*Environ. Sci. Technol.* **2007**, *41,* 4479−4485

# Larval Exposure to 4-Nonylphenol and 17$\beta$-Estradiol Affects Physiological and Behavioral Development of Seawater Adaptation in Atlantic Salmon Smolts

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Population declines of anadromous salmonids are attributed to anthropogenic disturbances including dams, commercial and recreational fisheries, and pollutants, such as estrogenic compounds. Nonylphenol (NP), a xenoestrogen, is widespread in the aquatic environment due to its use in agricultural, industrial, and household products. We exposed Atlantic salmon yolk-sac larvae to waterborne 10 or 100 $\mu$g $L^{-1}$ NP (NP-L or NP-H, respectively), 2 $\mu$g $L^{-1}$ 17$\beta$-estradiol ($E_2$), or vehicle, for 21 days to investigate their effects on smolt physiology and behavior 1 year later. NP-H caused approximately 50% mortality during exposure, 30 days after exposure, and 60 days after exposure. Mortality rates of NP-L and $E_2$ fish were not affected until 60 days after treatment, when they were 4-fold greater than those of controls. Treatment with NP-L or $E_2$ as yolk-sac larvae decreased gill sodium-potassium-activated adenosine triphosphatase ($Na^+$,$K^+$-ATPase) activity and seawater (SW) tolerance during smolt development, 1 year after exposure. Exposure to NP-L and $E_2$ resulted in a latency to enter SW and reduced preference for SW approximately 2- and 5-fold, respectively. NP-L-exposed fish had 20% lower plasma insulin-like growth factor I (IGF-I) levels and 35% lower plasma triiodothyronine ($T_3$). Plasma growth hormone and thyroxine ($T_4$) were unaffected. Exposure to $E_2$ did not affect plasma levels of IGF-I, GH, $T_3$, or $T_4$. Both treatment groups exhibited increased plasma cortisol and decreased osmoregulatory capacity in response to a handling stressor. These results suggest that early exposure to environmentally relevant concentrations of NP, and other estrogenic compounds, can cause direct and delayed mortalities and that this exposure can have long term, "organizational" effects on life-history events in salmonids.

## Introduction

Historically, Atlantic salmon were abundant in rivers from arctic regions of the North Atlantic south to the Iberian Peninsula and southern New England. Since the mid-1980s, worldwide populations of wild Atlantic salmon have declined by 45% (*1*). These declines are often attributed to anthropogenic disturbances including dams, commercial and recreational fisheries, and exposure to contaminants in freshwater (FW), estuarine, and marine environments. During the parr-smolt transformation, anadromous salmonids pass through some of the most polluted areas of larger rivers and estuaries during their downstream migration to the sea (*2*).

The parr−smolt transformation is a developmental process of morphological, physiological, and behavioral changes that prepares juvenile salmonids for downstream migration and ocean entry (*3*). This transformation is mediated by increases in endogenous hormones including growth hormone (GH), insulin-like growth factor I (IGF-I), thyroid hormones, and cortisol (*4*−*7*). Development of seawater (SW) tolerance, a critical part of smolting, results from reorganization of the osmoregulatory tissues including large increases in gill sodium-potassium-activated adenosine triphosphatase ($Na^+$,$K^+$-ATPase) activity (*8*). These modifications are vital for maintaining homeostasis during ocean residence where the aquatic environment is three times the osmolality of the fish's internal environment.

Nonylphenol (NP), a xenoestrogen, is used in the manufacture of a wide array of household and industrial products including paper, agricultural chemicals, commercial and household cleaning products, contraceptives, cosmetics, detergents, paint, pesticides, and plastics. Due to the widespread use of these products, NP is commonly released from industrial and municipal treatment plants into the aquatic environment (*9*) and is hypothesized to be responsible for declines in Atlantic salmon populations in New Brunswick, Canada (*10*). Recent research indicates that exposure to NP or 17$\beta$-estradiol ($E_2$) just prior to the parr−smolt transformation inhibits hypoosmoregulatory physiology (*11, 12*) and delays downstream migration of Atlantic salmon (*13*). However, the NP levels required for these impacts were higher than those often present in salmon rivers (*14*). In addition, previous work has not investigated the potential long-term impacts of exposure during early development. Exposure to estrogenic compounds during ontogeny can permanently modify the organization and future function of the endocrine system (*15*). In the present study, we investigated the effects of aqueous exposure of Atlantic salmon yolk-sac larvae to NP and $E_2$ on the physiological and behavioral development of the parr−smolt transformation.

## Materials and Methods

**Fish Rearing.** Atlantic salmon eggs were obtained from the White River National Fish Hatchery (Bethel, VT) and transferred to the Conte Anadromous Fish Research Center (Turners Falls, MA) in February 2002. Eggs were maintained until hatching in covered egg trays with a flow rate of 1−2 L $min^{-1}$ using dechlorinated city water at 7 ± 1 °C. Two replicate tanks were used for each treatment and for rearing until physiological and behavioral sampling. During aqueous exposure, fish were removed from experimental tanks every other day to maintain similar fish densities among tanks

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10.1021/es070202w CCC: $37.00 
Published on Web 05/17/2007

following treatment-related mortality. After treatment and upon absorption of the yolk sac, larvae were placed in 1-m diameter tanks, provided fresh water at a flow rate of 2−4 L $min^{-1}$, maintained under natural photoperiod, and fed to satiation daily using automatic feeders. Five months after treatment fish were transferred to 1.6 m diameter tanks with fresh water at a flow rate of 6−8 L $min^{-1}$ under natural photoperiod and fed to satiation twice daily. Temperatures were allowed to fluctuate with the ambient conditions of the rearing water which was provided directly from the Connecticut River. Animals were maintained in this manner until sampling.

**Chemical Exposure.** During March 2003, approximately 3400, 21-day post-hatch (dph) Atlantic salmon yolk-sac larvae were exposed to branched para-NP (CAS no. 84852-15-3, 95.3% pure; Schenectady, NY), 17$\beta$-estradiol ($E_2$) (Sigma Chemical, St. Louis, MO), or vehicle (0.0001% methanol). Concentrated solutions were delivered via a peristaltic pump (Cole-Parmer Instrument Co., Vernon Hills, IL) and mixed in head tanks with dechlorinated city water to deliver target concentrations of either 10 or 100 $\mu$g $L^{-1}$ NP (NP-L or NP-H, respectively), 2 $\mu$g $L^{-1}$ $E_2$, or vehicle on a continuous basis and under flow-through conditions for 21 d (until absorption of the yolk sac). This period of exposure was selected because it represents a reasonable period during which agricultural, forestry, or industrial activity might be expected to elevate environmental levels, and because previous work has established it as representative of long-term or chronic exposure (*16*). All effluent was passed though an activated carbon canister prior to discharge. During the exposure, water temperature was maintained at 7 ± 1 °C and mortality was recorded daily. After exposure, animals were held for 13 months as described above until sampling. Water temperatures were 10 ± 1 °C on the sampling date.

**Fish Sampling.** During the peak of smolt development in early May 2004, 12 fish from each treatment group were either transferred to 30‰ SW for 24 h to assess ion regulatory ability (SW challenge), assessed in a behavioral assay for their preference for SW, sampled in FW to examine potential effects on physiological indices related to smolting, or exposed to a handling stressor to examine the impact of additional stressful stimuli during development.

The SW challenge was conducted by rapidly netting fish from the FW tank and transferring them directly into 1.6 m diameter tanks with recirculating SW (30‰) at 10 °C with charcoal filtration and aeration. Fish were sampled according to the protocol below 24 h after transfer.

The SW preference tank was constructed of 1.25 cm thick PVC sheets supported by a wood frame. Lighting was provided by four 23 W, 5100 K (degrees Kelvin) full-spectrum bulbs producing 1600 lumens each. The tank was divided into 4 chambers (two sets of parallel chambers connected by a PVC bridge) allowing two different treatment groups to be tested simultaneously. Each of the two replicates per treatment was alternately paired with different groups to control for possible behavioral interactions due to treatments. The parallel chambers were separated by a perforated wall so that water was shared between them. To conduct the behavioral assay the tank was filled with FW on one side and SW (32‰) on the other. Each chamber was filled to just below the bridge. After netting into the FW chambers, 12 fish from each group were allowed to acclimate for 2 h. Additional FW was then added to the FW side and allowed to spill over into the SW chambers until there was 7.6 cm of water over the bridge. Once the aqueous bridge was formed, activity was videotaped from a central location above the chambers for 2 h. Water temperature, salinity, and dissolved oxygen (DO) was measured before the addition of fish and at the end of the assay. Air was bubbled into each of the four chambers to maintain DO. Both SW and FW were chilled to 11.5 ± 1 °C. Over the course of 2 h, water temperature increased by approximately 1.5 °C, the salinity of the FW chamber increased to approximately 2.5−3.5‰, while the surface and bottom salinity of the SW chambers decreased to approximately 31.5−28.5‰. Videotape was analyzed for presence of fish in SW at 30 s intervals for 120 min, and is presented as percent of fish in SW. Fish swimming activity (the number of fish that entered the FW−SW interface at the bridge regardless of whether they returned to FW or entered SW) was also quantified using videotape analysis. Trials were conducted in two replicates using 12 fish from each treatment and 12 vehicle controls.

The handling stress consisted of rapidly dewatering fish by holding them in a net for 45 s, followed by a 2 min crowding (fish are placed back into the tank and water levels maintained such that the dorsal fins are exposed). Fish were sampled 3 h post-stressor.

Sampled fish were anesthetized with 200 mg $L^{-1}$ tricaine methane sulfonate (neutralized and buffered with sodium bicarbonate, pH 7.0) and length and weight were measured. All fish were sampled within 6 min of first disturbing the tank to ensure that baseline (unstressed) levels of cortisol were measured. Blood was collected in heparinized syringes from the caudal vasculature, centrifuged at 3000*g* for 5 min, then plasma was removed and frozen at −80 °C. A gill biopsy (approximately 6−8 primary gill filaments) was taken and placed in 100 $\mu$L of SEI (250 mM sucrose, 10 mM $Na_2EDTA$, 50 mM imidazole, pH 7.3) on ice for determination of $Na^+$,$K^+$-ATPase activity. Samples were frozen within 30 min and stored at −80 °C until analysis.

**Analytical Methods.** Gill $Na^+$,$K^+$-ATPase activity was measured according to the microassay protocol of McCormick (*17*). Gill filaments were homogenized in SEI buffer containing 0.1% sodium deoxycholate. Following centrifugation at 3000*g* for 0.5 min, $Na^+$,$K^+$-ATPase activity of the supernatant was determined by linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH), measured at 340 nm for 10 min at 25 °C, in the presence and absence of 0.5 mM ouabain.

Protein content in the gill homogenate was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL), and specific activities were expressed as $\mu$mol ADP $mg^{-1}$ of protein $h^{-1}$.

Plasma GH levels were measured using a double-antibody salmon GH radioimmunoassay developed by Bolton et al. (*18*) and modified by Björnsson et al. (*19*). Plasma IGF-I levels were measured by a radioimmunoassay validated for salmonids (*20*). Plasma $T_4$ and $T_3$ were measured by a direct radioimmunoassay (*7*). Plasma cortisol was measured using an enzyme immunoassay as outlined in ref *21*.

Plasma chloride concentration was measured using silver titration chloridometry (Labconco, Kansas City, MO) with external standards.

Water samples were obtained from each tank on the 10th and 21st (last) day of exposure, frozen at −80 °C for determination of NP concentration (Spectrum Analytical Inc. Agawam, MA) using gas chromatography (EPA method SW-846 8270C). The surrogate compounds 2-fluorophenol and phenol-$d_5$, which have chemical composition and behavior similar to that of NP in the analytical process, were spiked into all blanks, standards, and samples prior to analysis. Percent recoveries of these compounds ranged from 15 to 110%.

**Statistics.** All statistics were analyzed using Statistica (Version 7; Statsoft Inc, Tulsa, OK). There were no obvious differences in values between replicate tanks nor were significant differences found among replicate tanks for any parameter. Data were statistically analyzed and reported based on combined replicates within treatment.

The Kaplan−Meier product-limit method was used in the survival analysis to compare cumulative mortality using life

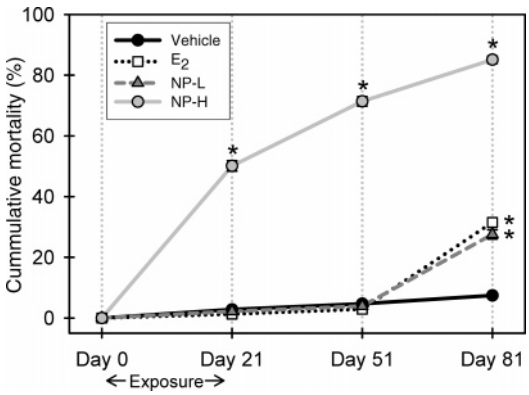


**FIGURE 1. Kaplan−Meier curves describing the effects of aqueous exposure to 10 or 100 $\mu$g $L^{-1}$ nonylphenol (NP-L or NP-H, respectively), 2 $\mu$g $L^{-1}$ 17$\beta$-estradiol ($E_2$), or vehicle on mortality of Atlantic salmon yolk-sac larvae during the 21-day exposure period and for 60 days thereafter. Values are means ± standard error. Asterisks indicate significant differences from vehicle controls (Wilcoxon test, $P < 0.001$).**

tables constructed from the proportions of fish surviving to three discreet time points (termination of exposure and 30 and 60 days after exposure). Pairwise comparisons were analyzed using Gehan's Wilcoxon Test ($P < 0.05$).

All values for physiological parameters are reported as means ± standard error. Analysis of variance (ANOVA) was used to examine the significance of treatment. When significant treatment effects were found, Newman−Keul's tests ($P < 0.05$) were used to determine which treatments were significantly different from vehicle control.

For SW preference, a binomial distribution was used to compute $z$ values for the observed number of cases where percent of vehicle controls in SW outnumbered treated. The associated tail probability for that $z$ value was used to estimate $P$ and significance assigned when $P < 0.05$. A two-way analysis of variance (ANOVA) was used to examine the effects of time, treatment, and their interaction on fish swimming activity.

## Results

To assess the impacts of early exposure to NP and $E_2$, Atlantic salmon yolk-sac larvae were aqueously exposed to either 10 or 100 $\mu$g $L^{-1}$ nonylphenol (NP-L or NP-H, respectively), 2 $\mu$g $L^{-1}$ $E_2$ as a positive control, or vehicle control for 21 days at 8 °C. Mean concentrations of aqueous NP measured from the treatment tanks were 6.5 ± 1.1 and 79.9 ± 9.1 $\mu$g $L^{-1}$ for NP-L and NP-H respectively. NP-H increased mortality to 50% within the exposure period, which continued at this rate for 60 days after exposure (Figure 1). Mortality reached 100% 120 days after treatment was terminated (data not shown). Mortality rates of NP-L and $E_2$ fish were not affected until 31−60 days after treatment was terminated, at which time they were approximately 4-fold greater than that of controls (Figure 1).

To ascertain exposure effects on seawater performance and associated mechanisms of ion regulation, we investigated the physiological effects of direct transfer from freshwater to 30‰ seawater. Treatment with NP-L or $E_2$ as yolk-sac larvae increased plasma chloride of smolts 1 year later approximately 5−10 mM over vehicle controls after 24 h in SW (Figure 2A). In addition, FW gill $Na^+$,$K^+$-ATPase activity was reduced 28% in fish previously treated with NP-L and 17% in $E_2$-treated fish (Figure 2B). SW gill $Na^+$,$K^+$-ATPase activity was not effected (data not shown). There was no mortality during the 24 h SW exposure.

To characterize the effects of early exposure on the behavioral preference for seawater, fish were transferred to the freshwater side of a two-chamber tank and allowed to

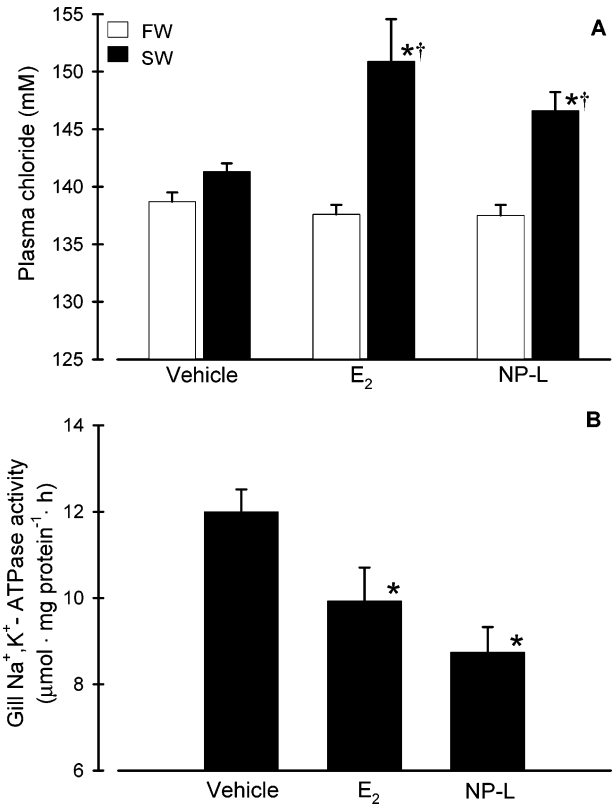


**FIGURE 2. Plasma chloride concentration in FW (white bars) and after 24 h in SW (black bars) (A) and Gill $Na^+$,$K^+$-ATPase activity in FW (B) of smolts 1 year after aqueous exposure to 10 or 100 $\mu$g $L^{-1}$ nonylphenol (NP-L or NP-H, respectively), 2 $\mu$g $L^{-1}$ 17$\beta$-estradiol ($E_2$), or vehicle as yolk-sac larvae. Values are means ± standard error. Asterisks indicate significant differences from vehicle and daggers indicate significant differences within treatment between FW (gray bars) and SW (black bars). For all groups $n = 12$.**

acclimate for 2 h prior to having volitional access to 32‰ seawater. Statistical comparison of the number of fish in SW over the 2-h bioassay indicated that preference for SW was markedly reduced by NP-L and nearly abolished by $E_2$ (Figure 3). Mean number of fish in SW over the entire bioassay was reduced approximately 2-fold and 5-fold for NP-L and $E_2$-treated fish, respectively. Time for the first fish to enter SW was significantly longer for NP-L and $E_2$ fish compared to vehicle controls groups (vehicle control = 9.0, NP-L = 33.5, and $E_2$ = 17.5 min; Figure 3). Analysis of the remaining period (34−120 min) demonstrated significantly reduced preference for SW in the $E_2$ and NP-L groups, independent of the observed latency. There was a significant impact of time on the number of fish reaching the FW−SW interface, but no effect of treatment ($P = 0.108$) or an interaction of time and treatment ($P = 0.232$) except during the last time interval where fewer $E_2$-treated fish were observed at the FW−SW interface (Table 1).

To identify which hormones may be involved in the observed disruptive effects of NP-L and $E_2$ on salinity tolerance and preference, plasma was obtained from all groups at the peak of smolting. Compared with vehicle controls, fish exposed to NP-L as yolk-sac larvae had 20% lower plasma IGF-I levels as smolts, whereas plasma GH was unaffected ($P = 0.327$) (Figure 4A). Prior, NP-L exposure also markedly reduced plasma triiodothyronine ($T_3$), but not thyroxine ($T_4$) (Figure 4B). Exposure of yolk sac-larvae to $E_2$ did not affect plasma levels of IGF-I, GH, $T_3$, or $T_4$ ($P = 0.605$, $P = 0.084$, $P = 0.306$, $P = 0.264$, respectively; Figure 4A and B).

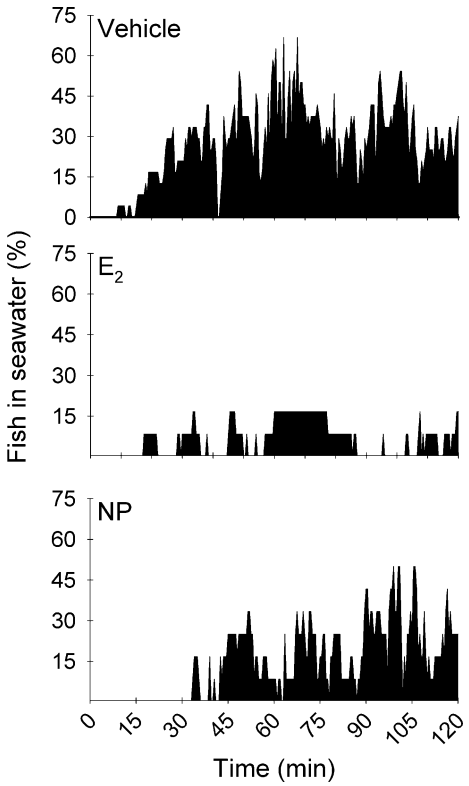


**FIGURE 3. SW preference behavior of Atlantic salmon smolts 1 year after aqueous exposure to 10 or 100 $\mu$g $L^{-1}$ nonylphenol (NP-L or NP-H, respectively), 2 $\mu$g $L^{-1}$ 17$\beta$-estradiol ($E_2$), or vehicle as yolk-sac larvae ($n \geq 24$ individuals per group in 2–4 replicate tests).**

**TABLE 1. Number of Fish Entering the FW–SW Bridge from FW during Each 30-min Interval**

| | time after formation of aqueous bridge (min) | | | |
|---|---|---|---|---|
| | 0–30 | 31–60 | 61–90 | 91–120 |
| vehicle control | 8.0 ± 0.8† | 32.0 ± 3.2 | 38.5 ± 5.2 | 35.5 ± 4.7 |
| $E_2$ | 7.5 ± 0.5† | 35.5 ± 5.5 | 28.0 ± 4.0 | 18.58 ± 2.5* |
| NP-L | 6.0 ± 1.0† | 26.5 ± 2.5 | 31.5 ± 6.5 | 32.5 ± 2.5 |

[a] Asterisk (*) indicates significant difference from vehicle control, and daggers (†) indicate significant differences within groups (2-way ANOVA). Values are means ± standard error; $n = 24$.

To establish whether early exposure to aqueous contaminants results in heightened sensitivity to future stressful events, we exposed juvenile Atlantic salmon to a handling stressor 1 year after exposure as yolk-sac larvae to NP-L or $E_2$. Early exposure to NP-L and a handling stressor increased plasma cortisol 68% more than the stress-induced increase in the vehicle control group (Figure 4C). The post-stressor increase of plasma cortisol in $E_2$-treated fish did not differ significantly from that of vehicle-treated controls ($P = 0.854$). However, the within-treatment increase in plasma cortisol due to the handling stressor was significantly elevated in NP-L- and $E_2$-treated fish, whereas there was no significant increase in that of vehicle controls (Figure 4C). Furthermore, treated fish (NP-L and $E_2$) demonstrated a greater post-stressor reduction in plasma chloride than vehicle controls and exhibited a greater magnitude of chloride loss in within-treatment comparisons (Figure 4D).

## Discussion

Here we demonstrate that NP and $E_2$ have delayed mortality effects when exposure occurs during ontogeny, and that there can be long-term detrimental effects on smolt development. $LC_{50}$ values for NP exposure of juvenile salmonids range from 130 to 920 $\mu$g $L^{-1}$ and these guidelines for allowable exposure levels of aquatic organisms to chemicals are largely based on short-term mortality rates while delayed mortality or developmental impacts are often overlooked (*22*). Our data indicate that delayed mortality during early development can occur after longer exposures to considerably lower concentrations of NP. In the present study, NP-H increased mortality to 50% within the exposure period which continued at this rate for 60 days after exposure, when significantly greater mortality rates of NP-L and $E_2$ fish were first observed. This period coincided with yolk absorption and the onset of exogenous feeding. While not directly tested in the present investigation, increased mortality during this transition suggests that early exposure to NP may have compromised the physical structure of the digestive system and metabolic processing of food or disrupted feeding behavior, or a combination of these factors. Alternatively, exposure to NP or $E_2$ may have suppressed the immune system, an effect seen in other teleost fish following exposure to $E_2$ or estrogen-like chemicals (*23*, *24*).

To ascertain exposure effects on SW performance and associated mechanisms of ion regulation, we investigated the physiological effects of direct transfer from FW to 30‰ SW 1 year after exposure. NP-L and $E_2$ impaired hypoosmoregulatory ability, assessed as capacity to tightly regulate plasma chloride after 24 h in SW. In addition, FW gill $Na^+$,$K^+$-ATPase activity was significantly reduced in fish treated with these estrogenic compounds. The activity of this ion regulatory pump is directly involved in salt secretion and is widely used as a marker of smolt development (*17*). Research on the effects of relatively higher concentrations of NP (repeated injections of 150 $\mu$g $g^{-1}$) injected during smolt development yield similar results with fish exhibiting reduced salinity tolerance (*11*, *12*). Reduced SW tolerance and poor smolt development, such as we have demonstrated here, are associated with lower survival and growth in SW, increased predation, and reduced adult returns (see references in *25*). Thus, we suggest that exposure to nonylphenol and other estrogenic compounds early in development will decrease seawater survival.

Aqueous exposure of yolk-sac larvae to $E_2$ and environmentally relevant levels of NP can compromise behavioral preference for SW during smolt development of Atlantic salmon. $E_2$ and NP have been shown to reduce FW, downstream migratory behavior of exposed smolts (*13*). Here we demonstrate that 1 year after exposure, preference for SW was markedly reduced by NP-L and nearly abolished by $E_2$. Similarly, researchers have shown long-term effects of exposure to PCBs during ontogeny on future development of juvenile or adult behaviors (*26–28*). To enter SW during the behavioral assay, fish must swim to the top of the water column and pass over a submerged bridge at the FW–SW interface. Therefore, the specificity of the SW preference test was validated by controlling for random swimming activity which did not differ among groups. The present data integrate physiological impacts mentioned above with a behavioral outcome indicating that early exposure to NP and possibly other estrogenic compounds will extend estuarine residence which is known to increase vulnerability to predators (*29*).

To identify which hormones may be involved in the observed disruptive effects of NP-L and $E_2$ on salinity tolerance and preference, plasma was obtained from all fish at the peak of smolting. Prior NP-L exposure reduced plasma IGF-I levels. Exogenous IGF-I can increase salinity tolerance and gill $Na^+$,$K^+$-ATPase activity (*in vitro* and *in vivo*), and the mechanism of action may be directly at the gill (*8*). The predominant source of circulating IGF-I is from the liver, controlled primarily by circulating GH binding to its hepatic receptor (*30*). Therefore, NP may negatively affect IGF-I by

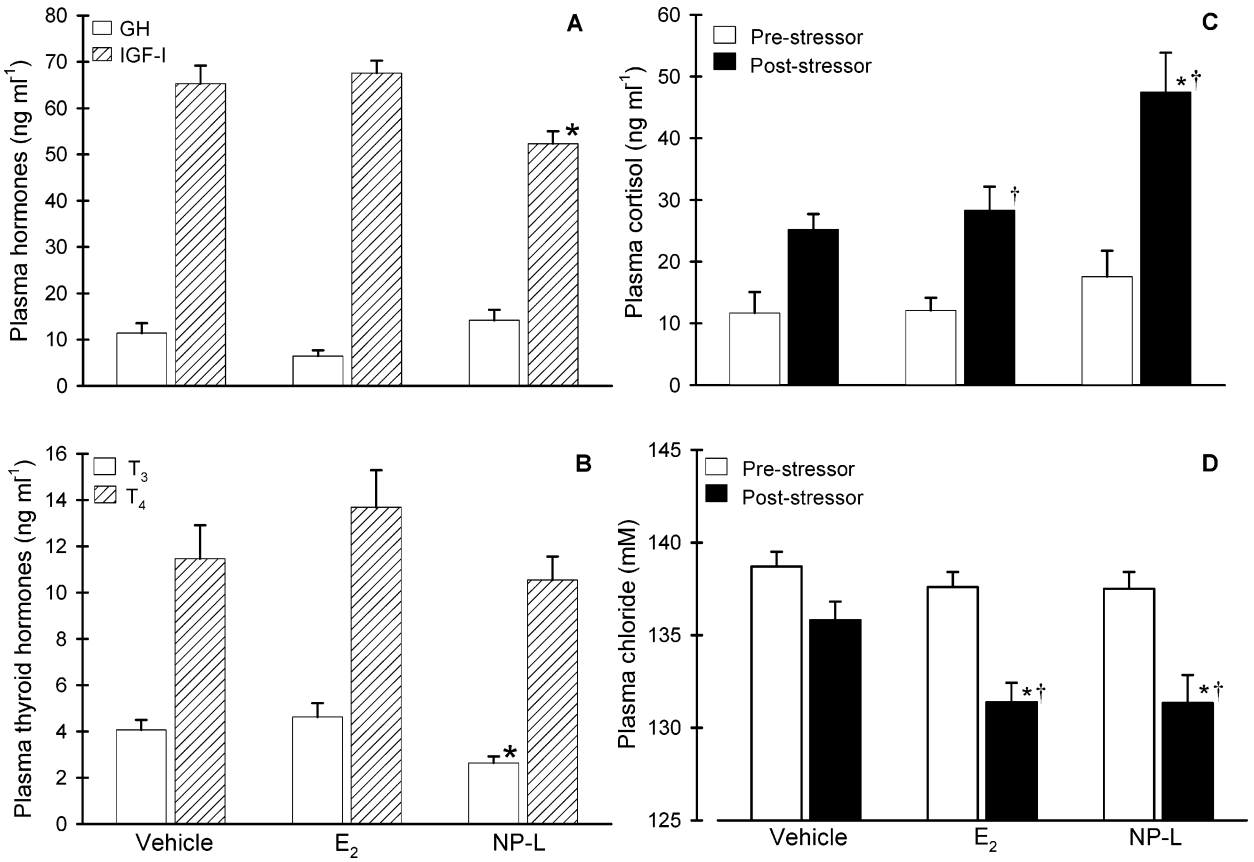


**FIGURE 4. Plasma hormone concentrations of Atlantic salmon smolts 1 year after aqueous exposure to 10 or 100 $\mu$g L$^{-1}$ nonylphenol (NP-L or NP-H, respectively), 2 $\mu$g L$^{-1}$ 17$\beta$-estradiol ($E_2$), or vehicle as yolk-sac larvae. Also shown are plasma levels of cortisol and chloride of previously treated smolts before and after a handling stressor. (A) Plasma levels of GH (white bars) and IGF-I (hatched bars). (B) Plasma levels of $T_3$ (white bars) and $T_4$ (hatched bars). (C) Plasma levels of cortisol before (white bars) and after (black bars) application of a handling stressor. (D) Plasma chloride concentrations before (white bars) and after (black bars) application of a handling stressor. Asterisks indicate significant differences from vehicle control and daggers indicate significant differences within treatments. For all groups $n = 12$.**

reducing the abundance or function of hepatic GH receptors (GHR). Estrogenic compounds can decrease hepatic GHR in some mammals (*31*), and recent work indicates an inhibitory effect of NP and $E_2$ on liver GHR mRNA expression in tilapia (Lerner et al., unpublished data). There is evidence that this inhibitory action operates through suppression of GH-induced JAK2 phosphorylation (*32*), although this mechanism has not yet been studied in fishes.

Interestingly, the effects of NP-L and $E_2$ on plasma IGF-I were dissimilar, in spite of the fact that $E_2$-exposed fish exhibited reduced salinity tolerance and preference. In light of these results, we hypothesize that the effects of estrogenic compounds on ion regulation may involve several pathways. There is evidence indicating that the estrogen receptor (ER) is promiscuous, and that binding of non-estrogen ligands to the ER may initiate diverse intracellular second messenger cascades, resulting in ligand-dependent variations in downstream products from a single receptor (*33*). Additionally, the three ER subtypes found in teleosts (ER$\alpha$, ER$\beta$a, and ER$\beta$b) exhibit differences in relative ligand-binding affinity and tissue distribution which could contribute to the selective action of ER agonists in different tissues (*34*). These potential mechanisms for effects are not mutually exclusive and provide possible explanations for the differences in endocrine effects of NP and $E_2$ in the present study.

Thyroid hormones are critical to development and growth at the embryonic and larval stages of many fishes and regulate morphological and behavioral changes during smolting (*35, 36*). The role of thyroid hormones in ion regulation is less certain. In the present study, exposure of yolk-sac larvae to NP-L markedly reduced plasma $T_3$ (but not $T_4$) during smolting. McCormick et al. (*12*) postulated that the actions of NP on circulating $T_3$ may result from direct interaction of NP with components of the thyroid axis rather than through the ER. There is evidence that NP interferes with the binding of $T_3$ to transthyretin in birds and amphibians, and that similar compounds can inhibit thyroid action by acting as an antagonist of $T_3$ binding to the thyroid hormone receptor in mammals (*37, 38*). The results of the present study, reduced plasma $T_3$ absent of changes in plasma $T_4$, and the lack of an $E_2$-effect on circulating thyroid hormones, support this hypothesis. Although not tested in the present study, our data and this line of evidence suggest that the disruptive effects of NP may also include negative impacts on thyroid-dependent processes such as imprinting and migratory behavior.

In the present study, cortisol levels following a handling stressor were greater in NP-L-treated fish than controls. This effect was not observed in fish previously treated with $E_2$. However, a comparison of the change in plasma cortisol within treatment groups indicates that NP-L and $E_2$ treated fish exhibit greater responses to a handling stressor than vehicle controls. In addition, the handling stressor reduced plasma chloride in FW, in smolts treated with estrogenic compounds as yolk-sac larvae. Exposure to estrogenic compounds during early development can sensitize the developing hypothalamic−pituitary−interrenal axis, producing an organism that exhibits increased sensitivity to stressful stimuli (*15, 24*). Smolts are inherently more sensitive to stress than are other salmon life-history stages (*21*), and during

downstream migration, may encounter numerous stressors including dams, predators, or toxic chemicals. These results suggest that early exposure to estrogenic compounds can further increase the stress response, potentially leading to maladaptive reactions to environmental disturbances.

Early disruption of normal hormonal signals by estrogen or estrogen mimics can permanently modify the organization and future function of the endocrine system in vertebrates (*15, 39*). Much of this research has focused on long-term effects on physiology and behavior associated with reproduction (*40, 41*), including the formation of intersex gonadal tissue. Although we did not find impacts on sex ratio or gonad morphology (data not shown), our results demonstrate that exposure can also impact osmoregulatory development and migratory behavior. We propose that the inhibitory effect of xenoestrogens on SW tolerance may be related to the anadromous life history of salmon in which endogenous sex steroids are extremely low during development, then increase during reproductive maturation and the accompanying movement back into FW (*42*). Confirmation of this biological link between sex steroids and osmoregulatory function would suggest that exposure to xenoestrogens during early development can have organizational effects acting to maintain a FW existence.

Considering that worldwide annual production of alkylphenol ethoxylates, of which the major degradation product is NP, is approximately 650 000 tons (*43*), the potential for the biological disruption of salmon development is high. Kolpin et al. (*14*) measured 95 contaminants in 139 rivers in the United States. NP, which bioaccumulates in fishes (*44*), was present in about half of those rivers at concentrations of 0.2−40 $\mu$g $L^{-1}$, ranking it as the seventh most abundant. Therefore, where NP is present in the aquatic environment it may exceed concentrations indicated by our results to have long-term detrimental effects on survival, the capacity for SW entry, and ultimately on the sustainability of salmon populations. NP can be present in drinking water and food including baby food and formula (*43*), and can increase growth of human breast cancer cells (*45*). Numerous contaminants act as environmental estrogens and there is evidence that these work additively at environmentally relevant concentrations to exert biological impacts (*46, 47*). Thus, measurement of NP (or any single xenoestrogen) alone may be a conservative estimate of potential deleterious effects on wildlife and human populations.

## Acknowledgments

We thank the White River National Fish Hatchery, U.S. Fish and Wildlife Service, for providing the fish used in these studies. This work could not have been completed without the technical assistance of B. Egnér, A. Moeckel, M. Monette, and M. O'Dea. We also thank L. Iwanowicz, B. Letcher, M. Mather, J. Podos, and R. T. Zoeller for critical reviews of this manuscript. This research was supported by the Woods Hole Oceanographic Institution (WHOI) Sea Grant Program, under grants from the National Oceanic and Atmospheric Administration, U.S. Department of Commerce, under Grant No. NA16RG2273, project no. *R/B-165*, and by a grant from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS).

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*Received for review January 25, 2007. Revised manuscript received April 9, 2007. Accepted April 17, 2007.*

ES070202W