, but these were measured in the range of micrograms per liter, relative to the milligrams per liter measured in Atlantic salmon. It is clear that Vtg induction is not an appropriate biomarker of response in Atlantic sturgeon at this life stage.

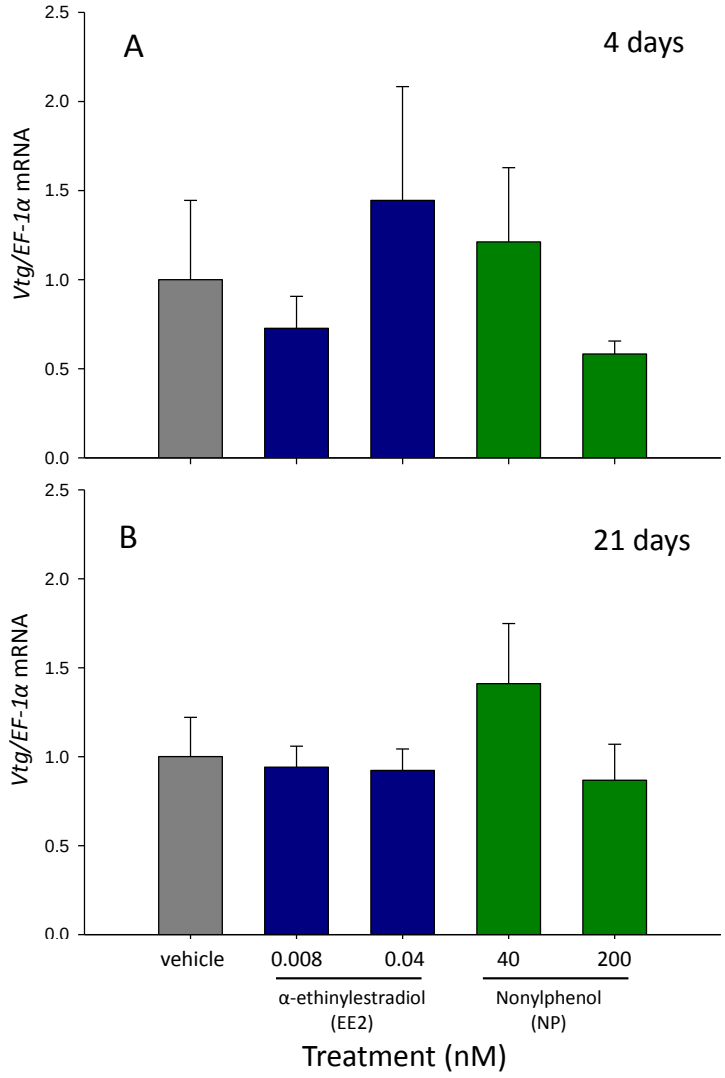

Figure 8. Liver Vtg mRNA levels in Atlantic sturgeon juveniles exposed to endocrine disrupting compounds. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, and 4-nonylphenol (NP) is in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

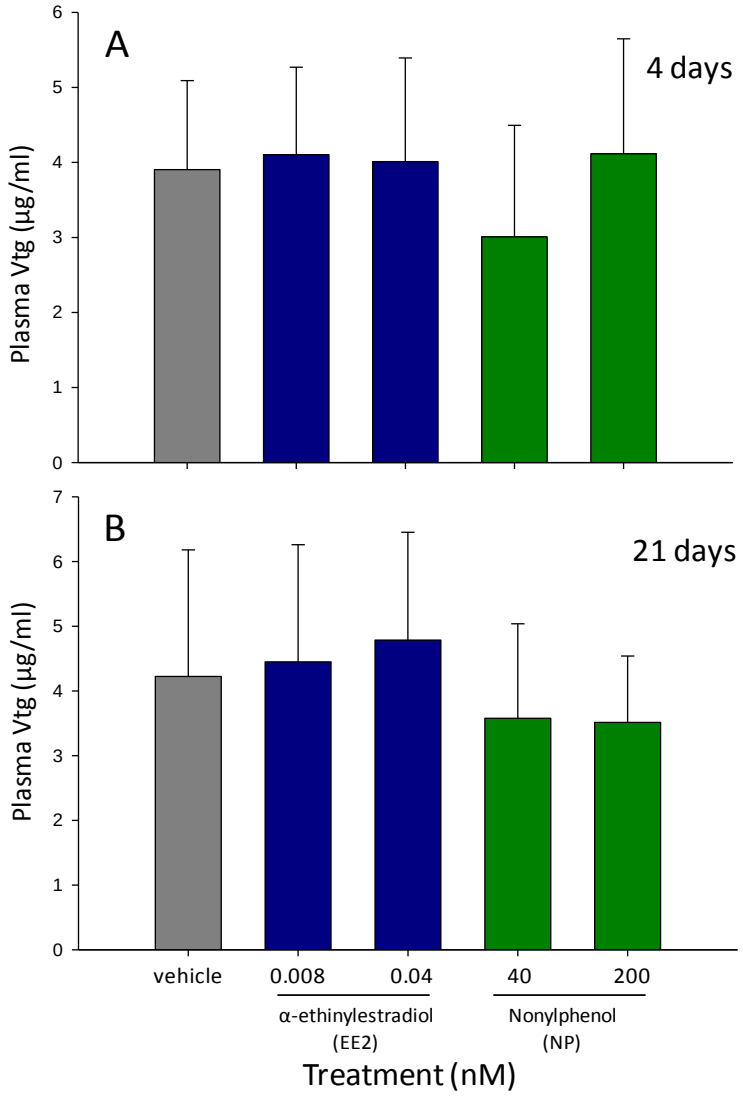

Figure 9. Plasma Vtg protein concentration in Atlantic sturgeon juveniles exposed to endocrine disrupting compounds. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, and 4-nonylphenol (NP) is in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

3.3 Objective #3 - Are the commonly used WET testing species, fathead minnow (*Pimephales promelas*) and brook trout (*Salvelinus fontinalis*) suitable surrogates for Atlantic sturgeon, shortnose sturgeon, and Atlantic salmon?

3.3.1 Fathead Minnow Surrogacy

Fathead minnow were originally discussed by the team as a possible surrogate for investigation, but a study by Dwyer et al. (2005) suggested that brook trout are more sensitive to

contaminants in wastewater effluent than fathead minnow. Further, a study by Cope et al. (2011) argues that fathead minnow are unlikely to be an appropriate surrogate for shortnose sturgeon, demonstrated by side-to-side cage studies in an urbanized river in North Carolina. These studies suggest that fathead minnow may be the least comparable surrogate species, so efforts were focused on brook trout.

3.3.2 Brook trout Vtg mRNA

> *Brook trout Vtg responses to the EDCs used in this study were similar to those of Atlantic salmon. Therefore, measurement of Vtg and other endocrine disrupting responses in brook trout may be appropriate surrogates for understanding impacts to Atlantic salmon.*

Brook trout demonstrate a similar pattern of responsiveness (Figure 10) in Vtg mRNA to the pattern seen in early life stages of salmon (in particular, yolk-sac larvae and feeding fry). In this experiment, brook trout juveniles that were approximately two weeks post first-feeding were exposed to two compounds (EE2 and NP) for 21 days. Eight fish were sub-sampled at day four for Vtg mRNA for comparison to the acute exposures. The remaining fish were exposed for an additional 18 days and demonstrated nearly identical patterns of Vtg upregulation. Interestingly, the total overall transcription decreased between day four and day 21, indicating possible downregulation of Vtg transcription during long-term exposure.

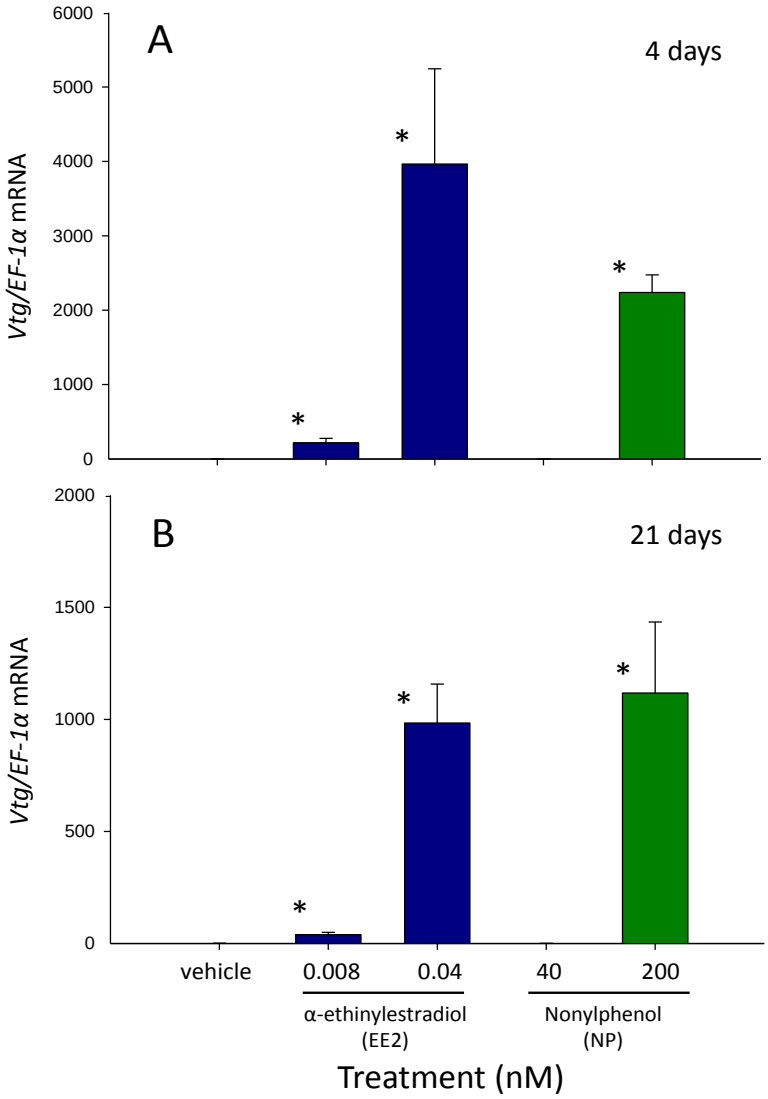

Figure 10. Plasma Vtg mRNA transcription in brook trout juveniles exposed to endocrine disrupting compounds for 21 days. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, and 4-nonylphenol (NP) is in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

Chronic exposure of Atlantic salmon results in Vtg mRNA transcription that is similar to that of brook trout (Figure 11). In brook trout, all doses elicit a positive response at four days of exposure (Figure 11A), but the low doses of both compounds show no significant Vtg response at 21 days (Figure 11B). These slight differences in response between brook trout and Atlantic salmon may indicate differences in sensitivity to these compounds or differences in downregulation of Vtg over time. Alternatively, these slightly differing responses may reflect slight differences in age/life-stage between the brook trout (two weeks post first-feed) and Atlantic salmon (six weeks post first-feed).

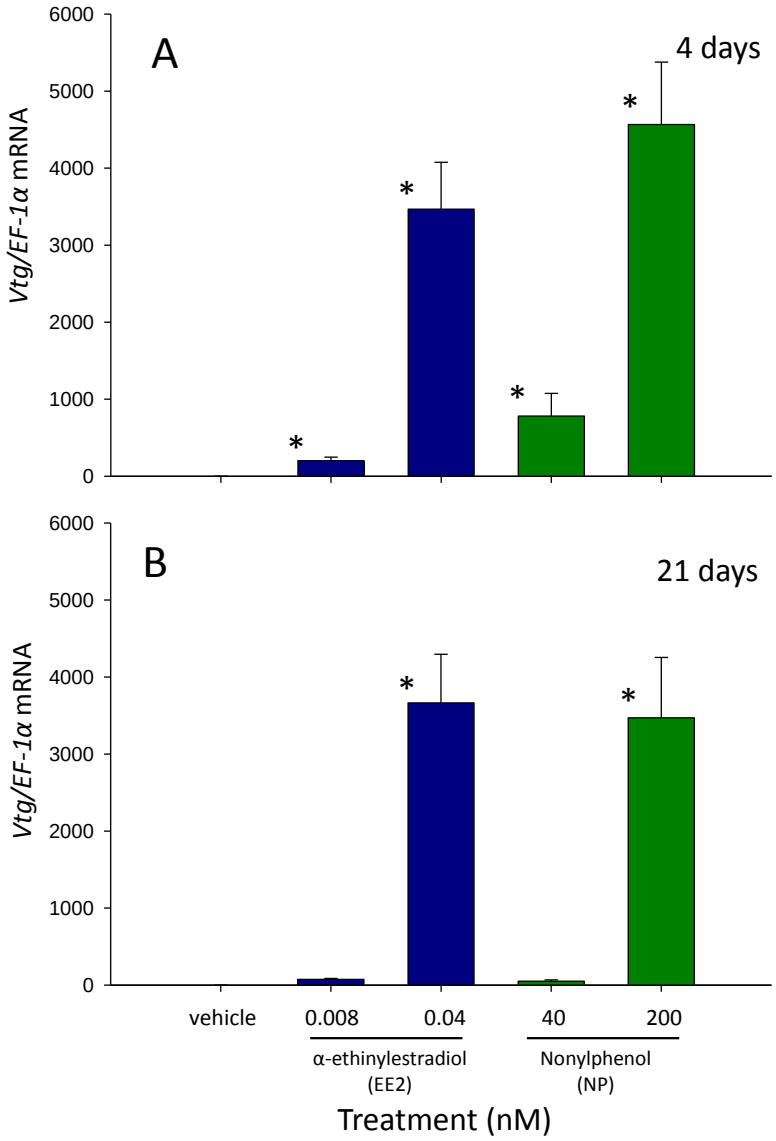

Figure 11. Plasma Vtg mRNA transcription in Atlantic salmon juveniles exposed to endocrine disrupting compounds for 21 days. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, and 4-nonylphenol (NP) is in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control.

3.3.3 Brook trout surrogacy

Differential responsiveness between brook trout and Atlantic sturgeon Vtg mRNA indicates that it is not an appropriate surrogate for EDC responses in sturgeon.

Vtg demonstrates very different patterns between the salmonids (brook trout and Atlantic salmon) and the sturgeon (shortnose sturgeon and Atlantic sturgeon). Because EDCs most

commonly elicit impacts through the estrogen receptors (ERs), it may be that the ER pathway is not fully developed in early stage sturgeon, but that this pathway develops quickly in salmonids. Dissimilarity in Vtg mRNA response (presumably due to differential development of the ER (or other) pathways) between brook trout and Atlantic sturgeon indicates that brook trout may not be an appropriate surrogate species for understanding impacts in Atlantic sturgeon and potentially other salmonids. Sturgeon and salmon have very different life-histories, with different ages at maturity possibly accounting for the different ages at which Vtg responsiveness "turns on" in an animal.

3.4 Mortality and growth

3.4.1 Mortality data

> *Mortality in all exposure experiments was minimal, and no treatment effects were observed. LD_{50} is therefore not an appropriate measure of impacts of these animals in response to common EDCs.*

In this collection of exposures, mortality was generally low and no treatment effects on mortality were seen in either the four-day or 21-day exposures (Table 5). Therefore, LD_{50}, the dose or dilution at which 50% mortality occurs (Chapman 2000) could not be determined for any compound because greater than 50% mortality must be observed in at least one treatment. This measure of mortality often overlooks sublethal effects which can alter long-term behavior, disease susceptibility, and overall fitness. For example, Lerner et al. (2007b) exposed Atlantic salmon to E2 and NP for 21 days during the yolk-sac fry stage and saw minimal mortality during exposure for E2 and the lowest dose of NP, both of which were closely approximated to doses used in this study. These fish did however exhibit reduced ability to increase gill Na^+/K^+-ATPase activity during preparation for seawater entry as smolts, indicating a decrease in ion regulatory ability and the potential for increased seawater mortality. Other studies have linked estrogenic compounds to reduced survival during smolting (McCormick et al. 2005, Bangsgaard et al. 2006), demonstrating long-lasting, sublethal effects that result in morbidity and mortality later in life.

Table 5. Summary of mortality data for all exposures carried out in this study.

Species	Exposure period	Life stage	Average experiment mortality	# fish per treatment	P-value*
Atlantic salmon	4-day	embryo	4.5 ± 0.8	40	0.22
Salmo salar		yolk sac larvae	1.1 ± 0.8	36	0.09
		feeding fry	1.1 ± 0.8	32	0.09
		smolts (1-year old)	0	12	--
	21-day	feeding fry	2.2 ± 1.4	90	0.43
Brook trout	21-day	feeding fry	0.9 ± 0.2	88	0.83
Salvelinus fontinalis					
Shortnose sturgeon	4-day	embryo	15.6 ± 2.6	32	0.19
Acipenser brevirostrum		yolk sac larvae	1.7 ± 1.1	12	0.49
		feeding fry	7.5 ± 2.8	8	0.35
		juveniles (2-years old)	0	8	--
Atlantic sturgeon	21-day	juveniles (4-months)	2.2 ± 1.4	90	0.095
Acipenser oxyrhynchus					

*statistical analyses carried out by one-way ANOVA on ranks

WET testing endpoints are often LD_{50} values for short-term, two-day or four-day exposures to wastewater effluent. The team saw no contaminant-related mortality in any of the studies on Atlantic salmon, shortnose sturgeon, brook trout, and Atlantic sturgeon exposed to endocrine disrupting compounds in four-day exposures.

The maximum concentration of each contaminant used was chosen to elicit a strong, positive vitellogenic response based on studies of other species. These higher concentrations represent contaminant levels that are ten to fifteen times higher than what fish would be exposed to in the wild with regard to the concentration of any single contaminant.

Our results indicate that the high doses of each of the compounds tested elicited a vitellogenic response, an indicator of long-term reproductive abnormality. Since LD_{50} levels are greater than the highest dose used for each compound, the results suggest that LD_{50} is not an adequate measure of single-contaminant impact on long-term fitness and development of Atlantic salmon, shortnose sturgeon, brook trout or Atlantic sturgeon.

3.4.2 Growth data

Acute exposures

> *Impacts to growth were nonexistent to minor in four-day exposures of Atlantic salmon and shortnose sturgeon.*

Atlantic salmon

Feeding fry size at the end of the four-day experiment showed some minor, but overall significant treatment effects for animals exposed to E2 and EE2 (weight-p=0.04, E2, length-p=0.004, E2 and p=0.008, EE2, one-way ANOVA). However, no differences between controls and treatments were observed with *post hoc* tests. Differences among treatments did not exhibit dose-dependence (although EE2 demonstrated a general decrease in size with increasing dose. No treatment effects on body size were seen in smolts following acute exposures.

Shortnose sturgeon

Shortnose sturgeon fry showed no treatment effects on length or weight for any exposures except for E2 (p=0.045, one-way ANOVA). However, no treatments were significantly different from the control. Two year old sturgeon also demonstrated no treatment effects on body size.

Chronic exposures

Impacts to growth (or size at age) were assessed by comparing the size (weight and total length) at day 21 of exposure. Individuals were randomly assigned to tanks, and size did not differ significantly among treatments at the start of each experiment. Therefore, one-way ANOVA was used to test for significant differences in size at the end of each chronic exposure (21-day) in Atlantic salmon, brook trout, and Atlantic sturgeon.

Atlantic salmon

Atlantic salmon feeding fry demonstrated small, but significant changes to growth in response to 21-day EDC exposure, with the greatest impacts from nonylphenol exposure. Marginally non-significant impacts to weight (p=0.051) were noted (Figure 12A), but lengths at day 21 differed significantly (p=0.002) with lengths at 200 nM NP significantly reduced compared to the lengths of control fish. Exposure to EDCs impaired growth over a three week period, but little is known about how growth may be impaired in the long term, especially for animals that may be exposed to these compounds for longer periods (>21 days).

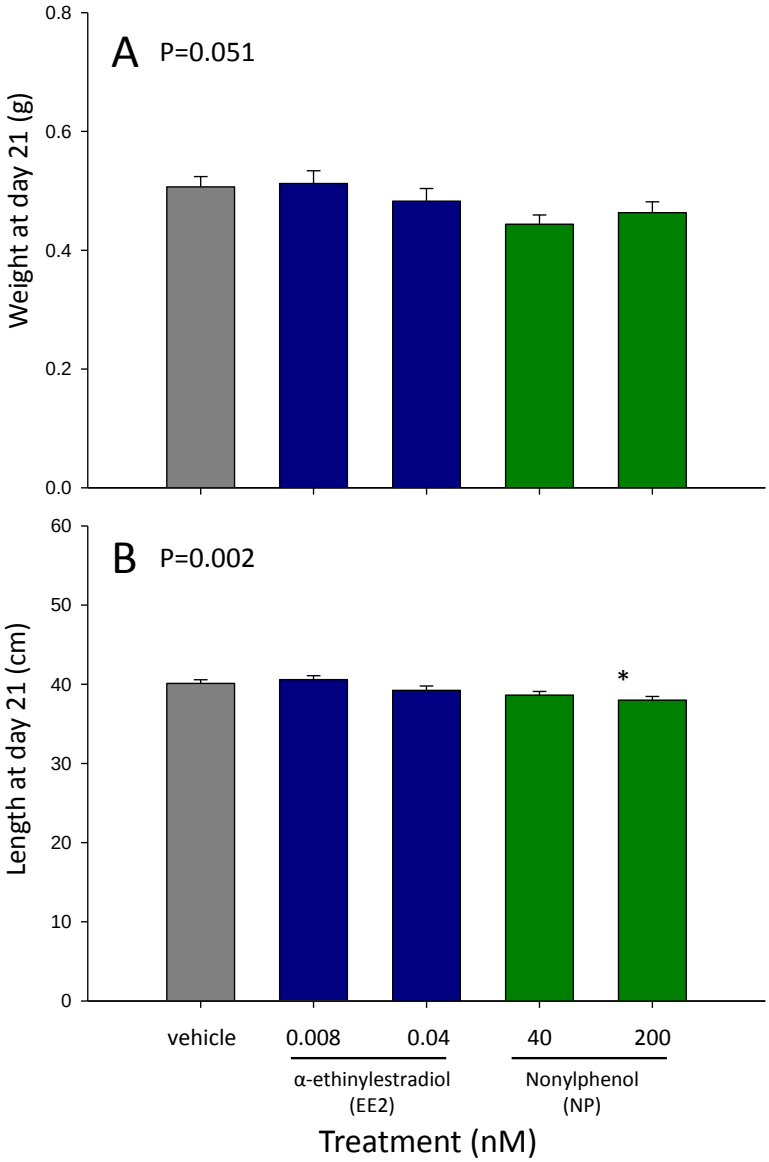

Figure 12. Weight and total length of Atlantic salmon after exposure to EDCs for 21 days. P-value represents an overall treatment effect (one-way ANOVA on ranks) and asterisks indicate significant differences between the treatment and control.

Brook trout

There were small, but significant differences in body size at the end of 21-day exposures of brook trout to EDCs (Figure 13). Both length and weight demonstrated a significant overall effect of EDC exposure (one way ANOVA on ranks, $p<0.001$ for both), with NP 200 nM significantly decreasing both the weight (Figure 13A) and total length (Figure 13B) relative to control animals. These results are similar to growth impacts seen in 21-day Atlantic salmon exposures and suggest that growth effects in these two salmonids may be comparable.

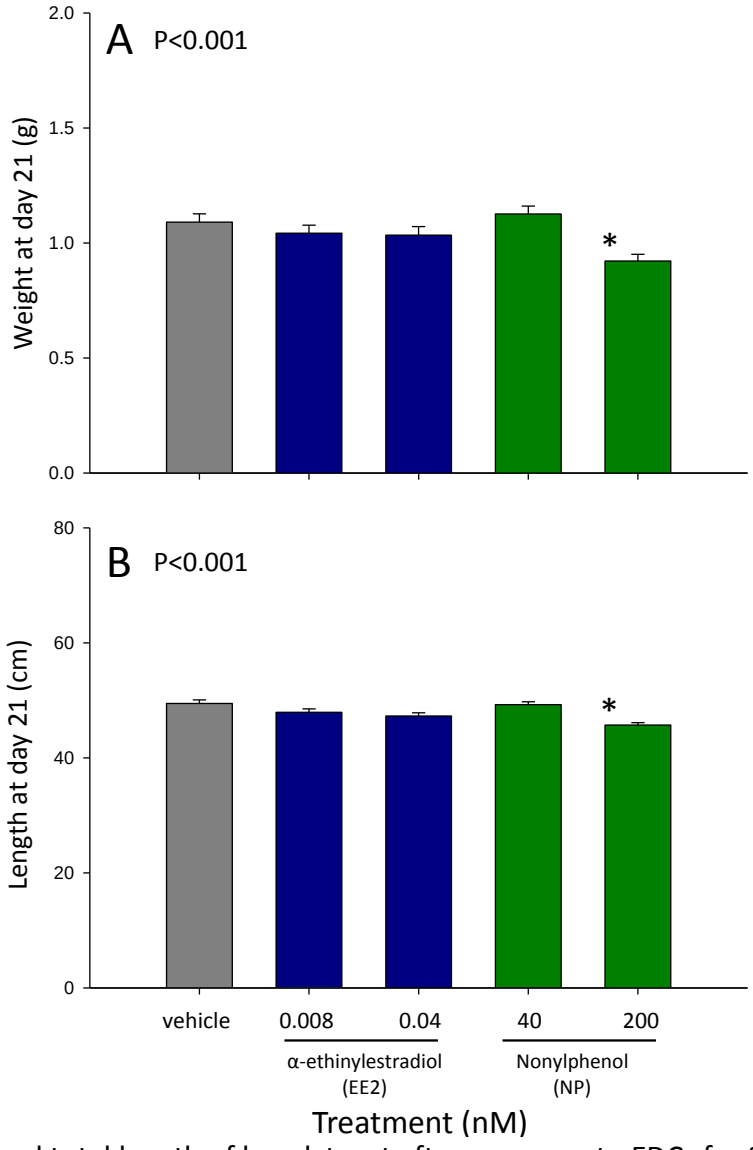

Figure 13. Weight and total length of brook trout after exposure to EDCs for 21 days. P-value represents an overall treatment effect (one-way ANOVA on ranks) and asterisks indicate significant differences between the treatment and control.

Atlantic sturgeon

No size differences among Atlantic sturgeon were observed after 21 days of exposure to EDCs (Figure 14). Sturgeon are slow-growing and demonstrate high variability in growth rates among individuals from the same cohort (personal observation). Therefore, this large variability in size at the start of the exposure may have masked differences among treatments. In order to determine if impacts to growth are likely in response to EDC exposure in Atlantic sturgeon, it may be necessary to expose animals for a longer period of time in order to determine impacts to growth.

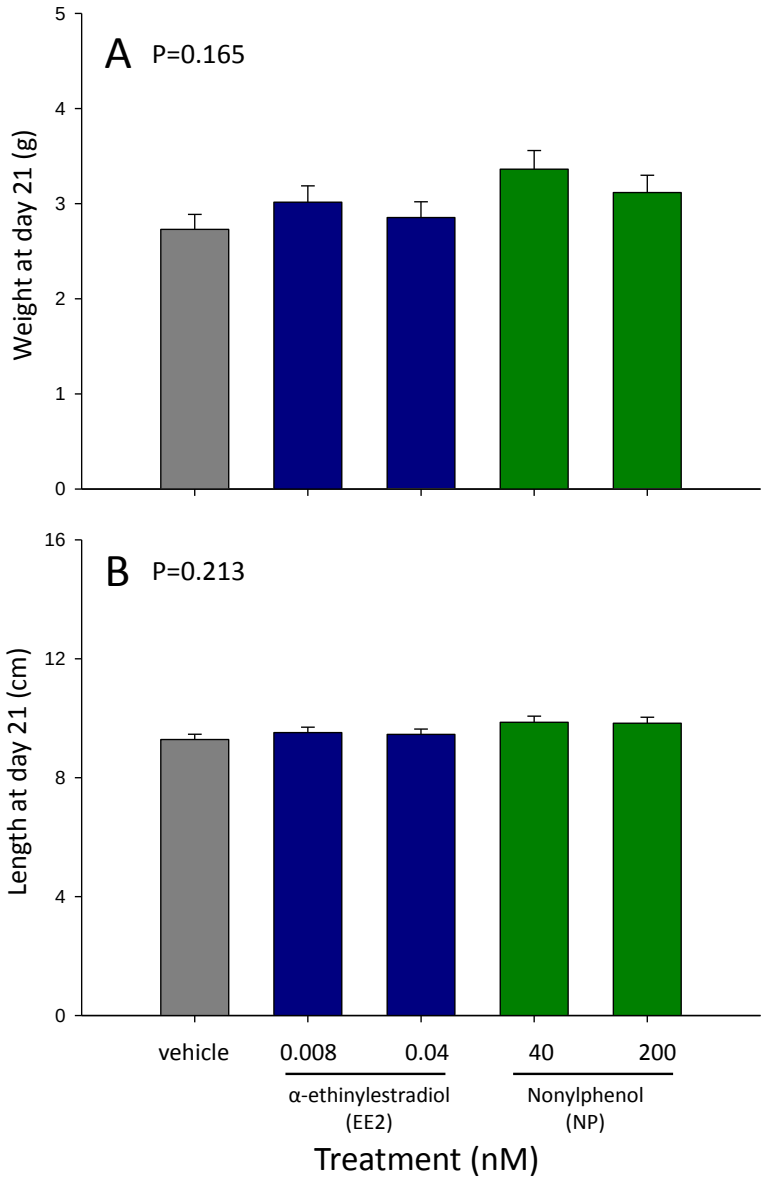

Figure 14. Weight and total length of Atlantic sturgeon after exposure to EDCs for 21 days. P-value represents an overall treatment effect (one-way ANOVA on ranks).

4 YEAST E-SCREEN ASSAY

Treated water from six wastewater treatment facilities in Massachusetts and Maine demonstrate low to high estrogenic activity at levels that could potentially impact Vtg regulation in salmon or sturgeon downstream from the wastewater outflow.

Background

The yeast estrogen screen (YES or E-screen) assay was developed in the mid 1990's to measure relative estrogen potency of effluent, receiving water, or other exposure medium. It is based on the principle that many estrogenic compounds in the environment compete with natural estrogen in a cell to bind to the estrogen receptor (Arnold et al. 1996). It is a useful tool because it can be used to determine combined estrogenicity of mixtures of compounds, as well as estrogenicity of single compounds present in water or other medium (e.g., sediment, sludge, etc.). The assay was developed using yeast cells as a relatively inexpensive and accurate alternative to estrogen receptor assays using mammalian cell lines (Arnold et al. 1996) and is used to determine estrogenic potential of different media (Leusch et al. 2010).

The E-screen assay was developed by expressing the human estrogen receptor (hER) and two estrogen response elements to the lacZ gene. When exposed to estrogenic compounds, activation of the lacZ gene increases beta-galactosidase activity which causes a color change (Arnold et al. 1996). Yeast cells with hER are exposed to the test medium (wastewater samples, etc.) and color change is monitored. In samples where the estrogenic activity is high, the binding of an estrogen or estrogen-mimic to the estrogen receptor results in a color change that can be measured with a spectrophotometer. Samples where no binding occurs (low estrogenic activity in the sample) will not undergo a color change. These absorbance readings are then compared to a 17 β-estradiol standard curve to estimate estrogen equivalence (EEQ) (Colosi and Kney 2011).

Literature indicates that the E-screen assay has been used by testing facilities and laboratories to demonstrate estrogenic activity of wastewater and single (or mixtures of) compounds. Although less abundant in the literature, it is also used to compare EEQs among different compounds or effluents that fish are exposed to in the laboratory. Kitamura et al. (2009) exposed Japanese medaka to two estrogenic compounds, river water, and treated wastewater to screen for Vtg induction. The water used in the exposures was analyzed using the E-screen assay, and the authors were able to determine a level of EEQ at which Vtg was upregulated (5 ng E2/l) in their study species.

Objectives

The purpose of introducing the E-screen assay to our plans for Option Year Two was to 1) determine baseline EEQ data for Vtg upregulation in our study species and 2) survey water sources for EEQ values where sturgeon and/or salmon are expected to occur. In collaboration with Dr. Luke Iwanowicz at the USGS Leetown Science Center Laboratory, we ran E-screen assays for the water samples collected during our 4-day and 21-day exposures, as well as water

samples collected from sturgeon overwintering grounds and other locations in Massachusetts and Maine where ESA-listed species occur. This allowed us to determine EEQs for Vtg upregulation for these species and compare our laboratory studies to preliminary baseline data from riverine systems in New England. (Note regarding item 1: Dr. Iwanowicz is currently analyzing the EEQ values from laboratory exposures and these will be provided to NOAA as an addendum as soon as possible. This data will be used in a publication of this research to demonstrate estrogenicity of water used in our exposures.)

Effluent water samples were collected between December 29, 2012 and January 4, 2013 between 9 AM and noon from six wastewater treatment facilities; two in Massachusetts and four in Maine. All samples were voluntary, anonymous, and non-regulatory. All samples came from moderately urbanized NPDES-permitted facilities which currently test for BOD, suspended solids, chlorine, and coliform bacteria but not EDCs. The community populations served by the facilities range from 8,500 to 59,000. Due to the anonymity, the data is coded (Table 6).

Table 6. Results from the E-screen assay. Estrogenic activity from six wastewater treatment plants is indicated as Estradiol Equivalent Concentration (EEQ). Due to anonymity, the six municipalities from Massachusetts (MA) and Maine (ME) are coded and population sizes are given as ranges. Dechlorinated city water (Turners Falls, MA) was the water used in all exposures in this study (controls and EDC exposures).

Water sample	EEQ (ng/L)	EEQ (nM equivalent)	population size (x thousand)
Dechlorinated city water	BD	BD	--
Distilled water	BD	BD	--
MA1	1.7	0.0062	5-10
MA2	2.12	0.0078	15-20
ME1	1.09	0.0040	55-60
ME2	3.24	0.0119	15-20
ME3	0.8	0.0029	15-20
ME4	4.5	0.0165	30-35

BD-below the limit of detection

E-screen assay data is reported as Estradiol (17 β-estradiol, E2) Equivalent Concentration, or EEQ. This represents an estrogenic signal that is equivalent to exposure to a particular

concentration of E2. For comparison, we have included EEQ values in nM equivalency for direct comparison to the concentrations used in this study. In this snapshot from WWTP effluent in MA and ME, all water samples induced estrogenic activity (Table 6). Further, all samples displayed estrogenic activity that were lower than or comparable to the lowest concentration of f E2 used in the short-term exposures of Atlantic salmon and shortnose sturgeon (0.04 nM). Water samples were collected from the wastewater treatment facility after treatment and immediately prior to discharge and therefore reflect an EEQ that is likely to be higher than the estrogenicity of the receiving waters due to dilution. It is important to note that the water samples analyzed were "grab" samples and do not reflect the mean estrogenicity of the effluent, nor do these EEQ values reflect what salmon or sturgeon are exposed to in adjacent waters. All samples assayed contained compounds that
were bound to the estrogen receptor and therefore potentially contain endocrine disruptors. A detailed analysis of both the wastewater from these facilities, along with receiving water is necessary to determine the range of estrogenic values and the potential for endocrine disruption in salmon or sturgeon.

5 ADDITIONAL BIOMARKERS OF EDC EXPOSURE

Acute (four-day) and chronic (21-day) exposure to common endocrine disrupting compounds alters transcription of genes involved in growth in Atlantic salmon.

Previous research suggests that genes involved in ion transport and regulation are impaired in response to EDCs in Atlantic salmon (Lerner et al. 2007a,b). Therefore, the team assessed the transcription of eight genes involved in ion regulation, homeostasis and general seawater preparedness in salmon smolt gill tissue from animals exposed to EDCs for four days (Figure 15). Overall, most genes were not induced by EDC exposure at the end of four days, but both $nka_{\alpha 1a}$ and *igf1* were induced by EDC exposure (Figures 15A and 15F, respectively). No treatment was significantly upregulated relative to the control, indicating that induction of these genes was variable among treatments. NKAs ($nka_{\alpha 1a}$ and $nka_{\alpha 1b}$) are ion transport proteins that pump sodium and chloride across the cell membrane to maintain homeostasis (McCormick et al. 2013), which is especially important as animals move between fresh and seawater.

In addition, the growth hormone (GH) and insulin-like growth factor (IGF) axis is involved in osmoregulation (McCormick et al. 2011, Yada et al. 2012) and may also be impaired by endocrine disruptors (Lerner et al. 2012). Growth hormone (GH) and IGF(-1) are polypeptides that are essential for regulation of growth, development, and differentiation in vertebrates (Wood et al. 2005). During positive growth, GH is released from the pituitary and binds to hepatic GH receptors. This in turn stimulates release of IGF-1 into the blood stream, which acts as a negative feedback on GH release and synthesis (Fruchtman et al. 2000, Wood et al. 2005).

Insulin-like growth factor-I promotes cell differentiation and cell growth and overall somatic growth (McCormick et al. 1992) via the IGF-1 receptor (IGF-1R) (Wood et al. 2005). In this study, short-term (four-day) exposure to EDCs does not appear to influence transcription of these genes in a dose-dependent manner in smolt gill tissue.

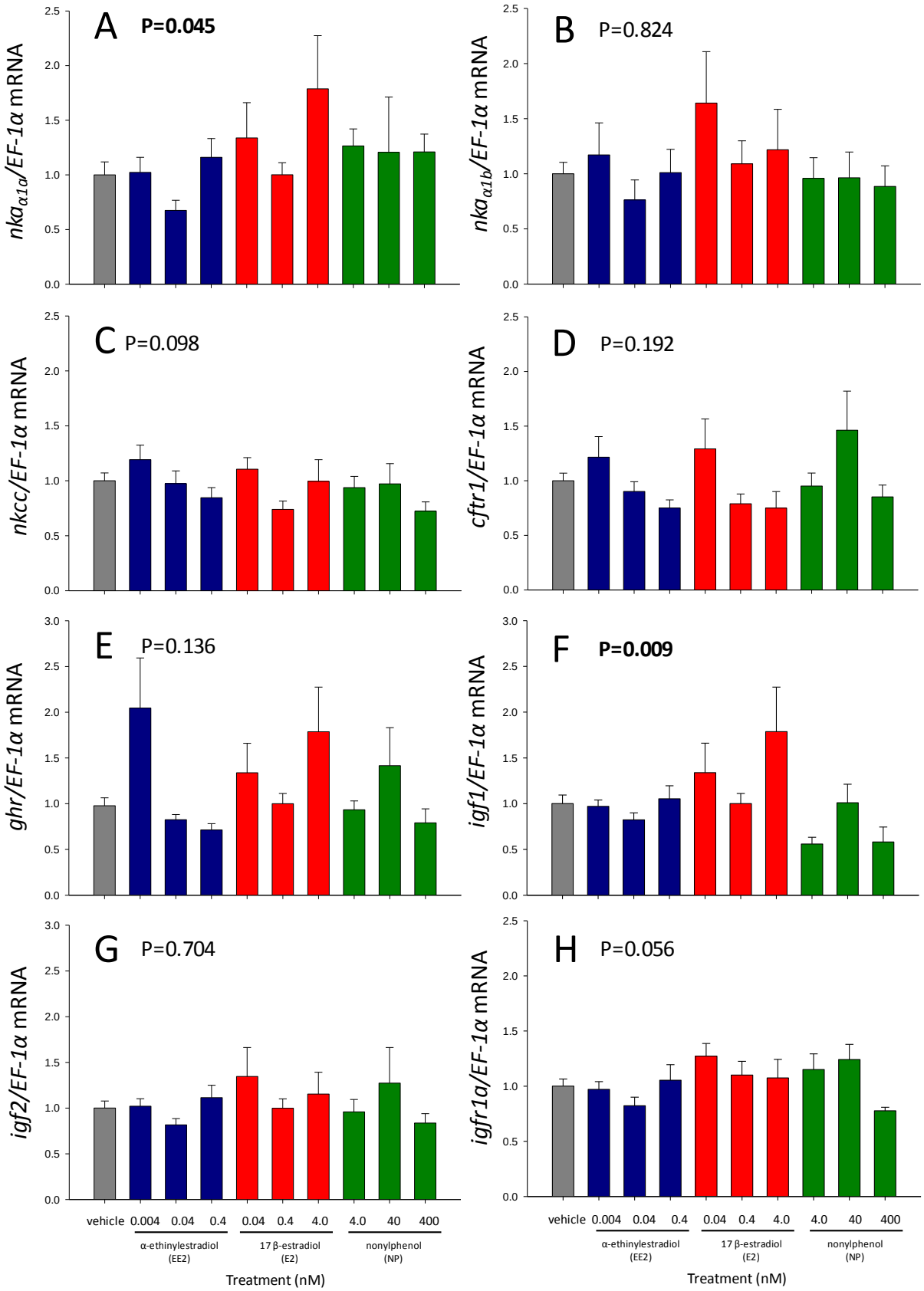
A P=0.045
B P=0.824
C P=0.098
D P=0.192
E P=0.136
F P=0.009
G P=0.704
H P=0.056
nkα1a/EF-1α mRNA
nkα1b/EF-1α mRNA
nkcc/EF-1α mRNA
cftr1/EF-1α mRNA
ghr/EF-1α mRNA
igf1/EF-1α mRNA
igf2/EF-1α mRNA
igfr1a/EF-1α mRNA
vehicle 0.004 0.04 0.4 0.04 0.4 4.0 4.0 40 400
α-ethinylestradiol (EE2)
17 β-estradiol (E2)
nonylphenol (NP)
Treatment (nM)

Figure 15. Transcription of eight genes involved in ion regulation and/or growth in gill tissue of Atlantic salmon smolts exposed to EE2, E2, or NP for four days. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. P-values indicate overall significance by one-way ANOVA (note, *post hoc* comparisons yielded no treatments that were statistically significant from the controls).

To determine impacts to gene expression in other tissues, the team investigated several genes involved in reproduction and growth, including estrogen receptor α (*erα*), growth hormone receptor (*ghr*) and two IGF isoforms (*igf1* and *igf2*) in liver of Atlantic salmon smolts exposed to EDCs for four days (Figure 16). All genes showed significant response to EDCs with increased transcription of *era* and a general trend of repression of genes in the GH-IGF growth axis. These changes to the GH-IGF axis indicate that these common EDCs act not only through the estrogen receptor pathway (erα), but have effects on expression of genes involved in growth (either directly on growth axis genes or through the estrogen receptor-mediated pathway).

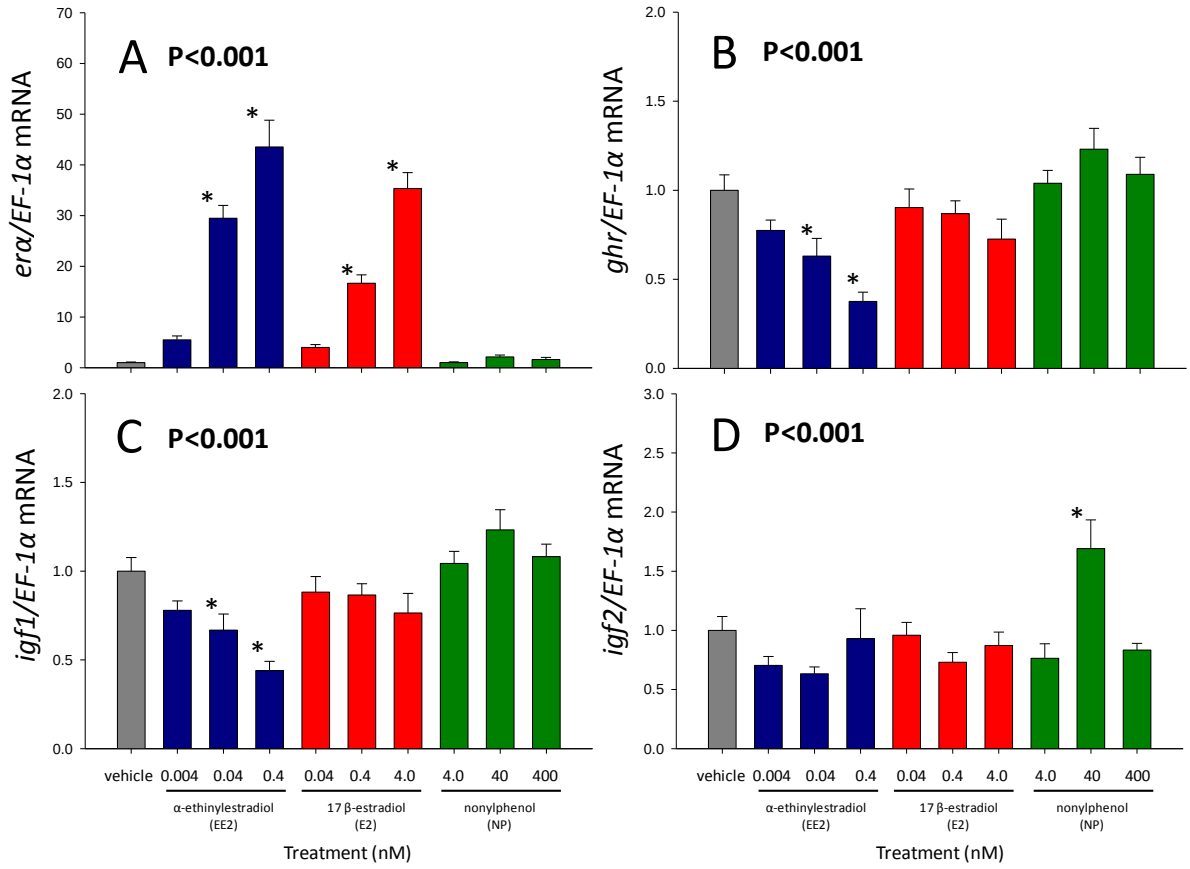

Figure 16. Transcription of four genes involved in reproduction, development and growth from the liver of Atlantic salmon smolts exposed to EE2, E2, or NP for four days. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control. P-values indicate overall significance by one-way ANOVA.

Interestingly, several of the numerous forms of insulin-like binding proteins (*igfbp 1-6*) were also influenced by EDC exposure in Atlantic salmon smolts (Figure 17). IGF binding proteins mediate the impacts of IGF by transporting IGF to IGF receptors and either facilitating or inhibiting binding (Shimizu et al. 2009). Several IGFbps were significantly downregulated in response to EDCs, with the exception of *igfbp5.2* that showed highly increased transcription in response to EE2 and E2. While the exact function of all these different binding proteins is unknown, it is clear that each of the three common EDCs used in this study are capable of altering expression patterns of one or more IGF binding proteins in Atlantic salmon, suggesting that impacts to growth or other physiological processes may be possible.

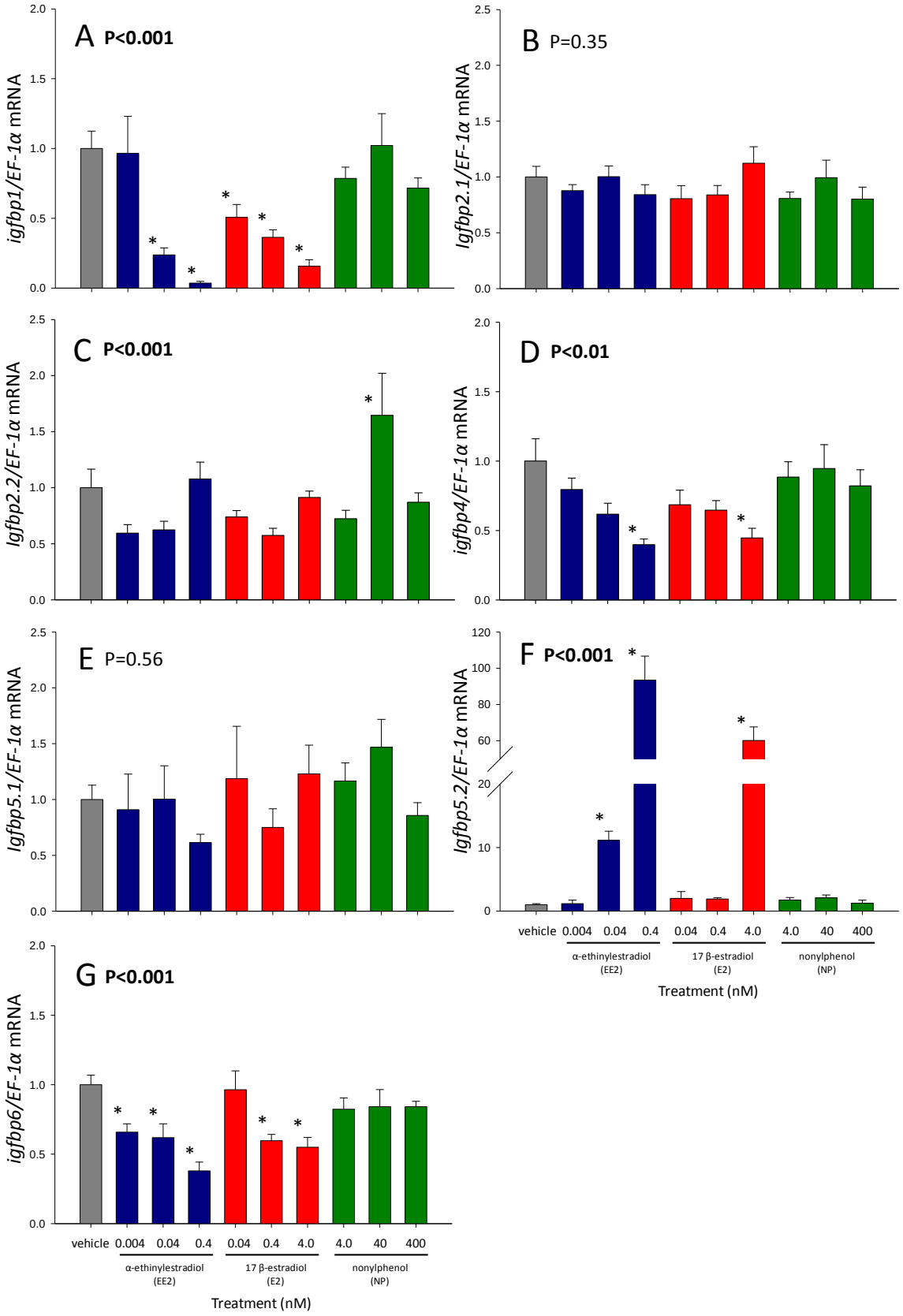
A P<0.001
B P=0.35
C P<0.001
D P<0.01
E P=0.56
F P<0.001
G P<0.001
igfbp1/EF-1α mRNA
Igfbp2.1/EF-1α mRNA
Igfbp2.2/EF-1α mRNA
igfbp4/EF-1α mRNA
Igfbp5.1/EF-1α mRNA
Igfbp5.2/EF-1α mRNA
igfbp6/EF-1α mRNA
vehicle 0.004 0.04 0.4 0.04 0.4 4.0 4.0 40 400
α-ethinylestradiol (EE2)
17 β-estradiol (E2)
nonylphenol (NP)
Treatment (nM)

Figure 17. Transcription of seven IGF binding protein (*igbbp1-6*) genes involved growth from the liver of Atlantic salmon smolts exposed to EE2, E2 or NP for four days. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control. P-values indicate overall significance by one-way ANOVA.

Because large changes to gene transcription were seen in salmon smolt livers exposed to EDCs for four days, the team investigated the same genes in livers from feeding fry exposed to EDCs for 21 days. Interestingly, *erα* was downregulated in response to EE2 and NP (Figure 18A) at 21 days compared to increased transcription seen after four days in smolts (Figure 16A). The downregulation of *erα* could help to explain the slight differences in Vtg transcription between day four and day 21 (Figure 11). As estrogen receptor is downregulated, Vtg transcription in the liver would also likely decrease over time.

Impacts to growth-related genes were also significant in 21-day exposed Atlantic salmon, with significant depression of transcription for ghr and the igf's in the liver (Figure 18B-D). Growth was significantly impacted in this experiment by EDC exposure, with significant impacts to Atlantic salmon fry exposed to a high dose of NP. This may suggest a possible link between impacts to the animals' growth phenotype and expression of genes in the GH-IGF axis.

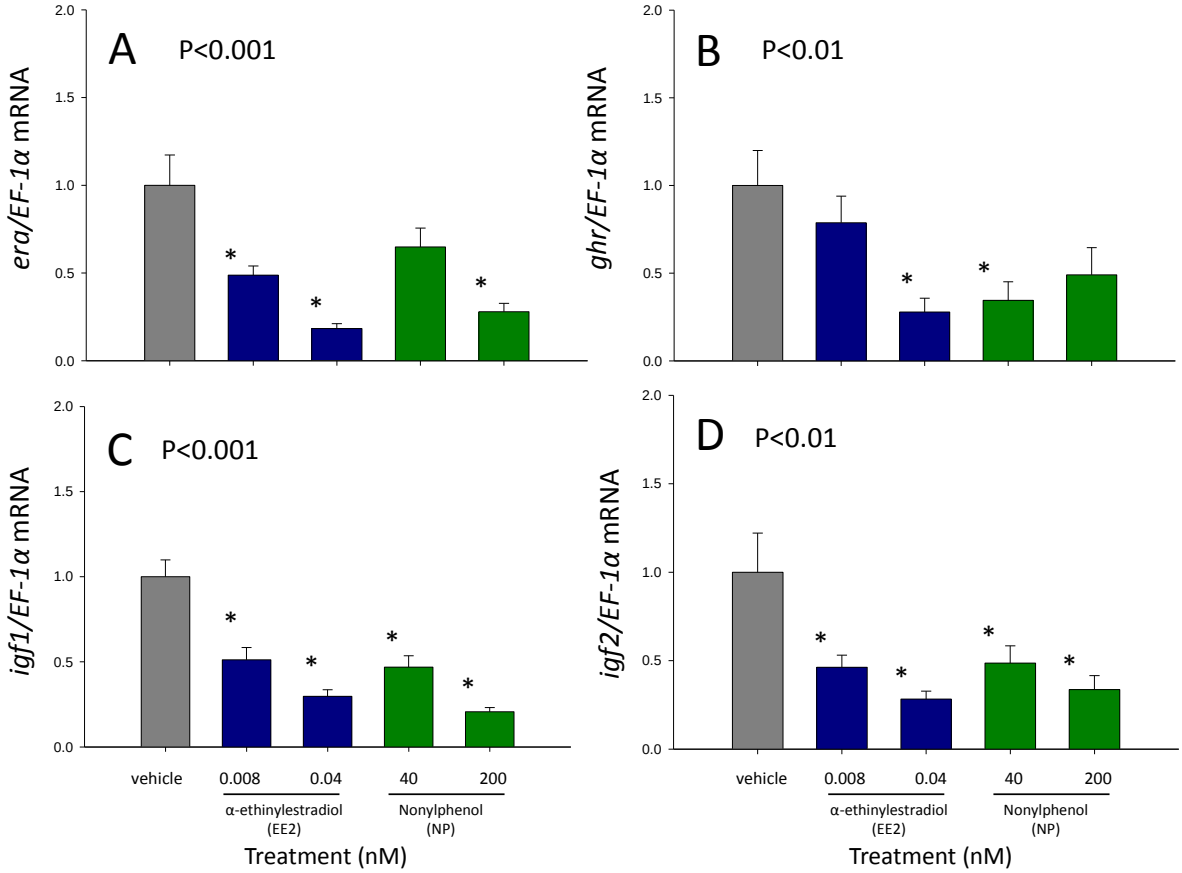

Figure 18. Transcription of eight genes involved in ion regulation and/or growth from the liver of Atlantic salmon feeding fry exposed to EE2, E2, or NP for 21 days. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control. P-values indicate overall significance by one-way ANOVA.

Transcription of binding proteins in juvenile Atlantic salmon livers was significantly altered by EDC exposure after 21 days (Figure 19). Patterns of transcription were in the same general direction as was seen in four-day exposed smolts, but these exposures demonstrated transcriptional responses in nonylphenol exposed animals that were not present in smolts. Here it is also important to note that because not much is known about the impacts of these individual binding proteins (Bower et al. 2008) it is necessary to determine what, if any, impacts to the fitness of fish these altered gene transcription patterns may convey.

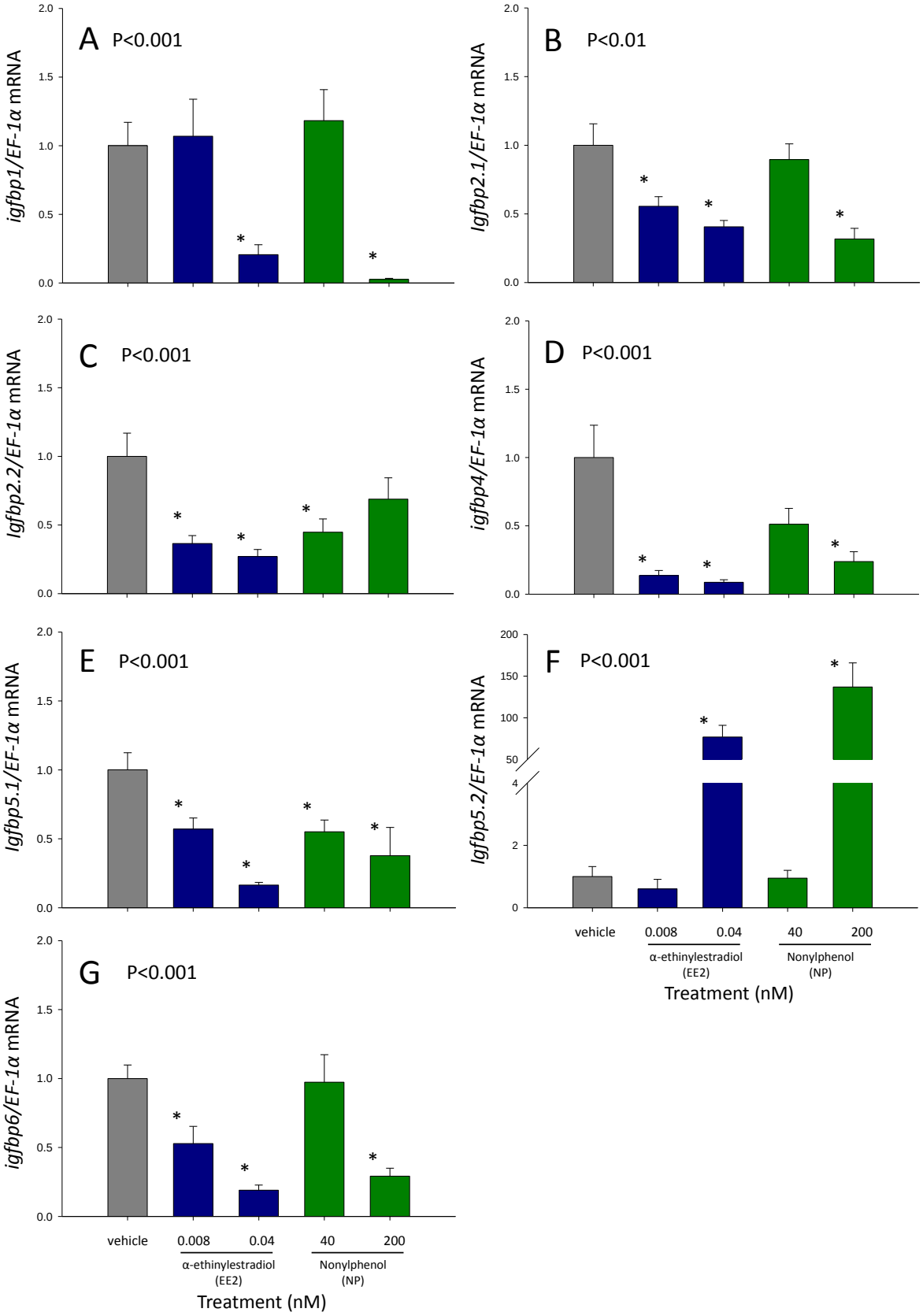
A P<0.001
B P<0.01
C P<0.001
D P<0.001
E P<0.001
F P<0.001
G P<0.001
igfbp1/EF-1α mRNA
Igfbp2.1/EF-1α mRNA
Igfbp2.2/EF-1α mRNA
igfbp4/EF-1α mRNA
Igfbp5.1/EF-1α mRNA
Igfbp5.2/EF-1α mRNA
igfbp6/EF-1α mRNA
vehicle
0.008
0.04
40
200
α-ethinylestradiol (EE2)
Nonylphenol (NP)
Treatment (nM)

Figure 19. Transcription of eight genes involved in ion regulation and/or growth from the liver of Atlantic salmon feeding fry exposed to EE2, E2, or NP for 21 days. Values are normalized to the housekeeping gene, EF-1α, and then normalized to the control. The control is indicated in grey, α-ethinylestradiol (EE2) is indicated in blue, 17 β-estradiol (E2) in red, and 4-nonylphenol (NP) in green. Bars represent treatment means ± standard error. Asterisks indicate significant differences between each concentration and the control. P-values indicate overall significance by one-way ANOVA (note, no individual statistically significant differences as compared to the controls).

6 SUMMARY OF CONCLUSIONS AND RECOMMENDATIONS

Current WET test methods are not appropriate for determining long-term impacts to growth and reproduction in Atlantic salmon, shortnose sturgeon and Atlantic sturgeon exposed to EDCs.

- All three endangered species used in this study demonstrated endocrine disruption in response to EDC exposure in the form of increased Vtg transcription. However, the most commonly used WET test endpoints used with surrogate fish species (mortality and growth) were not altered within the time-frame of a routine WET test (< 96 hours). This indicates that little information about EDC exposure and subsequent endocrine disruption (whether short- or long-term) can be gained from common WET test measures of mortality and growth.

Mortality in all exposure experiments was minimal, and no treatment effects on mortality were seen.

- LD_{50} is not an appropriate measure of potential long-term impacts or sublethal effects on fitness due to exposure to the common EDCs, E2, EE2, and NP.

Vtg is an appropriate and sensitive biomarker of acute EDC exposure in most life stages of Atlantic salmon.

- Vtg is a sensitive biomarker for common EDCs, but more research is necessary to determine negative impacts that are most likely the result of long-term exposure (21 days or more).

Vtg mRNA is not responsive to EDC exposure in early life stages of shortnose sturgeon, but may be a good biomarker in older animals.

- Vtg response is possible in older animals, but more research is needed to determine the age at which the Vtg response to EDCs is activated. Further research is needed to determine if shortnose sturgeon are not susceptible to these compounds in early life stages, or if these compounds affect physiological processes not measured in the current study.

Lack of Vtg response in shortnose and Atlantic sturgeon may indicate that sensitivity to EDCs may occur at older ages/life stages than Atlantic salmon and other fish species.

- Lack of Vtg response in early life stages may indicate that sensitivity to these compounds may be reduced until an age at which responsiveness develops, perhaps in association with sexual differentiation which occurs later in sturgeon than in salmonids.

Because brook trout are more responsive to EDCs than Atlantic sturgeon in early life stages, it may be a more useful surrogate species *if the precautionary principle is to be applied*.

- Sensitive biomarkers of brook trout to EDC exposure (Vtg induction) could be used to determine potential estrogenic effects of water where sturgeon (and salmon) are found. While sturgeon may not show a response in early life stages, brook trout are likely to respond with Vtg gene transcription to lower levels of EDCs, indicating the potential for long-term estrogenic impacts on sturgeon.
- Brook trout *should not be considered appropriate surrogates* for Atlantic salmon, Atlantic sturgeon or shortnose sturgeon if the endpoints of a WET test are growth and/or mortality.
- Further research is needed to determine whether longer-term (>21 days) exposure to environmentally relevant concentrations of EDCs induces long term impacts in brook trout, Atlantic salmon, Atlantic sturgeon and/or shortnose sturgeon.

Impacts to growth were undetectable or slight in four-day exposures of Atlantic salmon and shortnose sturgeon.

- Acute (four-day) exposures appear to not induce changes in growth among treatments, or this short period is not long enough to induce changes that are statistically detectable in a short time period.

Impacts to growth were detected in 21-day exposures of Atlantic salmon, brook trout, and Atlantic sturgeon.

- Chronic (21-day) exposures to EDCs induced reduced growth in animals. However, this happened only after long-term (21 days) of exposure, a time frame that is not used in WET tests. Therefore, growth is not an appropriate measure of sublethal, long-term impacts of EDC exposure, particularly for periods as short as 48 to 96 hours.

Treated water from six wastewater treatment facilities in Massachusetts and Maine demonstrate estrogenic activity

- Wastewater samples from MA and ME WWT facilities that discharge into rivers where Atlantic salmon, Atlantic sturgeon, and shortnose sturgeon are found show elevated estrogenicity. The levels of estrogenicity in these water samples are comparable to or lower than the low dose of E2 used in this study, indicating the potential for Vtg induction in fish that may be exposed to this effluent.

Acute (four-day) and chronic (21-day) exposure to common endocrine disrupting compounds alters transcription of genes involved in growth in Atlantic salmon.

- Genes involved in the GH-IGF axis are significantly altered by both four-day and 21-day exposure to common EDCs. Further research is needed to determine whether transcription of these genes persists in animals exposed for greater than 21 days, and if these alterations in genes affects long-term growth or fitness of the animals.

7 SUMMARY OF NOVEL PROJECT ACCOMPLISHMENTS

7.1 Development of novel assays for the detection and quantification of Vtg in Atlantic salmon, shortnose sturgeon, and Atlantic sturgeon

Prior to this study, no published assay for shortnose sturgeon Vtg mRNA existed. The team developed a qRT-PCR assay for assessing gene transcription of shortnose sturgeon Vtg mRNA. The team developed several other novel assays for the detection of Vtg mRNA and/or Vtg protein for used with the four species described in this study (Table 7). Assay details are available in the Supplemental Information section, or will be made available upon request.

Table 7. Summary of assays used in this study. Novel assays resulting from this work (previously unpublished in the format used in this study) are indicated in bold*. Publications indicating the primer sets used are in parentheses.

	Assay used	**Experiments completed**
Atlantic salmon	Vtg mRNA qRT-PCR (*Vtg*-Arukwe and Roe, 2008; *EF-1α*-Ingerslev et al., 2006)	Acute (4-day) exposures of embryos, yolk-sac larvae, feeding fry and smolts. Chronic (21-day) exposures of juveniles.
	Vtg protein ELISA (*Peck et al., 2011)	Acute (4-day) exposures of smolts.
Shortnose sturgeon	**Vtg mRNA qRT-PCR** (**Vtg-cloned in-house**), β-actin-Roy et al., 2011)	Acute (4-day) exposures of embryos, yolk-sac larvae, feeding fry and juveniles.
	Vtg protein ELISA (†D. Papoulias, USGS)	Acute (4-day) exposures of 2+ year old juveniles.
Atlantic sturgeon	Vtg mRNA qRT-PCR (**Vtg sequence from shortnose sturgeon**), β-actin-Roy et al., 2011)	Chronic (21-day) exposures of juveniles.
Brook trout	**Vtg mRNA qRT-PCR** (*Vtg*-from GenBank sequence AF454752.1, *EF-1α*-Ingerslev et al., 2006)	Chronic (21-day) exposures of juveniles.
Other	Yeast E-screen (Arnold et al., 1996)	**EEQs measured for six wastewater treatment plants that discharge into known sturgeon and salmon habitat in ME and MA

*This publication provided a template for assay development-see Appendix for details.
†Diana Papoulias, USGS, provided a lake sturgeon primary antibody for assay development.
**EEQ data for these locations is novel, to our knowledge.

8 *PROJECT OUTREACH ACTIVITIES ACCOMPLISHED*

An additional, yet vital, dimension of the project was to maintain communications, outreach, and information transfer among Gulf of Maine agencies, researchers, and entities permitted to discharge wastewater into public waterways under the terms of the National Pollution Discharge Elimination System (NPDES). This outreach was pivotal to the investigation's larger purpose which is to determine whether federally recognized water quality monitoring procedures required of wastewater managers are providing adequate protection for salmon and sturgeon species as required by the Endangered Species Act. As such, Field Coordinator and Project Technical Advisor, Barbara Arter, conferred with numerous parties to identify relevant literature, to brief colleagues and researchers, and attend meetings of the Maine Department of Environmental Protection, Penobscot River NPDES Stakeholder Group, and the Penobscot River Restoration Science Exchange. The Kier Associates team outreach activities during the investigation included:

2010-2011

- Dr. Duffy and Ms. Arter worked with Christine Vaccaro (NMFS) to identify and upload project-relevant documents to NMFS' shared-access FTP site. The posted scientific literature focuses on developing immunochemical assays with which to measure vitellogenin, experimental designs for exposure to several contaminants, and assessing the endpoints of exposure in anadromous species.
- Information was gathered concerning other endocrine disruptors as well as studies of sturgeon habitat and migration underway in the principal area of interest, Maine's Penobscot and Kennebec watersheds.
- The Maine Department of Environmental Protection (DEP) provided information identifying WET test contractors for the three major cities in the target region (Bangor, Brewer, and Augusta), Maine surface water ambient toxins (SWAT) reporting, Maine surface water criteria, and EPA Publicly Owned Treatment Works (POTW) studies.
- Ms. Arter reviewed the project with Barry Mower of Maine's DEP, Steve Mierzykowski of the U.S Fish and Wildlife Service, Adria Elskus of the U.S Geological Survey/ Orono, and Gayle Zydlewski of the University of Maine.
- Ms. Arter presented a briefing about the project to the Penobscot River Science Exchange at the University of Maine in Orono on 4 March 2011. The presentation generated interest regarding possible testing for other contaminants (the presentation can be viewed at: http://www.umaine.edu/searunfish/penobscotexchange/PSE_Spring2011mtg.html.
- Ms. Arter also attended Maine DEP Penobscot River MEPDES Stakeholder Group Meetings in November 2010 and March 2011 in Bangor to learn more about the MEPDES process and river discharge issues in general. She also participated in a statewide MEPDES Stakeholder group focused on marine (bay and estuary) discharge criteria in June 2011 in Augusta, Maine.

- Volunteer project co-investigator Dr. Stephen McCormick attended the NOAA sturgeon conservation workshop in February 2011 in Virginia where he met with sturgeon experts to discuss conservation strategies and current sturgeon research. Dr. McCormick presented a brief overview of the project to US Geological Survey staff at the Conte Lab on April 2011. The presentation was well received; no significant concerns were raised.
- Dr. Duffy discussed the goals of this project with a group of high school students at the Women in Science summer camp at Smith College.
- Ms Arter discussed the goals of the project at the Diadromous Species Restoration Research Network Natural Variability Workshop at the University of Maine, Orono in May 2011.

2011-2012

- Dr. Duffy presented the salmon exposure data and preliminary shortnose sturgeon exposure data at the Society for Environmental Toxicology and Chemistry conference in Boston on November 17, 2011.
- Ms. Arter and Dr. Duffy discussed the acquisition of brook trout used in WET testing with representatives from Lotic, Inc and Barry Mower of the Maine DEP. After much discussion, it was determined that obtaining brook trout samples from municipal WET tests was unfeasible.
- Dr. Duffy presented the salmon exposure data and preliminary shortnose sturgeon exposure data at the Atlantic Salmon Research Forum meeting held January 10 and 11th in Bangor, ME.
- Dr. Duffy presented preliminary data from the salmon and shortnose sturgeon exposures at the ICES Oceans of Change conference in Majorca, Spain in April.
- Dr. Duffy presented the Atlantic salmon exposure work as well as some preliminary shortnose sturgeon exposure work at the Fish Biology Congress Meeting in Madison, WI.
- Dr. McCormick, Dr. Duffy, Barbara Arter, and Kimberly Damon-Randall met to discuss project plans and progress while attending the Atlantic Salmon Research Forum meeting.

2012-2013

- Much of fall and winter 2012-2013 was spent coordinating the collection of wastewater treatment samples for the purpose of E-screen testing. This required researching and developing an appropriate sampling protocol, identifying sturgeon overwintering sites in Maine and Massachusetts, and coordinating with Maine and Massachusetts DEPs and 6 wastewater treatment facilities to collect and ship water samples.
- Ms. Arter was contacted by a Colby College professor who is conducting EDC studies in Maine area lakes and streams. Ms. Arter explained the project and the protocols being used.
- Ms. Arter discussed the project with a Ph.D. candidate from Worcester College who is conducting a similar study on the presence of EDCs in wastewater treatment facilities in southeastern Massachusetts. Ms. Arter assisted the student in determining which

facilities might be amenable to sampling for research and the protocol that should be used for obtaining such samples.

- Ms. Arter forwarded the recently released NOAA report, Biological Assessment of Shortnose Sturgeon (*Acipenser breviorstrum*), to the team.
- Ms. Arter presented a talk titled, "Penobscot Bay and Estuary Point Source Pollutant Studies," for the Penobscot Bay Stewards on May 28, 2013 in Belfast, ME. The presentation included a synopsis of the study and some of its preliminary results.

9 ACKNOWLEDGEMENTS

The team would like to thank the following for their contributions to this project: Barry Mower (ME DEP), Clarisa Trasko (Maine DEP), Kimberly Damon-Randall (NMFS), who served as the project's Contracting Officer's Technical Representative, Christine Vaccaro (NMFS), Trent Liebich (NMFS), Lynn Lankshear (NMFS), Gayle Zydlewski (UMaine), Bryant Firmin (MA DEP), James Crowley (ME DEP), Adria Elskus (USGS), Diana Papoulias (USGS), Steve Mierzykowski (USFWS), Ken Gillette (USFWS), Dan Wong (USFWS), Stephen Gephard (CT DEP), Ken Simmons, (MA DFW), Cornel Ceapa (Acadian Sturgeon and Caviar, Inc.), Ben Wiinikind (Wiining Aquaculture), Mark Fisk (Hy-on-a-Hill Trout Farm) and Chuck Wall (MWCC). We also thank several employees of MA and ME wastewater treatment plantsm whose names we didn't capture for supplying the team with municipal water samples. Lastly, the team would like to thank the following employees and consultants at the Conte Anadromous Fish Research Laboratory (USGS) for their direct input and expertise with the material presented here: Amy Regish, Michael O'Dea, Jason Breves, Rolando Garcia, Matt O'Donnell, Todd Dubreuil, Micah Kieffer, Erica Parker, Kathleen Pietryka, Barnaby Watten, Andrew Weinstock, Kasie Auger, and Alex Haro.

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SUPPLEMENTAL INFORMATION

S.1. Assay Development and Methodology

S.1.2 Atlantic salmon Vtg mRNA assay development and exposures

Assay development and exposures

Note: All acute exposures were carried out with identical methods to those described for Atlantic salmon, unless otherwise noted.

The salmon Vtg mRNA assay was developed using the methods for qRT-PCR as described in Arukwe and Roe (2008) (Table S1). The primers for the housekeeping gene (EF-1α) were taken directly from Ingerselv et al. (2006) (Table S1). Both primer sets were validated using the Roche 480 LightCycler. Each set yielded optimal melting curves indicating the production of one amplicon (~200 base pairs) each.

Experiments were carried out using Atlantic salmon (*Salmo salar*) encompassing four life stages: late-stage embryos, newly hatched larvae, feeding fry, and smolts. All life stages were the progeny of sea run adults from the Connecticut River stock of Atlantic salmon, which is under active ESA restoration. Treatments with embryos, yolk-sac larvae, and feeding fry were carried out in 1.1 L aerated flow-through chambers with a 19 L head tank. Twenty embryos were allocated to each treatment, which were carried out in duplicate chambers. Embryos were acclimated to 7 ^{o}C (to minimize the chance of temperature-induced malformations that occur at temperatures $\geq$ 10 ^{o}C) over 72 hours before exposures were carried out. Aerated, covered head tanks were refilled daily with dechlorinated city water and the compound of interest, ensuring a turnover of 7 times d^{-1} and an average flow rate of 0.25 L h^{-1}. All exposures were carried out in a cold room to maintain temperature. Compounds (α-ethinylestradiol (EE2), 17 β-estradiol (E2) and 4-nonylphenol (NP)) were purchased from Sigma-Aldrich (St. Louis, MO) and all concentrations were converted to nanomolar for comparison (Table S1). All compounds used were solubilized in methanol (used as a vehicle for each compound) at a final concentration of <0.0001% to minimize solvent toxicity (Lerner et al. 2007a). Control treatments received solvent only. At the end of 96 h embryos were anesthetized with MS-222 (Argent Laboratories, Redmond, WA), briefly weighed, then immediately snap-frozen on dry ice, and stored at -80 ^{o}C prior to analysis.

Yolk-sac larvae and feeding fry were exposed as above with the following changes. Both stages were exposed at 15 ^{o}C. Yolk-sac larvae exposures were carried out in March, 2011 at 18 fish per replicate and feeding fry exposures in early May, 2011 at 15 fish per replicate. Both life stages were acclimated in the exposure chambers for 72 h prior to the beginning of the experiment. Feeding fry were acclimated to pelleted salmon feed in the hatchery, and fish were fed daily. In addition, feces and uneaten food were siphoned from experimental tanks daily to minimize bacterial degradation of contaminants. In order to obtain length data, fry where anesthetized and photographed lying next to a ruler for scale.

Data analysis

The housekeeping gene, EF-1α, demonstrated stable transcription during the fry and smolt stages (one-way ANOVA, p=0.12 and p=0.25, respectively). Some differential transcription of EF-1α was noted during the embryo and larval stages, evidenced by a few significant differences in *post hoc* comparisons among compounds. Our analysis was restricted to comparisons of dosage within a single compound for each life stage (one-way ANOVA, Holm-Sidak *post hoc* comparisons). One significant difference was noted between EF-1α in the control vs. NP 40 nM treatment, but statistical analyses of *Vtg* transcription normalized to cDNA input yielded identical statistical interpretations when compared to the data normalized to EF-1α. All statistical analyses were considered significant at $p \leq 0.05$.

Vtg mRNA data did not differ statistically among replicates compared for yolk-sac larvae, feeding fry, or smolt exposures. A significant tank effect was noted for embryos, but this was limited to EE2 (one-way nested ANOVA, p= 0.002). This effect was likely driven by high variability in responses and low numbers of fish analyzed from each replicate tank (n=4). All other replicate treatments demonstrated no statistically significant differences. Therefore, replicate tanks were combined for all analyses. All data were log-transformed for normality and homogeneity of variance where necessary. Treatment effects for all physiological measures were examined with one-way ANOVA with Holm-Sidak *post hoc* comparisons among individuals from duplicate tanks unless otherwise stated.

Table S1. Primer sequences used in this study. See supplemental information for details.

Species	Gene of interest	Primer sequence*		qPCR reaction conditions (40 cycles each)
		Forward	Reverse	
Atlantic salmon	Vtg	AAGCCACCTCCAATGTCATC	GGGAGTCTGTCCCAAGACAA	95C-30s, 57C-30s, 72C-30s
Salmo salar	EF1α	GCTGTGCGTGACATGAGG	ACTTTGTGACCTTGCCGC	95C-30s, 60C-1m
Brook trout	Vtg	TGCAGGAGTCTGGAGTTCAGGGA	GTCCTTCCTGATTCTCTCCTGGCA	95C-30s, 60C-30s, 72C-30s
Salvelinus fontinalis	EF1α	GCTGTGCGTGACATGAGG	ACTTTGTGACCTTGCCGC	95C-15s, 57C-15s, 72C-1m
Shortnose sturgeon	Vtg	AGGAACATCGCCAAGAAGGAACCA	TTCAAGCAGAACCATGGAGGCAAC	95C-15s, 60C-30s, 72C-30s
Acipenser brevirostrum/ Atlantic sturgeon *Acipenser oxyrhynchus*	β-actin	CATTGTCACCAACTGGGATGAC	ACACGCAGCTCATTGTAGAAGGT	95C-15s, 60C-30s, 72C-30s

*all primer sequences are listed as 5'-3'

S.1.3 Atlantic salmon Vtg Enzyme Immunoassay (EIA) development and exposures

Assay development and exposures

The team developed a quantitative and sensitive EIA from the methods outlined in Peck *et al*. (2011) using a commercially available antibody, polyclonal, rabbit anti-Atlantic salmon Vtg antibody, AA-1 (Biosense Laboratories AS, Bergen, Norway). Costar, 96-well microtiter plates (#3369, Corning Life Sciences, Tewksbury, MA) were coated with 60 ng/ml Atlantic salmon vitellogenin (#9902, Biosense Laboratories, Bergen, Norway) in 0.05 M Na_2CO_3 and incubated overnight at 4 ^{o}C. The following day, plates were washed with cold phosphate-buffered saline (PBS) with 0.05% Tween-20 (Sigma-Aldrich), pH 7.2 (PBST) and blocked with PBST with 2% milk powder overnight at 4 ^{o}C. Samples or standards were mixed with the primary antibody (in PBST with 1% milk powder). Samples were diluted a minimum of 1:100 to minimize interference and false-positive readings where Vtg was low (Peck et al. 2011) up to 1:10,000 for samples with high Vtg content. These were mixed at 4 ^{o}C overnight on a rotary shaker. On the final day, the plate was washed with PBST, incubated with samples or standards for 2 h at 37 ^{o}C. Plates were washed, then incubated with secondary antibody (Goat anti-rabbit horseradish peroxidase conjugate (G(H+L)) (KPL, Inc., Gaithersburg, Maryland) at 1:2500 for 45 min at 37 ^{o}C. Color development was carried out with TMB for 30 minutes then quenched with 1N H_2SO_4. Optical density was measured with a BioTek Synergy 2 spectrophotometer (BioTek, Winooski, VT) at 450 nm. Parallelism was demonstrated between diluted samples and Vtg standard. Interassay variation was 4.9 $\pm$ 1.5% S.E. Assay was sensitive to Vtg concentration $\leq$ 50 ng/ml, indicated by significant difference from the zero standard. Protocol is available upon request.

Atlantic salmon smolts were also exposed to all compounds in three consecutive, four-day experiments between April 5 and April 19, 2011, during the predicted peak of smolting. Smolts were placed in 30 L circular tanks at six fish per tank on the morning the exposures were initiated. Fish were not fed during the four-day period. Large, covered 560 L circular tanks served as head tanks, which were filled just prior to the beginning of the exposure period. Head tanks were replenished daily with the appropriate volume of stock contaminant in methanol mixed with dechlorinated city water, yielding a turnover time of at least 2 times d^{-1} and flow rate of approximately 10.5 L hr^{-1}.

Following the 96-h exposures, smolts were removed from experimental tanks, anesthetized with a lethal dose of MS-222, and immediately bled with heparinized syringes following collection of length and weight data. Hematocrit (measure of total red blood cell volume) was measured and blood was centrifuged at 5000 x g for 5 minutes at 4 ^{o}C and plasma was aliquoted into tubes and stored at -80 ^{o}C. Fish were immediately placed on ice, and gill and liver tissues were removed, snap-frozen and stored at -80 ^{o}C until analysis.

Data analysis

Atlantic salmon Vtg protein concentration was log-transformed for normality and treatments were compared with a two-way ANOVA with sex as the second factor.

S.1.4 Atlantic salmon, additional physiological parameters

Hepatosomatic index (HSI) was significantly elevated in response to the highest doses of α-ethinylestradiol and 17 β-estradiol, but no significant differences were noted for nonylphenol (Fig. 4). Additionally significant differences were seen for hematocrit and T_3, but T_4 and cortisol did not vary among treatments (Table 2). Nonylphenol exposure (4.0 and 400 nM) significantly reduced hematocrit relative to the control, and T_3 was significantly reduced by exposure to α-ethinylestradiol.

Na^+/K^+ -ATPase (hereafter, NKA) activity was measured in smolt gill tissue to determine potential impacts to seawater preparedness. Controls from each set of exposures differed significantly, with nonylphenol control NKA activity being significantly lower than control NKA values for α-ethinylestradiol. Within a given experimental (all doses of a single compound) no significant differences were noted among concentrations. Therefore, NKA activity did not exhibit compound- or dose-dependent responses.

Data analysis

Hepatosomatic Index data did not meet assumptions of normality and equal variance following transformation, and data were compared using a one-way ANOVA on ranks with Dunn's *post hoc* comparisons. All statistical analyses were carried out with SigmaPlot version 11.0 (Systat Software, San Jose, CA).

S.1.5 Shortnose sturgeon Vtg RNA assay development and exposures

Prior to this study, no published assay for shortnose sturgeon Vtg mRNA existed. The team developed a qRT-PCR assay for assessing gene transcription of shortnose sturgeon Vtg mRNA. To do this, the team partially cloned a portion of the sturgeon vitellogenin gene. To accomplish this, sturgeon Vtg sequences in GenBank were aligned with Vtg sequences from other fish species. Mixed base primers were designed based on regions of the Vtg gene that are conserved across lineages. These primers then amplified putative Vtg sequences which were sequenced at the University of Massachusetts-Amherst genomic sequencing facility. One primer set yielded two partial sequences (amplicons) which closely matched the sequences the primers were intended to amplify. Next, these amplicons were cloned into vectors (circular rings of DNA) and inserted into *E. coli* bacteria. The bacteria were grown on plates containing X-gal, allowing for blue/white screening of bacterial colonies with the insert of choice. Colonies containing the putative insert (the vector containing the partial Vtg gene) were grown overnight, cleaned, and sequenced.

These sequences yielded matches to other Vtg genes, including ~98% homology to other known sturgeon Vtg genes. The product from the cloning reactions is a 627 base pair partial sequence that contains two exons and a putative intron. This partial sequence will be published in GenBank immediately prior to publication of this work, as is customary with cloned molecular sequences. Sequence data is available upon request.

Primers for qRT-PCR assays were designed to amplify small fragments of the cloned Vtg gene (<200bp). One primer set yielded excellent results for an amplicon of 119 base pairs that spans a single intron. This primer set results in qRT-PCR efficiencies of 100% ± 5%, single melt curve peaks that indicate one amplified product (Figure S1). The housekeeping gene primer set for β-actin was taken from Roy et al. (2011). Reaction conditions for qRT-PCR were used as indicated in Roy et al. (2011) (Figure S1).

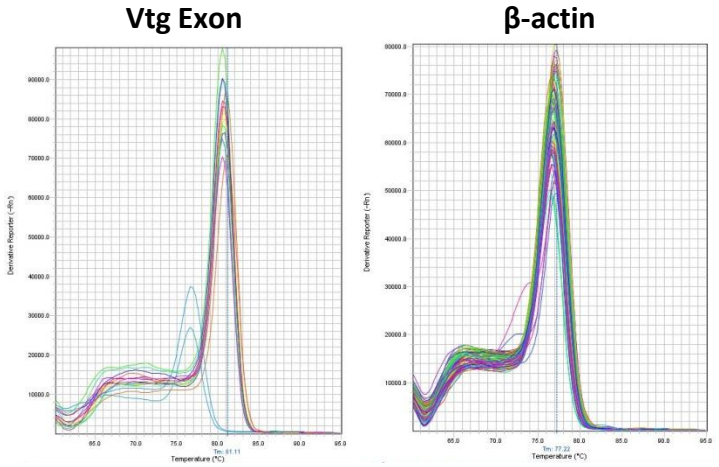

Figure S1. Melt curves from new shortnose sturgeon primer set (Vtg Exon, left) and housekeeping gene (β-actin, right). The melt curves for each primer set show one uniform peak for both standards and samples verifying that the primers are targeting only our gene of interest and that there are no additional amplicons being produced from a single primer set (as may happen if there is genomic DNA contamination or if assay conditions are suboptimal). The outlying peaks are negative controls and are not expected to fall within the curve.

Exposures were carried out with two-year old sturgeon using the same experimental methods as the Atlantic salmon smolts (above). Four sturgeon were used per duplicate tank.

Data analysis

All samples were normalized to β-actin (the housekeeping gene), then normalized to the control value (equal to 1). Any samples that amplified with a Cycle Threshold (CT) value that differed by >0.5 were re-analyzed by qRT-PCR. β-actin showed stable transcription among treatments. All data were non-normal and simple transformation did not meet either assumption of normality or homogeneity of variance and were therefore analyzed by one-way ANOVA on ranks.

S.1.6 Shortnose sturgeon Vtg ELISA assay development

The team developed a sensitive, quantitative Vtg protein ELISA for determining shortnose sturgeon plasma Vtg. The first step in this process was to purify shortnose sturgeon Vtg, as this product was commercially unavailable. To accomplish this, two five-year old shortnose

sturgeon were injected intraperiotenially with 5 mg/kg body weight estradiol (E_2). Plasma and liver tissue were collected from both fish at eight days post injection and stored with cOmplete, Mini, EDTA-free protease inhibitors (Roche Applied Science). Plasma and liver tissue was collected from two-year old shortnose sturgeon to serve as control samples. All samples were stored at -80C.

The team purified shortnose sturgeon Vtg to be used in the development of the Vtg ELISA. To do this plasma from E2-injected shortnose sturgeon was applied to a Sepharose-6B or Sepharose-2B column and eluted in fractions of phosphate-buffered saline (PBS) with aprotinin to protect protein degradation with additional treatment to remove contaminants (Figure S2).

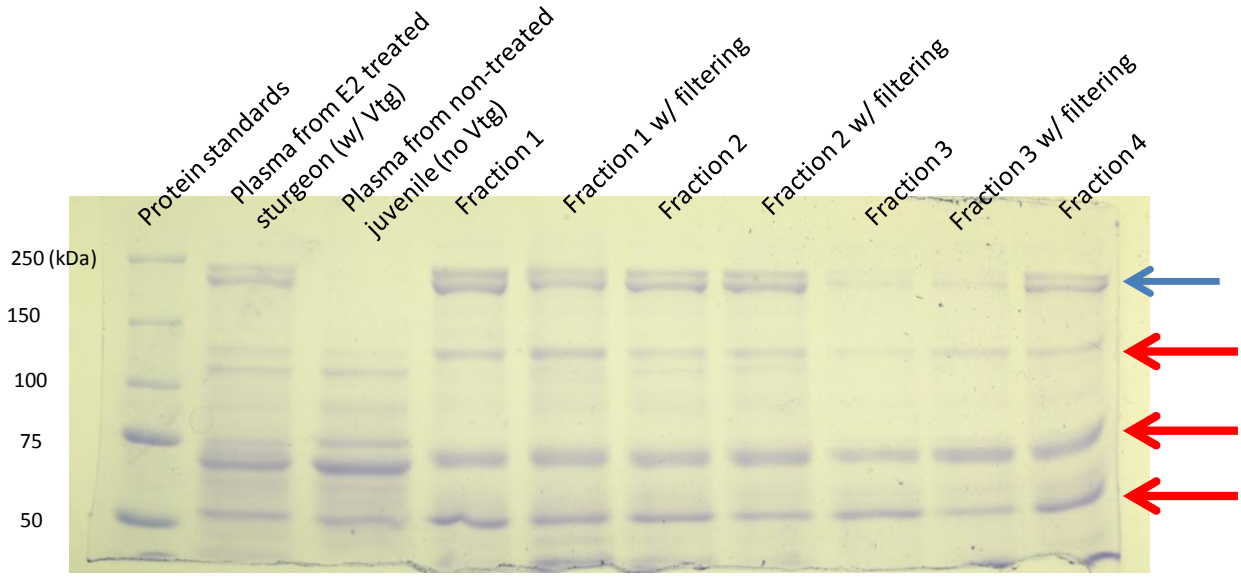

Figure S2. SDS-PAGE Protein Coomassie gel used to visualize size of proteins present in a sample. Samples are compared to protein standards of known size (in kilo Daltons, kDa) in the left hand column. The bands of interest for purification are approximately 220 kDa and are indicated by the blue arrow. Unknown protein bands are indicated by red arrows.

Figure S2 displays several samples from E2 injected fish (expected to have Vtg present) and plasma from a juvenile not treated with E2 (expected to have low or no Vtg present). The remaining columns are different treatments used to purify Vtg. Filtering indicates attempts to pass the unwanted proteins through a filter to eliminate proteins that are 100 kDa or less. However, filtering did not eliminate the additional proteins (not Vtg) that are still present in the samples after multiple treatments to remove them, indicating that these proteins are tightly associated with Vtg. This suggests that in an ELISA where proteins are in their folded, native form the smaller proteins are likely bound to the Vtg. In the SDS-PAGE Coomassie stain example above, proteins are denatured (unfolded), any bonds are broken, and protein fragments are segregated by size and visible in a SDS-PAGE Coomassie gel. These proteins were determined not to interfere in the ELISA, due to very low interference for plasma without Vtg. Therefore, several aliquots of shortnose sturgeon Vtg were chosen for the ELISA and stored in 50% glycerol with aprotinin at -20^{o}C for use.

The team made several attempts at using a commercially available antiobody for Gulf sturgeon (*Acipenser oxyrinchus desotoi*, a subspecies of Atlantic sturgeon) Vtg detection. This antibody (Monoclonal mouse anti-Gulf sturgeon Vitellogenin, ND-1H2, Biosense Laboratories, Bergen, Norway) worked well for western blot analysis of shortnose sturgeon Vtg (Figure S3), but was not compatible as an antibody for use in a sensitive, quantitative ELISA.

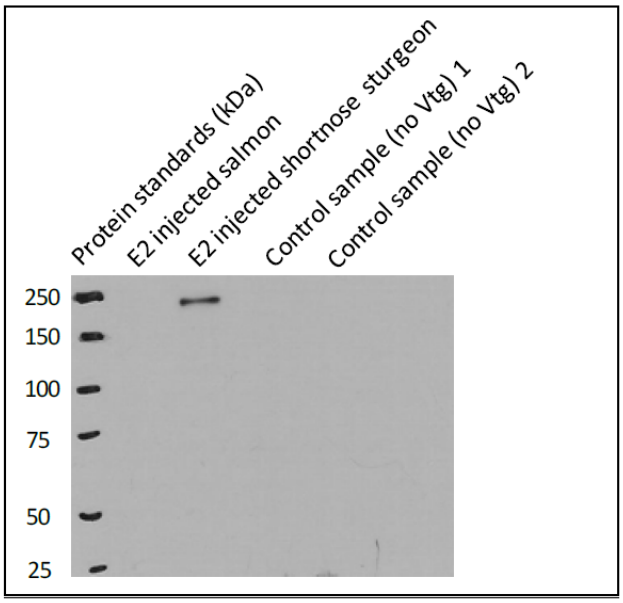

Figure S3. Western blot analysis of a commercially available Gulf sturgeon antibody. Samples analyzed include E2-injected salmon plasma (for cross-reactivity), E2-injected shortnose sturgeon plasma, and two control samples which do not contain sturgeon Vtg. Plasma from E2 injected shortnose sturgeon demonstrated cross-reactivity with the antibody in a band of Vtg approximately 220 kDa which is characteristic of Vtg in other sturgeon species. Protein standards (left) have been highlighted for clarity.

The team contacted Dr. Diana Papoulis of the USGS' Columbia Environmental Research Center who works with several sturgeon species. Dr. Papoulis kindly donated a suitable lake sturgeon (*Acipenser fulvescens*) rabbit polyclonal primary antibody for the sensitive detection of Vtg in shortnose sturgeon plasma. The team proceeded to develop an ELISA for shortnose sturgeon Vtg using the Atlantic salmon ELISA template as previously described (Figure S4). Shortnose sturgeon purified Vtg was used to coat the 96-well plate at 1000 ng/ml. The primary antibody was used at a final concentration of 1:90,000 and the secondary antibody (goat anti-rabbit HRP-labeled, KPL, Inc. Gaithersburg, MD) was used at a concentration of 1:7500. Samples were diluted 1:50 to 1:100 for the assay. Protocol is available upon request.

Assay validation showed that the detection limit at the lower end of the range (including our control, non-exposed samples) is slightly reduced compared to the Atlantic salmon Vtg assay, but is not expected to interfere with the ability. This does not, however, interfere with the ability of the assay to detect physiologically relevant differences among treatments.

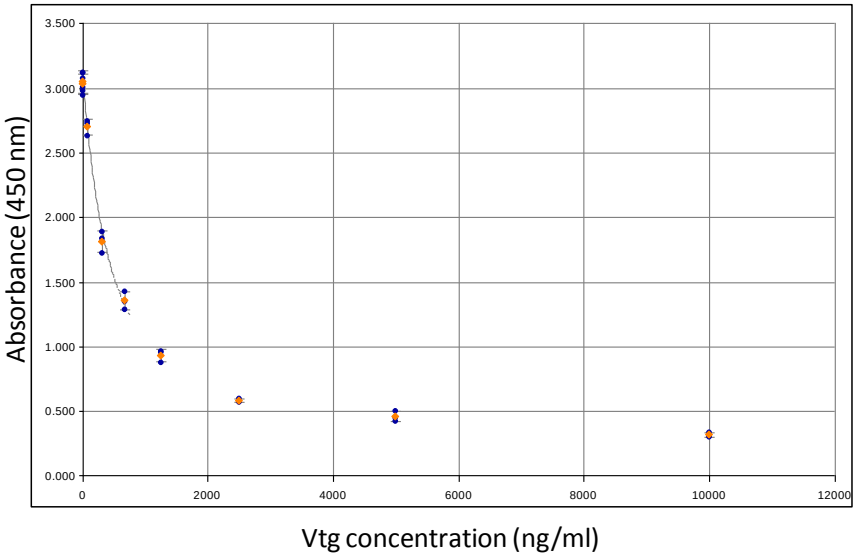

Figure S4. Representative standard curve for shortnose sturgeon Vtg ELISA indicating clean separation of standards across a good range of absorbance readings reflecting concentrations between 20 ng/ml-2,500ng/ml.

Data analysis

Shortnose sturgeon Vtg protein concentration was log-transformed for normality and treatments were compared with a one-way ANOVA (as sex could not be accurately determined).

S.1.7 Atlantic sturgeon Vtg RNA assay development

Because the primer set for shortnose sturgeon Vtg was designed to amplify a highly conserved region of the gene of interest, the team attempted to use the same primer set to amplify Vtg in Atlantic sturgeon. Initially, a PCR product was amplified, visualized on a gel to determine the size of the amplicon, and then purified for sequencing. This amplicon appeared as a band of around 120 base pairs (119 base pairs for the shortnose sturgeon amplicon) and a partial sequence of 75 base pairs of this amplicon demonstrated 100% sequence homology to two other sturgeon sequences on GenBank. qRT-PCR analysis with this primer set results in optimal PCR cycle efficiencies (90-110%), R^2 values $\geq$ 0.98 (Figure S5) and a single peak during melt curve analysis. This primer set is listed in Table S1. The housekeeping gene, β-actin, was also used for Atlantic sturgeon, as this primer set was designed for both species (Roy et al, 2011).

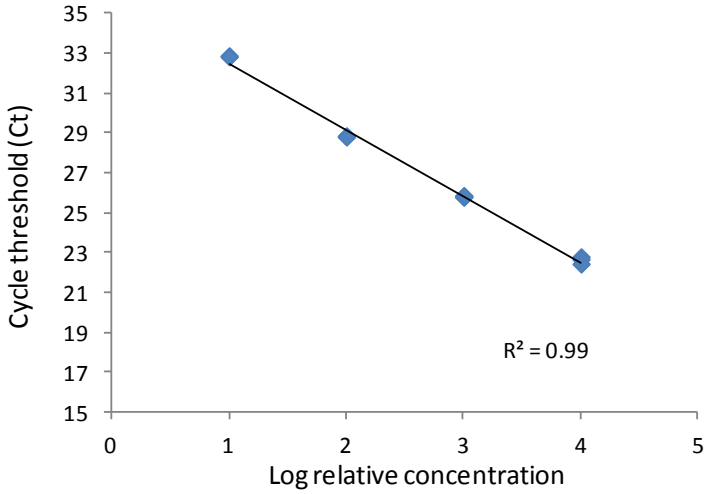

Figure S5. Representative standard curve from the Atlantic sturgeon qRT-PCR assay using cDNA made from 6-month old E2-injected Atlantic sturgeon.

Data analysis

Atlantic salmon Vtg mRNA was compared with a one-way ANOVA.

S.1.8 Atlantic sturgeon Vtg ELISA assay development and exposures

The team injected several six-month old Atlantic sturgeon juveniles with17 β-estradiol (5 mg/kg body weight) to use as a positive control for qRT-PCR and ELISA assays. The team used this plasma from the injected Atlantic sturgeon samples to determine the validity and cross-reactivity of the shortnose sturgeon ELISA with Atlantic sturgeon plasma. These validations demonstrate that the assay is compatible with Atlantic sturgeon plasma (parallelism among vitellogenic samples) with similar limits of detection (Figure S6). Therefore, Atlantic sturgeon Vtg was measured using the shortnose sturgeon ELISA.

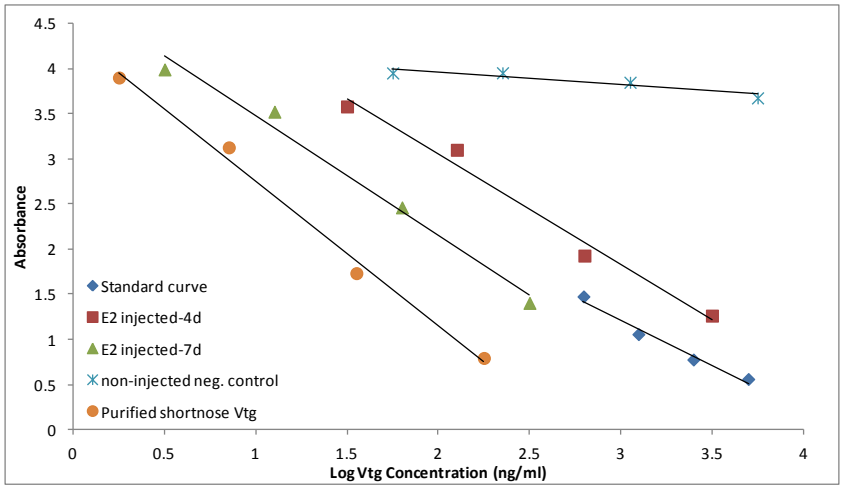

Figure S6. ELISA assay validation using the existing shortnose sturgeon protocol with Atlantic sturgeon plasma. Parallelism of the sample dilutions indicates assay compatibility between species. Non-injected control plasma (blue asterisks) dilutions do not show parallelism, as anticipated.

In early analysis, very low to undetectable Vtg concentrations were detected in fish that had been exposed to both EE2 and NP for 21 days. In order to determine whether this was due to a failure of the assay or low levels of Vtg in samples, several samples of plasma from E2-injected fish were run side-by-side in the ELISA (Figure S7). E2-injected fish demonstrated a heightened Vtg response relative to the fish that had been exposed during the 21-day experiment (waterborne exposure). This indicates that the assay was working properly, but it should be noted that the positive samples (from E2-injected fish) demonstrated low levels of Vtg relative to what was seen in both the Atlantic salmon juveniles (1-year old) and shortnose sturgeon juveniles (2-years old) after short-term exposure to the compounds used in this study.

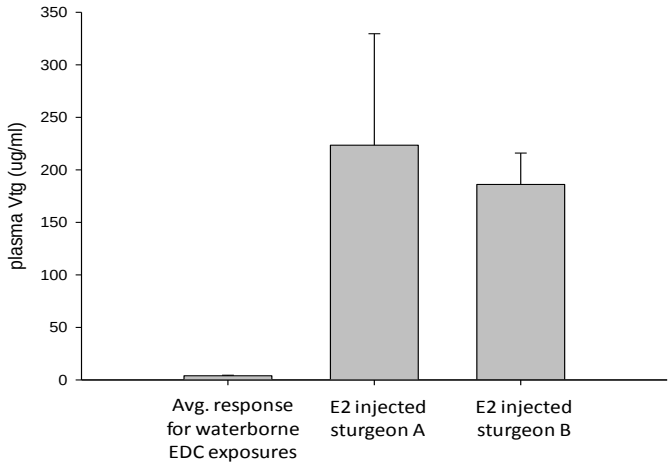

Figure S7. Plasma Vtg in Atlantic sturgeon exposed to EE2 and NP for 21 days and Atlantic sturgeon injected with high concentrations of E2 for positive confirmation of Vtg production. Experiments were carried out in the same manner as the Atlantic salmon and shortnose sturgeon juvenile exposures with slightly altered concentrations. All exposures (NP and EE2) were conducted concurrently.

Data analysis

Atlantic salmon Vtg protein concentration was log-transformed for normality and treatments were compared with a one-way ANOVA.

S.1.9 Brook trout Vtg RNA assay development

The Vtg mRNA assay was designed from a sequence of the brook trout (*Salvelinus fontinalis*) Vtg gene (GenBank accession number AF454752.1). Trials were carried out with several primer sets, but a single primer set spanning an exon-intron boundary (Table S1) produced reproducible results with optimal efficiencies (~100%) and a single peak during melt-curve analysis (Figure S8). Additionally, the primer set previously used as the Atlantic salmon housekeeping gene, EF-1α, was determined to be appropriate for analyses with brook trout.

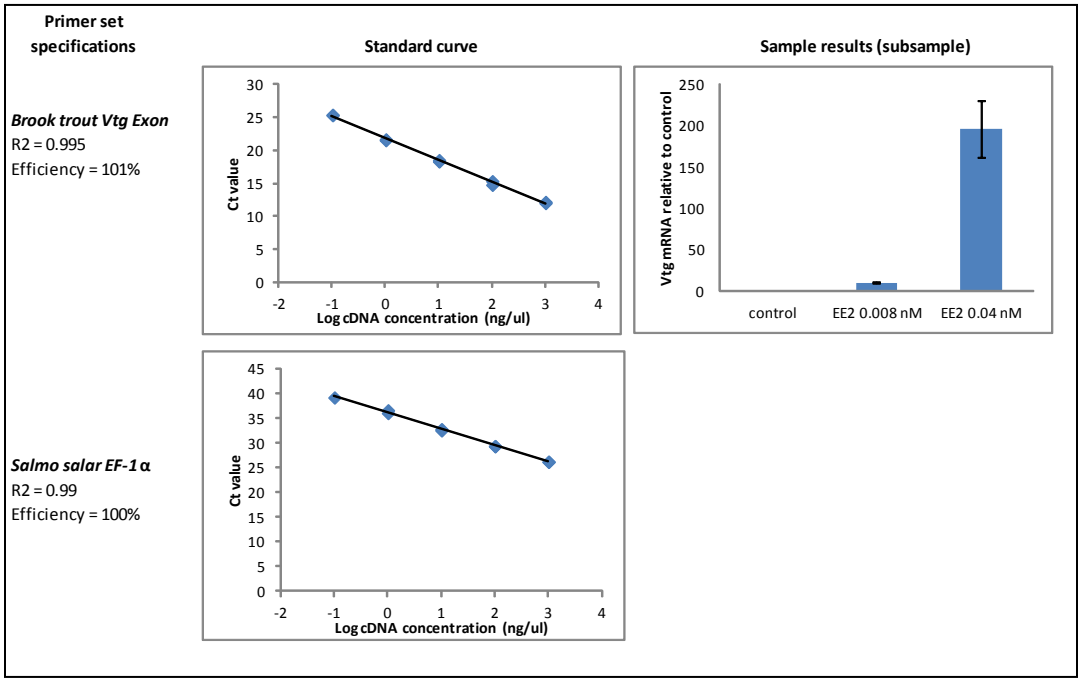

Figure S8. Validation data for design of the qRT-PCR assay to measure brook trout Vtg mRNA. The left side of the figure contains primer specifications for two primer sets and representative standard curves (left-hand column of figures). The primer set, Vtg Exon, amplifies an exon-intron boundary. Ct value (cycle threshold) refers to the cycle at which the fluorescence signal crosses an assay-based "threshold" for comparison among samples. The right-hand figure contains a subsample of the Vtg mRNA transcription levels quantified with the Vtg primer set. These are based on four fish from each treatment analyzed in duplicate.

Data analysis

Brook trout Vtg RNA transcription was compared with a one-way ANOVA.

S.1.10 Additional biomarkers of EDC exposure

Genes of interest were carried out with identical qRT-PCR methods as described above. All mRNA assays were carried out with a Life Technologies (Carlsbad, CA) StepOnePlus Real-Time PCR system. No differences among the controls from each experiment were detected, and therefore all control data was combined for analysis. All analyses were carried out by one-way ANOVA followed by Dunnett's test for *post hoc* comparison.

Table S2. Primer sequences and sources for genes of interest in Atlantic salmon gill and liver tissue.

Gene of interest	Primer sequence*		Source publication
	Forward	Reverse	
EF1α	GCTGTGCGTGACATGAGG	ACTTTGTGACCTTGCCGC	Ingerslev et al. 2006. Molec Immunol 43:1194–1201
ERα	GCATGGAGCACCTTTACAGCA	TGGAGGTGGTAGTGGTGGTAGA	Luo et al. 2005. Comp Biochem Phsiol C 140: 123-130
GHR	TGACTTTAAATGCCAGCACAAGGA	TGGTCACCAAATACTTCCCTCTTGA	Kiilerich et al. 2007. Gen Comp Endo 152: 295-303
IGF1	CCTGTTCGCTAAATCTCACTTC	TACAGCACATCGCACTCTTGA	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGF2	GGAAAACACAAGAATGAAGGTCAA	CCACCAGCTCTCCTCCACATA	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFR1a	GGGGCTCTCCTTCTGTCCTA	AGAGATAGACGACGCCTCCTA	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFBP1	AGGACCAGGGACAAGAGGAAG	CTGTTCCACCAGTTTCTTGC	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFBP2.1	CGGTGAGGAAGGCCACTAAGG	ATATCACAGTTGGGGATGT	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFBP2.2	TTCCATGATAACAGGGGACCAG	GACCGTGGGTGGACATGTGG	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFBP4	ACTTCCATGCCAAGCAGTGC	GGTCCCATCCTCACTCTCTC	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFBP5.1	ATCACGGAGGACCAACTGC	TGCTTGTCAATGGGTAGTGG	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFBP5.2	TTCTCCAGAGGAAGCTATGTTAG	TCAAGGCTGCTGACAGAGTG	Bower et al. 2008. J Exp Bio 211: 3859-3870
IGFBP6	GCTGCGTGCCTCTTCCTCA	TTACGGCAGGGTGCCTTTTC	Bower et al. 2008. J Exp Bio 211: 3859-3870
NKAα1a	CCAGGATCACTCAATGTCACTCT	GCTATCAAAGGCAAATGAGTTTAATATCATTGTAAAA	Nilsen et al. 2007. J Exp Bio 210: 2885-2896
NKAα1b	GCTACATCTCAACCAACAACATTACAC	TGCAGCTGAGTGCACCAT	Nilsen et al. 2007. J Exp Bio 210: 2885-2896
NKCC	CGAGACCAAGGCATTCTACA	ATGTCTCCGTCCTTCCAGTC	Kiilerich et al. 2011. J Endocrinol 209: 221-235
CFTR1	CCTTCTCCAATATGGTTGAAGAGGCAAG	GAGGCACTTGGATGAGTCAGCAG	Nilsen et al. 2007. J Exp Bio 210: 2885-2896

*all primer sequences are listed as 5'-3'

Table S3. Potential biomarkers of endocrine disruption in Atlantic salmon and Shortnose sturgeon. Quantitative, real-time PCR assays have been validated and are easily reproducible.

Species	Gene name	Abbreviation	Function	Publication
Atlantic salmon	Insulin-like growth factor-1	IGF-1	Regulator of growth hormone production, essential for skeletal/muscle growth	Filby, Thorpe, Tyler; "Multiple molecular effect pathways of an environmental oestrogen in fish'
	Insulin-like growth factor-1 receptor	IGF-1R	Receptor of IGF-1, mediates IGF-1 action	Filby, Thorpe, Tyler; "Multiple molecular effect pathways of an environmental oestrogen in fish'
	Growth hormone receptor	GHR	Receptor that mediates growth hormone action, cell reproduction, homeostasis	Tipsmark, Madsen; "Distinct hormonal regulation of NA, K-atpase genes in the gill of Atlantic salmon" Filby, Thorpe, Tyler; "Multiple molecular effect pathways of an environmental oestrogen in fish'
	Cytochrome P450 Aromatase 19a1	Cyp19a1	Regulates steroid synthesis (conversion of testosterone to estrogen) in gonad. Essential for reproductive development	Kortner, Mortensen, Hansen, Arukwe; "Neural aromatase transcript and protein levels in atlantic salmon (salmo salar) are modulated by the ubiquitous water pollutant, 4-nonylphenol"
	Cytochrome P450 Aromatase 19b1	Cyp19b1	Regulates steroid synthesis (conversion of testosterone to estrogen) in brain. Important in behavioral sex development.	Kortner, Mortensen, Hansen, Arukwe; "Neural aromatase transcript and protein levels in atlantic salmon (salmo salar) are modulated by the ubiquitous water pollutant, 4-nonylphenol" Crespo, Mananos, Roher, MacKenzie, Planas; "Tumor Necrosis Factor Alpha May Act as an Intraovarian Mediator of Luteinizing hormone-Induced Oocyte Maturation in Trout"
Shortnose sturgeon	Cytochrome P450 Aromatase 19a1	Cyp19a1	Regulates steroid synthesis (conversion of testosterone to estrogen) in gonad. Essential for reproductive development	Roy,Walker, Chambers, Wirgin; "Characterization and expression of cytochrome P4501A in Atlantic sturgeon and shortnose sturgeon experimentally exposed to coplanar PCB 126 and TCDD"