

## AQUEOUS EXPOSURE TO 4-NONYLPHENOL AND 17 $\beta$ -ESTRADIOL INCREASES STRESS SENSITIVITY AND DISRUPTS ION REGULATORY ABILITY OF JUVENILE ATLANTIC SALMON

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**Abstract**—Population declines of wild Atlantic salmon have been attributed to an array of anthropogenic disturbances, including dams, commercial and recreational fishing, habitat loss, and pollution. Environmental contaminants in particular, can act as environmental stressors on fish, typically causing disruption of ion homeostasis due to their close association with the aquatic environment. To examine the effects of the xenoestrogen 4-nonylphenol (NP) or 17 $\beta$ -estradiol (E<sub>2</sub>) on stress sensitivity and ion regulation, we exposed juvenile Atlantic salmon continuously for 21 d to either 10 or 100  $\mu$ g/L NP (NP-L or NP-H), 2  $\mu$ g/L E<sub>2</sub> (positive control), or vehicle control during the parr-smolt transformation in April. After treatment, fish were sampled in freshwater (FW), transferred to 30‰ seawater (SW) for 24 h, or subjected to a handling stress. Estradiol and NP-H increased plasma vitellogenin in males and females, and E<sub>2</sub> increased gonadosomatic index only in males. In FW, E<sub>2</sub> reduced sodium potassium-activated adenosine triphosphatase activity as well as plasma levels of growth hormone, insulin-like growth factor I, and triiodothyronine. Both E<sub>2</sub> and NP-H reduced plasma sodium in FW and increased plasma chloride in SW. Plasma cortisol levels pre- and poststressor were significantly elevated by all treatments relative to controls, but only E<sub>2</sub> increased plasma glucose before and after the stressor. These results indicate that exposure of anadromous salmonids to environmental estrogens heightens sensitivity to external stressors, impairs ion regulation in both FW and SW, and disrupts endocrine pathways critical for smolt development.

**Keywords**—Atlantic salmon Stress Osmoregulation 17 $\beta$ -Estradiol Nonylphenol

### INTRODUCTION

Anadromous fish are exposed to point-source pollution such as industrial sites and sewage treatment plants as they pass through some of the most polluted areas of larger rivers and estuaries during their downstream migration to the sea. Population declines of anadromous salmonids have been attributed to anthropogenic disturbances, including exposure to contaminants that act as endocrine disruptors. Frequently, these aquatic contaminants are estrogens or compounds that mimic the action of estrogens (xenoestrogens), which are often studied for their impacts on reproductive processes [1]. Xenoestrogens may have other sublethal effects on salmon, including alterations in development, growth, and stress. Nonylphenol (NP), a xenoestrogen, is a breakdown product of nonylphenol ethoxylate, which is more persistent, toxic, and estrogenic [2]. Nonylphenol ethoxylate 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. Because of the widespread use of these products, NP is commonly released from industrial and municipal treatment plants into the aquatic environment.

Sublethal stress results in an adaptive response elicited to restore physiological homeostasis. In fish, stressful stimuli include environmental disturbances, such as changes in salinity and temperature, increased predation risk, or competition for resources, and anthropogenic disturbances, such as dams, han-

dling, and pollution [3–5]. The classic stress response includes elevated circulating cortisol or corticosterone, which are commonly used as indicators of stress in vertebrates. In fish, cortisol has a dual role as a mineralocorticoid controlling hydromineral balance and as a glucocorticoid regulating glucose availability [3]. The presence of contaminants in the aquatic environment may therefore elicit a stress response affecting both metabolic and ion regulatory processes.

Parr-smolt transformation and subsequent downstream migration of anadromous salmonids is a critical life stage transition that involves numerous morphological, physiological, and behavioral changes that prepare juvenile salmonids (parr) for migration to and residence in the ocean [6]. An essential component to the parr-smolt transformation is a marked increase in seawater (SW) tolerance. Key physiological parameters of this enhanced osmoregulatory ability include increases in gill sodium potassium-activated adenosine triphosphatase (Na<sup>+</sup>,K<sup>+</sup>-ATPase) activity, which is critical for maintaining homeostasis in an environment that reaches three times the osmolality of the fish's internal milieu [7]. This developmental transformation is mediated by increased plasma levels of growth hormone (GH), insulin-like growth factor I (IGF-I), thyroid hormones, and cortisol [8]. All these changes likely contribute to the intensified sensitivity to stress compared to other salmon life history stages [9].

During smolting, endogenous estrogens of these juvenile fish are extremely low [10] and exogenous administration of 17 $\beta$ -estradiol (E<sub>2</sub>) is inhibitory to the development of salinity tolerance [11] and migratory behavior [12]. Nonylphenol has also been linked to delays in downstream migration and re-

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duced survival of Atlantic salmon smolts [12,13]. Although previous studies have shown that NP injection just prior to the parr-smolt transformation results in inhibitory effects on hypo-osmoregulatory physiology [11], it is not clear that aqueous exposure has the same impact. In fact, Moore et al. [14] found no effect of aqueous exposure of NP (5–20 µg/L) on salinity tolerance of Atlantic salmon. In the present study, we hypothesized that aqueous exposure to E<sub>2</sub> or environmentally relevant levels of NP would have three effects: elicit a stress response, affect the response to additional stressors, and negatively impact SW tolerance and other aspects of smolt development in Atlantic salmon.

## MATERIALS AND METHODS

### *Fish rearing*

Atlantic salmon were obtained from the White River National Fish Hatchery (Bethel, VT, USA) and transferred to the Conte Anadromous Fish Research Center (Turners Falls, MA, USA) in October 2001. Fish were maintained in 1.6-m-diameter tanks with Connecticut River, USA, water at a flow rate of 6 to 8 L/min under natural photoperiod and ambient temperature and fed a commercial dry pellet salmon diet (Zeigler Brothers, Gardners, PA, USA) twice daily to satiation until April 2002.

### *Chemical exposure*

For chemical exposure, juvenile male and female Atlantic salmon large enough to become smolts (25–50 g) were measured for fork length (the length from the rostral end to the fork of the caudal fin), total length (the length from the rostral end to the tip of the dorsal caudal fin), and weight and placed in each of eight 1-m-diameter tanks (18 per tank). Each tank was supplied with aerated freshwater at a flow rate of 2 to 4 L/min under natural photoperiod. Fish were exposed to branched para-nonylphenol (CAS No. 84852-15-3, 95.3% pure; Schenectady, NY, USA), E<sub>2</sub> (Sigma Chemical, St. Louis, MO, USA), or vehicle control (0.0001% methanol). Concentrated solutions were delivered via a peristaltic pump (Cole-Parmer Instrument, Vernon Hills, IL, USA) and mixed in head tanks with dechlorinated city water to deliver target concentrations of either 10 or 100 µg/L NP (NP-L or NP-H, respectively), 2 µg/L E<sub>2</sub>, or vehicle to replicate tanks on a continuous basis and under flow-through conditions for 21 d. Water temperature was maintained at 10 ± 1°C. Fish were fed a maintenance diet every other day until 24 h prior to sampling.

Water samples were obtained from each tank on the 10th and 21st day of exposure and frozen at –80°C for analysis of NP concentration by gas chromatography/mass spectrophotometry (U.S. Environmental Protection Agency method SW846 8270C; <http://www.epa.gov>).

### *Fish sampling*

Immediately following the chemical exposure, 12 fish from each treatment group (six from each replicate tank) were sampled directly from their freshwater (FW) exposure tanks. In addition, 12 fish from each treatment group (six from each replicate tank) were subjected to the following two treatments: transferred to 30‰ SW at 10 ± 1°C for 24 h to assess ion regulatory ability in SW or exposed to a handling stressor to examine the impact of an external stressor. 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 poststressor.

Sampled fish were anesthetized with 200 mg/L tricaine methane sulfonate (neutralized and buffered with sodium bicarbonate, pH 7.0) and length and weight measured. All fish were sampled within 6 min of first disturbing the tank. Blood was collected in heparinized syringes from the caudal vasculature, stored on ice for less than 30 min, and centrifuged at 3,000 g for 5 min, and then plasma was removed and frozen at –80°C. A gill biopsy (~6–8 primary gill filaments) was taken and placed in 100 µL of SEI buffer (sucrose; 250 mM, disodium ethylenediamine tetracetate; 10 mM, imidazole; 50 mM, pH 7.3) on ice for determination of Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. Samples were frozen within 30 min and stored at –80°C until analysis.

### *Analytical methods*

Gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was measured according to the microassay protocol of McCormick [15]. Gill filaments were homogenized in SEI buffer containing 0.1% sodium deoxycholate. Following centrifugation (3,000 g for 5 min) to remove insoluble material, Na<sup>+</sup>,K<sup>+</sup>-ATPase activity was determined by linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide, measured at 340 nm for 10 min at 25°C, in the presence or absence of 0.5 mM ouabain. Protein content in the gill homogenate was measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA), and specific activities were expressed as µmol adenosine diphosphate per milligram of protein per hour.

Plasma GH levels were measured using a specific double-antibody salmon GH radioimmunoassay [16]. Plasma IGF-I levels were measured by a radioimmunoassay validated for salmonids [17]. Plasma triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) were measured by a direct radioimmunoassay [18]. Plasma cortisol was measured using an enzyme immunoassay as outlined in Carey and McCormick [9].

Plasma vitellogenin (VTG) was measured by Western blot. Ten micrograms of total plasma protein were heated to 70°C for 15 min in Laemmli sample buffer and run on an 8% sodium dodecylsulfate/polyacrylamide gel electrophoresis. Following electrophoresis, the proteins were transferred to a polyvinylidene fluoride Immobilon membrane (Millipore, Bedford, MA, USA) at 30 V for 14 h in 25 mM Tris, 192 mM glycine buffer at pH 8.3. Membranes were blocked in phosphate-buffered saline with 0.5% triton (PBST) and 5% powdered milk for 1 h at room temperature. Blots were probed with rabbit anti-salmon VTG polyclonal antibody (Biosense Laboratories, Bergen, Norway) at a dilution of 1:1,000 in PBST for 1 h at room temperature. After washing in PBST, the blots were probed with horseradish peroxidase labeled goat anti-rabbit secondary antibody at a dilution of 1:2,000 in PBST for 1 h at room temperature. The blots were developed using the horseradish peroxidase colorimetric substrate diaminobenzidine.

Plasma sodium levels were measured with an atomic absorption spectrophotometer (AAAnalyst 100, Perkin-Elmer, Norwalk, CT, USA). Plasma chloride levels were measured using silver titration chloridometry (Labconco, Kansas City, MO, USA) with external standards.

### *Statistics*

All values are reported as means ± standard error. Two-way analysis of variance (ANOVA) was used to examine the significance of treatment, tank, and their interaction ( $p < 0.05$ ).



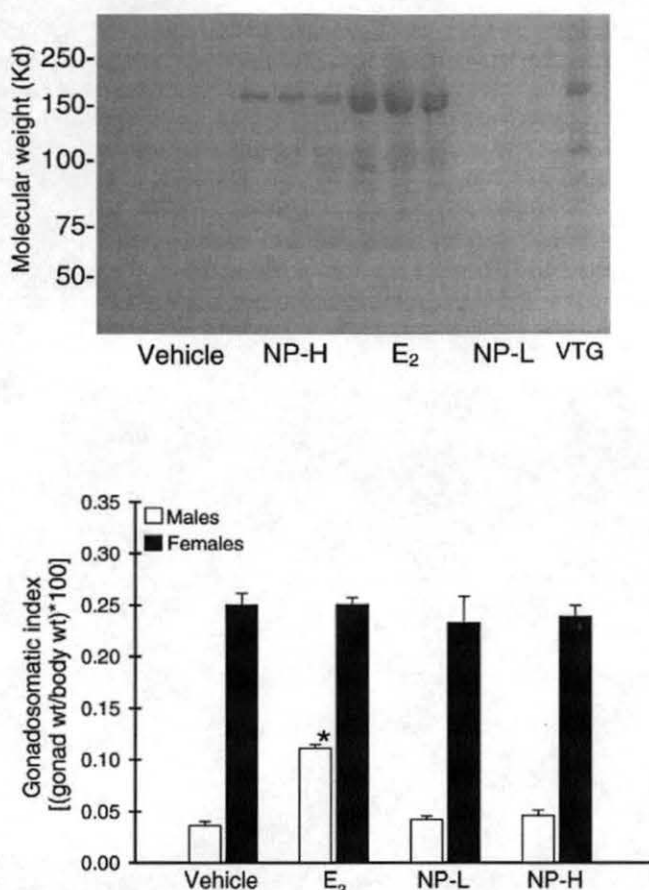


Fig. 1. Representative Western blot of plasma vitellogenin (VTG; top) and gonadosomatic index (GSI; bottom) of male and female juvenile Atlantic salmon aqueously exposed to estradiol ( $E_2$ ; 2  $\mu\text{g/L}$ ) or a low or high dose of nonylphenol (NP-L or NP-H; 10 or 100  $\mu\text{g/L}$  NP, respectively) for 21 d in freshwater. Shown in the Western blot are three lanes each for vehicle control,  $E_2$ , NP-L, and NP-H treatments. A single lane of VTG was used as an internal control. Values for GSI are means  $\pm$  standard error. Asterisks indicate a significant difference from vehicle control ( $p < 0.001$ ).

Significant tank effects were not found for any parameter; therefore, data were statistically analyzed and reported on combined replicates within treatment (one-way ANOVA,  $n = 12$  per group). When significant treatment effects were found, Newman-Keuls test ( $p < 0.05$ ) was used to determine which treatments were significantly different from vehicle control. Growth data were analyzed by linear regression analysis of the homogeneity of slopes, and a Newman-Keuls test ( $p < 0.05$ ) was used to determine which treatments were significantly different. All statistics were analyzed using Statistica® (Ver 7; Statsoft, Tulsa, OK, USA).

## RESULTS

No mortalities were observed in any group over the course of the study. Nominal aqueous concentrations were  $6.9 \pm 0.5$   $\mu\text{g/L}$  and  $73.9 \pm 9.4$   $\mu\text{g/L}$  for NP-L and NP-H, respectively. Plasma VTG levels of male and female fish were elevated in response to  $E_2$  and NP-H after 21 d of treatment (Fig. 1), whereas there was no detectable VTG in vehicle controls or NP-L-treated fish. No differences were observed in plasma VTG attributable to sex. Gonadosomatic index (GSI; [gonad wt/body wt]  $\times 100$ ) of males was increased by 37.5% in response to  $E_2$  (Fig. 1). Gonadosomatic index of both NP-L and

NP-H were similar to controls, and there was no effect of any treatment on female GSI.

Mean resting plasma cortisol levels were significantly elevated by all treatments compared with vehicle control (four- to ninefold; Fig. 2). A 50% increase in plasma glucose levels was observed in response to  $E_2$  treatment, whereas neither dose of NP had an effect (Fig. 2). All groups exhibited an increased plasma cortisol levels 3 h after a handling stressor. This increase was significantly greater in all treatments compared with controls (48–98%; Fig. 2). Only  $E_2$  elicited a significant increase in plasma glucose levels (73%; Fig. 2) and a significant decrease in plasma ion levels following a stressor (Fig. 3). The change in plasma cortisol levels within groups due to the handling stressor was 75, 23, and 36% greater than vehicle control for  $E_2$ , NP-L, and NP-H, respectively.

Final weight after 21 d of treatment was significantly lower for  $E_2$ -treated fish compared with vehicle control ( $p = 0.039$ ), whereas NP-L and NP-H were not significantly different from vehicle control (Table 1). The change in body weight was 83, 25, and 28% lower than controls for  $E_2$ , NP-L, and NP-H, respectively (Table 1).

Gill  $\text{Na}^+, \text{K}^+$ -ATPase activity was 30% lower in  $E_2$ -treated fish and was not significantly affected by either dose of NP (Fig. 4). Plasma sodium levels were reduced by 10 mM ( $E_2$ ) and 5 mM (NP-H) in FW, but there was no effect on plasma chloride levels (Fig. 3). After 24 h in SW, plasma chloride levels were increased 13 mM ( $E_2$ ) and 19 mM (NP-H), but there was no effect on plasma sodium levels (Fig. 3). Plasma levels of sodium or chloride ions were not affected in FW- or SW-fish treated with NP-L.

No effect of any treatment on plasma  $T_4$  levels was observed. Plasma  $T_3$  levels were 50% lower in  $E_2$ -treated fish compared to controls (Fig. 5) but not affected by NP-L or NP-H. Plasma GH levels were 83% lower than controls in  $E_2$ -treated fish and not affected by either dose of NP (Fig. 5). Similarly, compared to controls,  $E_2$  treatment reduced plasma IGF-I levels by 61%, but NP had no effect at either dose (Fig. 5).

## DISCUSSION

The present study indicates that environmental exposure to  $E_2$  or NP heightens stress sensitivity and impairs osmoregulatory ability during the parr-smolt transformation of Atlantic salmon. In this study, exposure to  $E_2$  or NP elicited a primary stress response as measured by increased plasma cortisol, which is a commonly used indicator of exposure to stressful stimuli in teleost fish [4]. An additional handling stressor further increased this hormonal response. Only  $E_2$  treatment elevated plasma glucose levels, a component of the secondary response to stress, before and after the handling stressor, as well as decreasing growth, indicative of tertiary responses to the stressor(s). In addition to their capacity to mobilize energy, increases in corticosteroids during acute or chronic stress are associated with negative impacts on growth, reproduction, disease resistance, and survival [5]. These data indicate that the magnitude of the response elicited by NP exposure, while significant, is less than that imposed by  $E_2$  treatment.

Reduced baseline cortisol levels and failure to elicit a cortisol response have been demonstrated following exposure to mixtures of environmental pollutants including heavy metals, aromatic hydrocarbons, and polychlorinated biphenyls in vitro and in vivo [19]. It was hypothesized that prolonged hyperactivity of cortisol in response to relatively long-term expo-

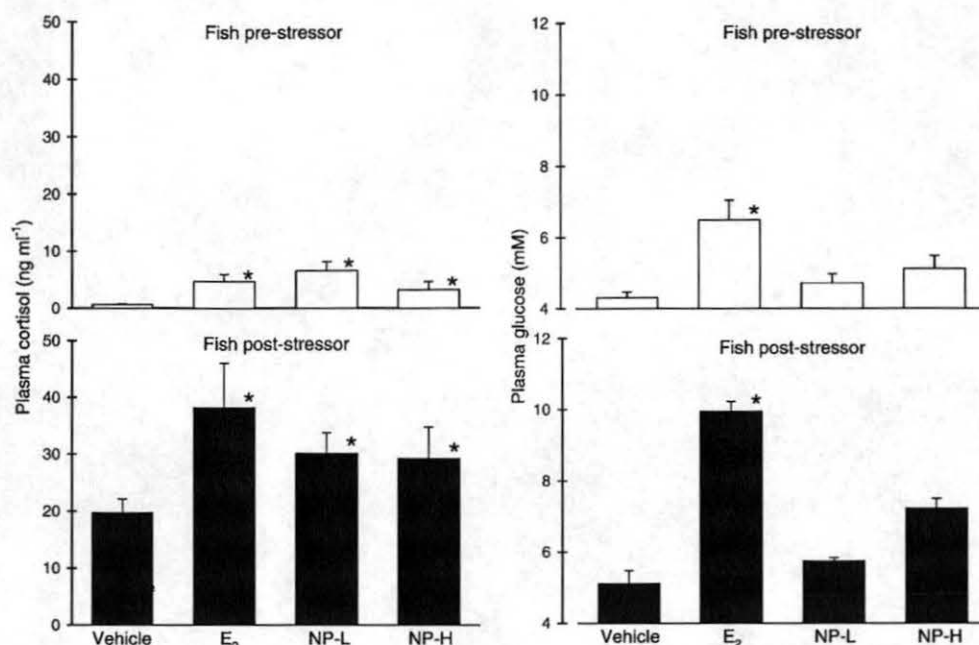


Fig. 2. Plasma cortisol and glucose before (top left and right, respectively) and after (bottom left and right, respectively) juvenile Atlantic salmon were subjected to a handling stressor subsequent to aqueous exposure to estradiol (E<sub>2</sub>; 2 µg/L) or a low or high dose of nonylphenol (NP-L or NP-H; 10 or 100 µg/L NP, respectively) for 21 d in freshwater. Values are means ± standard error. Asterisks indicate a significant difference from vehicle control ( $p < 0.05$ ).

tures to contaminants will lead to exhaustion of the pituitary–interrenal axis. In contrast, the present study and previous work on rainbow trout (*Oncorhynchus mykiss*) [20] indicate that E<sub>2</sub> exposure results in increased baseline cortisol and an increased response to a handling stressor. In addition, increased E<sub>2</sub> has been implicated in hyperactivation of the hypothalamus–pituitary–interrenal (HPI) axis culminating in postspawning death in mature salmon [21]. During the stress response, corticotropin-releasing factor (CRF) stimulates the production of cortisol via adrenocorticotrophic hormone release from the pituitary [22]. In mammals, E<sub>2</sub> has been demonstrated to promote an increase of corticotropin-releasing hormone (CRH) in vitro [23], indicating a possible mechanism of E<sub>2</sub> stimulation of the HPI axis. In mammals and fish, the CRH/CRF and glucocorticoid response during stress is regulated by negative feedback on glucocorticoid receptors (GR) in the brain, typically returning plasma cortisol levels to resting levels within 24 h [22,24]. During chronic stress, this regulatory mechanism is disrupted by down-regulation of GR, resulting in elevated secretion of cortisol [24]. Further investigations are necessary to determine whether estrogenic compounds impact GR, thereby promoting elevated cortisol in fish.

Fish exposed during early development to environmental estrogens demonstrate long-term disruption of physiological and behavioral tolerance for SW normally associated with smolt development (Lerner et al., unpublished data). During the parr-smolt transformation, exposure to relatively high concentrations of E<sub>2</sub> or NP can impair hypoosmoregulatory physiology [11,25]. In the current study, aqueous exposure to E<sub>2</sub> and environmentally relevant levels of NP reduce salinity tolerance, measured by increased plasma chloride levels after SW transfer, and disrupted ion regulatory ability in FW (decreased plasma sodium levels). The reduced capacity to tightly regulate ion fluxes in SW may be the result of reduced gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity, although the reported detrimental effects of these estrogenic compounds on the activity of this ion regu-

latory pump are not consistent across studies [11,14]. In the present study, aqueous E<sub>2</sub>, but not NP, reduces gill Na<sup>+</sup>,K<sup>+</sup>-ATPase activity. The effects of E<sub>2</sub> and NP on salinity tolerance may include impacts on other mechanisms of ion regulation, such as the number or function of chloride cells or other ion transporters [25].

The effects of stressors on fish typically include disturbance of ion homeostasis [5,26]. Handling, pollutants, and other environmental stressors can cause increased net ion effluxes when in FW and influxes when in SW [3]. This holds true for the present study, where fish treated with E<sub>2</sub> or NP-H had increased resting levels of plasma cortisol, reduced plasma sodium levels in FW, and increased plasma chloride levels in SW. This response may be due to increased catecholamine levels that reduce the vascular resistance of the gills and increase the diffusion rate of ions [3]. However, it is not clear whether the observed changes in plasma ion levels are the result of these contaminants acting as acute stressors. During a normal response to an acute stressor, rising circulating cortisol levels are thought to act as a compensatory mechanism, increasing ion uptake in FW [27]. Elevated cortisol levels are also integral to the development of salinity tolerance during smolting [7]. The observed increase in plasma cortisol is therefore not consistent with reduced osmoregulatory ability and suggests that these effects are more likely due to disruption of other endocrine pathways. Because of its association with reproduction and upstream migration of anadromous fish, estrogen signaling may be involved in determining set points for ion regulation. One mechanism by which this may occur is by decreasing GR, as discussed previously, thereby antagonizing the effects of cortisol in promoting ion uptake and SW tolerance.

Thyroid hormones clearly have a role in the morphological changes that occur during smolting and have been hypothesized to play a part in imprinting, downstream migration, and behavioral preference for SW [6,28]. In the present study, T<sub>4</sub>

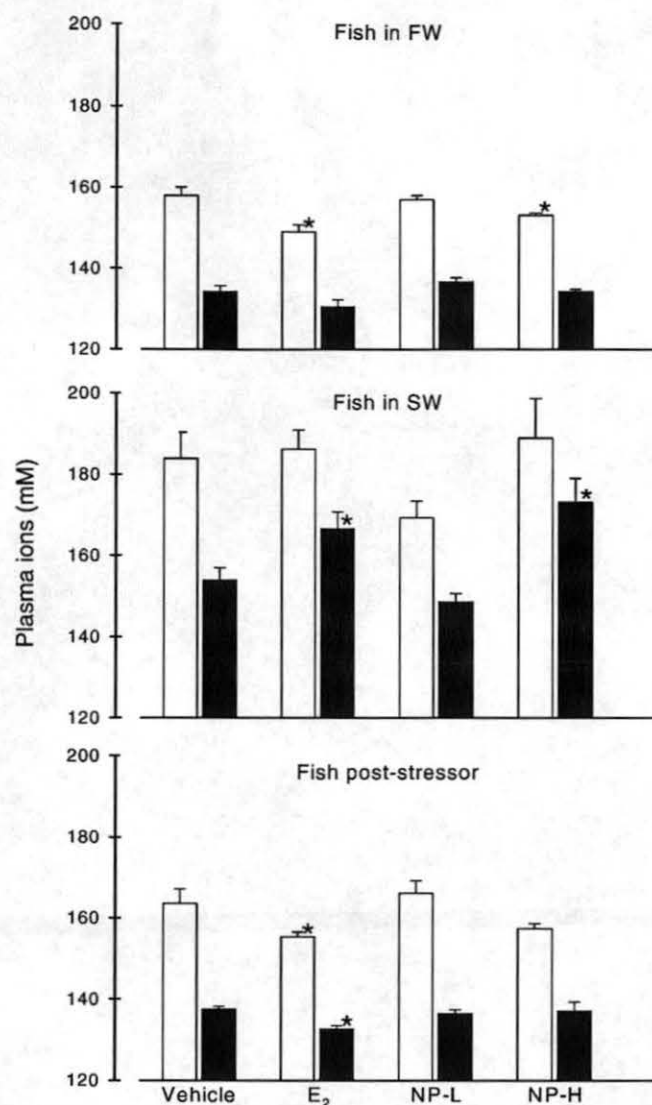


Fig. 3. Plasma sodium ( $\square$  Na<sup>+</sup>) and chloride ( $\blacksquare$  Cl<sup>-</sup>) in freshwater (FW, top), after 24 h in seawater (SW, middle), or 3 h after a handling stressor (bottom) of juvenile Atlantic salmon aqueously exposed to estradiol (E<sub>2</sub>; 2  $\mu$ g/L) or a low or high dose of nonylphenol (NP-L or NP-H; 10 or 100  $\mu$ g/L NP, respectively) for 21 d in freshwater. Values are means  $\pm$  standard error. Asterisks indicate a significant difference from vehicle control ( $p < 0.05$ ).

levels were not affected by E<sub>2</sub> or NP, but a reduction in plasma T<sub>3</sub> levels was associated with decreased salinity tolerance of E<sub>2</sub>-treated fish. Although the mechanisms of action of E<sub>2</sub> on the thyroid axis are not fully understood, it has been suggested that E<sub>2</sub> inhibits T<sub>3</sub> by decreasing hepatic T<sub>3</sub> conversion from T<sub>4</sub> and increasing renal T<sub>3</sub> degradation [29,30]. In agreement with the present data, several studies support the suppressive role of E<sub>2</sub> on circulating T<sub>3</sub> in salmonids [29–31]. Stress also suppresses the thyroidal activity of fish [32], and cortisol, like E<sub>2</sub>, reduces plasma T<sub>3</sub> levels by lowering the rate of T<sub>4</sub> to T<sub>3</sub> conversion at target tissues [33]. This E<sub>2</sub>-induced up-regulation of cortisol could have direct effects on the thyroid axis and/or indirect effects through GH suppression, which stimulates hepatic 5'-monodeiodinase activity and T<sub>3</sub> levels in rainbow trout [34]. Therefore, several endocrine pathways may have been involved in the observed decrease in plasma T<sub>3</sub> in the present study.

In addition to changes in interrenal and thyroid homeosta-

Table 1. Growth of juvenile Atlantic salmon exposed to a low or high dose of nonylphenol (NP-L or NP-H, respectively) or 17 $\beta$ -estradiol (E<sub>2</sub>) for 21 d in freshwater<sup>a</sup>

	Fish wt (T <sub>0</sub> )	Fish wt (T <sub>1</sub> )	Growth rate (%/d)
Vehicle	46.18 $\pm$ 1.98	51.40 $\pm$ 1.55	0.36
E <sub>2</sub>	46.04 $\pm$ 1.61	46.90 $\pm$ 1.42*	0.06*
NP-L	46.13 $\pm$ 1.87	51.80 $\pm$ 1.50	0.27
NP-H	45.58 $\pm$ 1.91	49.50 $\pm$ 2.00	0.26

<sup>a</sup> Values are means  $\pm$  standard error for fish weight one week before aqueous exposure began (T<sub>0</sub>) and at the end of the 21-d exposure (T<sub>1</sub>). Growth rate was calculated as the percent growth per day. E<sub>2</sub>, 2  $\mu$ g/L 17 $\beta$ -estradiol; NP-L, 10  $\mu$ g/L nonylphenol; NP-H, 100  $\mu$ g/L nonylphenol. E<sub>2</sub> had a negative impact on growth (one-way analysis of variance,  $p < 0.05$ ;  $n = 12$ ). Asterisk indicates significant difference from vehicle control.

sis, E<sub>2</sub> and NP-H treatment increased plasma VTG levels, a widely used biomarker of xenoestrogen exposure. Vijayan et al. [35] have demonstrated that E<sub>2</sub> treatment of tilapia (*Oreochromis mossambicus*) increases VTG and T<sub>3</sub> levels, has no effect on plasma cortisol, and decreased the activity of phosphoenolpyruvate, aspartate aminotransferase, malate dehydrogenase, and 6-phosphogluconate dehydrogenase, hepatic enzymes involved in gluconeogenesis. These data on tilapia suggest a physiological shift toward anabolic metabolism. This is in contrast with the present study and others [31] indicating that E<sub>2</sub> decreases T<sub>3</sub> levels in salmonids and increases cortisol levels, reflecting an increase in catabolic potential. Although this discrepancy may be attributed to a species differences, an E<sub>2</sub>-induced shift to catabolic metabolism is similar to what occurs when sex steroid production is high during maturation and reproduction [22].

The present study shows that aqueous exposure to E<sub>2</sub>, but not NP, reduces growth and plasma GH levels after 21 d of treatment. Inhibition of growth has been reported for a wide array of stressors, including handling and exposure to contaminants [5,36,37]. Similar to the present study, stress-induced elevation of plasma cortisol concentration is associated with reduced plasma GH in teleosts [38,39]. It is not clear from the present data whether E<sub>2</sub> reduces GH levels by reducing the rate of secretion, by increasing clearance rate, or both. Growth hormone is also involved in SW acclimation of salmonids [40]. In the present study, the reduction in circulating GH by E<sub>2</sub> treatment is probably involved in the observed decrease in salinity tolerance.

Plasma levels of IGF-I were reduced in E<sub>2</sub>-treated fish of the present study but not in fish exposed to NP. This effect of E<sub>2</sub> is consistent with other recent studies indicating that estrogenic compounds decrease circulating IGF-I during smolt development [11,36]. Insulin-like growth factor-I is a mediator of many of the effects of GH on growth and osmoregulation, and the predominant source of circulating IGF-I is hepatic, with GH acting as a major secretagogue [41]. This suggests that the GH-IGF-I axis is a likely mechanistic route for the decreased growth and ion regulatory capacity observed after E<sub>2</sub> treatment. Although changes in GH or IGF-I levels did not occur in response to NP-H treatment, which did cause decreased salinity tolerance, it is possible that changes in these hormones occurred prior to the terminal sampling at 21 d [11].

Estrogenic compounds can decrease hepatic GH receptor (GHR) density in some mammals [42], and an inhibitory effect of E<sub>2</sub> on liver GHR mRNA levels is indicated in tilapia [43]. Evidence exists that this inhibitory action occurs through sup-



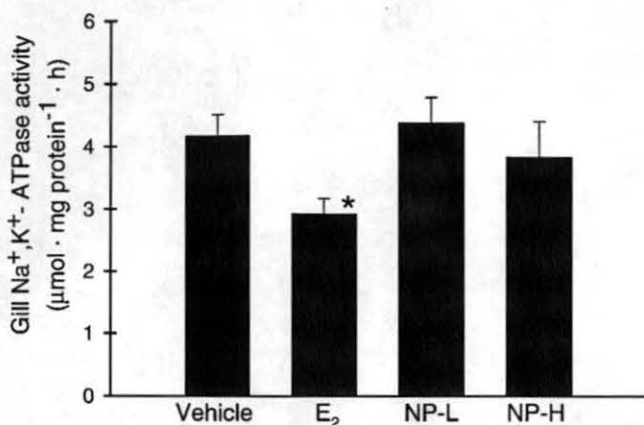


Fig. 4. Gill sodium potassium-activated adenosine triphosphatase (Na<sup>+</sup>,K<sup>+</sup>-ATPase) activity of juvenile Atlantic salmon aqueously exposed to estradiol (E<sub>2</sub>; 2 μg/L) or a low or high dose of nonylphenol (NP-L or NP-H; 10 or 100 μg/L NP, respectively) for 21 d in freshwater. Values are means ± standard error. Asterisks indicate a significant difference from vehicle control ( $p < 0.05$ ).

pression of GH-induced janus kinase-2 phosphorylation [44], although this mechanism has not yet been studied in fishes. Additionally, E<sub>2</sub> can increase IGF binding protein-1 in teleosts, which is known to inhibit the activity of IGF-I [45]. It is not presently known whether NP affects IGF binding protein-1 in a similar manner. If it does, then the ability of NP in the present study to reduce ion regulatory capacity in the absence of changes in IGF-I levels may be due to circulating IGF-I being prevented from interacting with its receptor.

The present data indicate that aqueous exposure to NP during smolting increases baseline cortisol and the cortisol response to a stressor (primary response) without initiating secondary or tertiary responses. These data also demonstrate that exposure to NP during smolting impairs ion homeostasis without detectably affecting the hormones and proteins involved in their regulation. It cannot be ruled out that the different impacts observed between NP and E<sub>2</sub> may be due to the greater estrogenic potency of E<sub>2</sub> (e.g., greater induction of vitellogenesis). These differences suggest that diverse pathways are involved in the observed impacts of these estrogenic compounds. It is known that environmental estrogens such as NP can interact with the native estrogen receptor (ER) to adversely affect reproductive development [46]. Evidence also exists that other estrogen mimics (some pesticides, alkylphenolic chemicals, phthalates, bisphenol A), including NP, have negative effects on osmoregulation by interacting with the ER [1]. However, the ER is promiscuous, and the binding of nonestrogen ligands may initiate diverse intracellular second-messenger cascades, resulting in ligand-dependent variations in downstream products from a single receptor [47]. In addition, the three ER subtypes found in teleosts (ERα, ERβa, and ERβb) exhibit differences in relative ligand-binding affinity and tissue distribution that could contribute to the selective action of ER agonists in different tissues [48]. These potential mechanisms for ligand-dependent effects are not mutually exclusive and provide possible explanations for the observed differences between the impacts of E<sub>2</sub> and NP in the present study.

In addition to the measured impacts on stress, growth, and ion regulation, exposure to xenoestrogens may have negative impacts on disease resistance. Stress and contaminant exposure can have dramatic and diverse effects on the immune system [49]. Growth hormone may also play a role in maintaining a

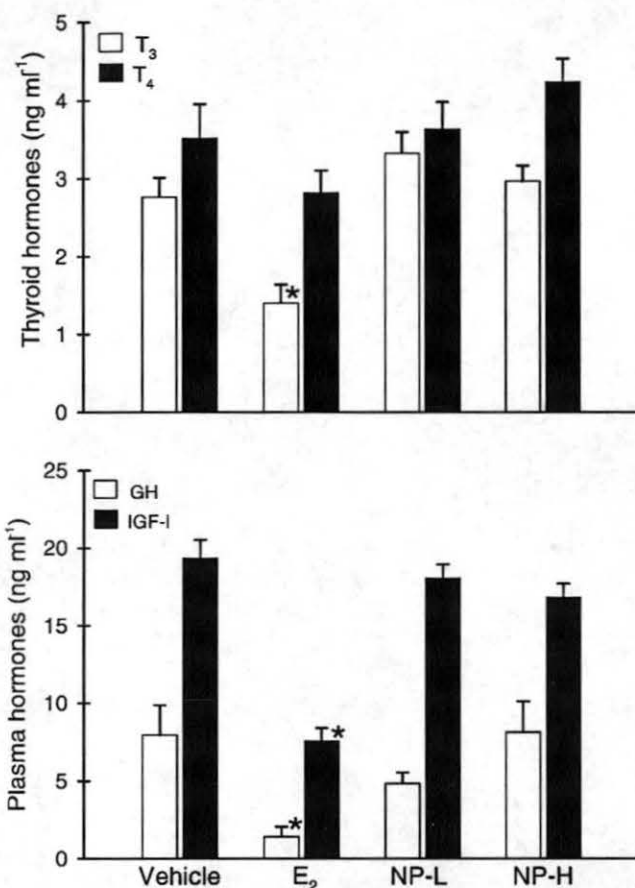


Fig. 5. Plasma thyroid hormones, triiodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) (top), growth hormone (GH), and insulin-like growth factor I (IGF-I) (bottom) of juvenile Atlantic salmon aqueously exposed to estradiol (E<sub>2</sub>; 2 μg/L) or a low or high dose of nonylphenol (NP-L or NP-H; 10 or 100 μg/L NP, respectively) for 21 d in freshwater. Values are means ± standard error. Asterisks indicate a significant difference from vehicle control ( $p < 0.05$ ).

robust immune system in fish [50], and contaminants that reduce circulating GH levels, as observed in the present study, may exacerbate vulnerability to disease. Therefore, these impacts in response to environmental stressors could increase susceptibility to disease and new pathogens encountered in the river, estuary, or marine environments.

In conclusion, we have found that exposure of juvenile Atlantic salmon to aqueous E<sub>2</sub> and NP elicits elevated cortisol, increases the response to external stressors, and impairs osmoregulatory physiology. In the case of E<sub>2</sub>, we also observed impacts on circulating levels of T<sub>3</sub>, GH, and IGF-I. The basis of the effects of aqueous NP-H on salinity tolerance is less clear but may occur through divergent mechanistic pathways not assessed in the present study. The doses of E<sub>2</sub> and NP-H used in the present study are likely greater than expected in a single exposure of these individual compounds in nature. It is possible that fish exposed to multiple xenoestrogens will respond cumulatively such that measurement of all xenoestrogens will be necessary to determine whether such effects occur in nature. In addition, the sensitivity of smolt development to xenoestrogens may change during smolting or at other life history stages. Based on our results, however, it seems that only in rare circumstances will exposure to nonylphenol and other xenoestrogens be sufficiently high to directly affect SW tolerance. The more environmentally relevant aqueous dose of

10 µg/L (NP-L, 6 µg/L actual) did impact basal cortisol and the cortisol response to an external stressor. Cortisol affects a wide variety of physiological responses, including feeding, growth, behavior, disease resistance, and ion regulation [4]. It is difficult to determine with certainty what impacts alterations on the stress axis may have in nature, but this is an area that deserves increased attention, as it may impact the long-term sustainability of salmon populations.

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