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Estrogenic compounds decrease growth hormone receptor abundance and alter osmoregulation in Atlantic salmon

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ABSTRACT

Exposure of Atlantic salmon smolts to estrogenic compounds is shown to compromise several aspects of smolt development. We sought to determine the underlying endocrine mechanisms of estrogen impacts on the growth hormone (GH)/insulin-like growth factor I (IGF-I) axis. Smolts in freshwater (FW) were either injected 3 times over 10 days with 2 $\mu g\, g^{-1}$ 17β-estradiol (E2) or 150 $\mu g\, g^{-1}$ 4-nonylphenol (NP). Seawater (SW)-acclimated fish received intraperitoneal implants of 30 $\mu g\, g^{-1}$ E2 over two weeks. Treatment with these estrogenic compounds increased hepatosomatic index and total plasma calcium. E2 and NP reduced maximum growth hormone binding by 30–60% in hepatic and branchial membranes in FW and SW, but did not alter the dissociation constant. E2 and NP treatment decreased plasma levels of IGF-I levels in both FW and SW. In FW E2 and NP decreased plasma GH whereas in SW plasma GH increased after E2 treatment. Compared to controls, plasma chloride concentrations of E2-treated fish were decreased 5.5 mM in FW and increased 10.5 mM in SW. There was no effect of NP or E2 on gill sodium–potassium adenosine triphosphatase (Na^+/K^+-ATPase) activity in FW smolts, whereas E2 treatment in SW reduced gill Na^+/K^+-ATPase activity and altered the number and size of ionocytes. Our data indicate that E2 downregulates the GH/IGF-I-axis and SW tolerance which may be part of its normal function for reproduction and movement into FW. We conclude that the mechanism of endocrine disruption of smolt development by NP is in part through alteration of the GH/IGF-I axis via reduced GH receptor abundance.

1. Introduction

In anadromous salmonids, endogenous sex steroids are extremely low during development until reproductive maturation, when increases accompany movement back into freshwater (FW) [50,51]. During smolting, administration of sex steroids is inhibitory to the development of salinity tolerance [21,33,44,45,62]. In Atlantic salmon, administration of exogenous estradiol (E2) can compromise physiological and behavioral parameters associated with normal smolt development, such as elevated gill sodium–potassium activated adenosine triphosphatase (Na^+/K^+,-ATPase) activity, increased chloride cell or ionocyte density, and improved salinity tolerance and preference [28,29]. Similar effects occur after exposure to environmental estrogens such as nonylphenol (NP) [26,28,29]. This suggests that exposure to estrogenic compounds in the environment can have deleterious effects on the physiological and behavioral changes necessary for maintaining osmoregulatory homeostasis in seawater. Indeed, Fairchild et al. [16] found that Atlantic salmon populations in New Brunswick experienced declines following spraying for spruce budworm, and concluded that NP present in the spray formulations may have affected survival of smolts soon after seawater entry. E2 and NP are present in both domestic and industrial wastewater and are two of the most widely distributed estrogenic compounds detectable in aquatic ecosystems [28].

Smolting is a developmental process involving morphological, physiological, and behavioral changes that prepare juvenile salmonids for migration to, and residence in, the ocean [20]. Increased gill Na^+/K^+-ATPase activity, which occurs during seawater (SW) acclimation in Atlantic salmon (*Salmo salar*) [61] and other euryhaline teleosts [14] is an essential component to the parr-smolt transformation. Gill Na^+/K^+-ATPase activity is localized to ionocytes [23,38], and its elevation is directly related to an increase in size and number of these specialized cells that are responsible for salt secretion [40,52]. While the parr-smolt transformation typically occurs in Atlantic salmon during the first or second spring

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after hatching, it is recognized that some aspects of smolt development are reversible. Smolts retained in fresh water in the laboratory exhibit a decrease in hypoosmoregulatory capacity and concurrent decreases in gill Na^+/K^+-ATPase activity [64,66] and ionocyte abundance [52].

The physiological changes that occur during smolt development in salmon are influenced by the endocrine axis of growth hormone (GH) and insulin-like growth factor I (IGF-I) [43,60,65]. GH levels rise during smolting [41] and exogenous GH treatment increases gill Na^+/K^+-ATPase activity and numbers of branchial ionocytes [7,32], resulting in increased salinity tolerance [8,24,32]. Treatment with GH can also increase seawater preference, a behavioral indicator of salinity tolerance, in pre-smolting coho salmon (*Oncorhynchus kisutch*) [22]. Plasma levels of IGF-I also increase during smolting [41]. In addition, IGF-I mRNA expression is elevated in the gill and kidney following exposure of rainbow trout (*O. mykiss*) to seawater [59], and administration of exogenous IGF-can increase salinity tolerance in this same species [41].

Environmental disruption of salinity tolerance during smolting has been linked to negative effects of estrogenic compounds on the GH/IGF-I axis, particularly reduced plasma levels of IGF-I [1,45]. Production of circulating IGF-I is largely due to GH binding the hepatic growth hormone receptor (GHR) [13]. GHR abundance has been demonstrated to increase during acclimation to seawater in rainbow trout [59], providing evidence for its role in increasing salinity tolerance. Estrogenic compounds can decrease hepatic GHR in some mammals [12], however this estrogenic effect is not consistent among all mammals [2]. While Davis et al. [11] demonstrated decreased hepatic GHR mRNA expression following injection of E2 in Mozambique tilapia, to date, the effects of E2 on GHR abundance and binding capacity in fish has not been examined. We hypothesize that E2 is an endogenous regulator of hyperosmoregulatory ability during maturation and upstream migration of adult salmon promoting ion uptake in freshwater, but acts as an exogenous disrupter of hypoosmoregulatory ability during smolt development and downstream migration. To address this, we investigated the impacts of exogenous E2 on salmon GH (sGH) binding capacity and ionregulatory status of freshwater and seawater-acclimated Atlantic salmon.

2. Materials and methods

2.1. Fish rearing

Atlantic salmon parr 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, 2004. Fish were maintained in 1.6 m diameter tanks supplied with Connecticut River water at a flow rate of 6–8 L min^{-1} under natural photoperiod and ambient temperature, and fed to satiation twice daily.

2.2. Treatments

During the peak of smolting in May 2005, 36 smolts in FW were implanted with uniquely coded passive integrated transponders (PIT; Biomark, Boise, ID) and received intraperitoneal injections with either 150 μg g^{-1} branched para-nonylphenol (CAS No. 84852-15-3, 95.3% pure; Schenectady, NY, USA), 2 μg g^{-1} E2 (Sigma Chemical, St. Louis, MO), or vehicle (vegetable oil). These concentrations were chosen based on their demonstrated ability to elicit physiological concentrations in previous injection studies [21,27,29,33,45]. Injections were repeated on the 4, and 8th day. Fish were not fed during treatment and were sampled on day 10. An additional 20 fish were transferred to recirculating, seawater (SW; 20‰ for 5 days, then 35‰ thereafter) at 10 °C with charcoal filtration and aeration for 8 weeks and fed to satiation once daily. After 8 weeks in SW, fish were paint marked on their anal fin for treatment identification and received intraperitoneal implants of vehicle (1:1 Crisco:vegetable oil) or 30 μg g^{-1} E2, which has been shown to elicit a physiological concentration in previous studies [50]. After injection of the implant, fish were held in seawater for 14 days until sampling. The concentration of the implant used was chosen based on the demonstrated ability to elicit physiological concentrations in previous studies [15,50,63].

2.3. Fish sampling

After treatment, 12 fish from each group in FW and 10 fish from each group in SW were sampled directly from their treatment tanks and measured for body weight and length. Fish were anesthetized with 200 mg l^{-1} tricaine methane sulphonate (neutralized and buffered with sodium bicarbonate, pH 7.0). All fish were sampled within six minutes of first disturbing the tank. Blood was collected in heparinized syringes from the caudal vasculature, stored on ice for less than 30 min, centrifuged at 3000*g* for 5 min, then plasma removed and frozen at −80 °C. Livers were removed, weighed and immediately frozen on dry ice for the receptor binding assay. A gill biopsy (approximately six to eight primary gill filaments) was placed in 100 μl of SEI (250 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.3) on ice for and then stored at -80 °C for determination of Na^+/K^+-ATPase activity. Additional gill tissue from SW fish was placed in 2% paraformaldehyde, stored overnight at 4 °C, and then transferred to 70% EtOH for immunocytochemistry. The remaining gill tissue was trimmed off the arches and frozen at −80 °C for the GH receptor binding assay.

2.4. Enzyme and hormone analyses

Gill Na^+/K^+-ATPase activity was measured according to the microassay protocol of McCormick, 1993 [39]. Gill filaments were homogenized in SEI buffer containing 0.1% sodium deoxycholate. Following centrifugation (3000*g* for 5 min) to remove insoluble material, Na^+/K^+-ATPase activity 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 or absence of 0.5 mM ouabain. Protein content in the gill homogenate was measured using a bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA), and specific activities were expressed as μmol ADP mg^{-1} of protein h^{-1}.

Plasma GH levels were measured using a specific double-antibody salmon GH radioimmunoassay [4]. Plasma IGF-I levels were measured by a radioimmunoassay validated for salmonids [48]. Plasma chloride concentration was measured using silver titration chloridometry (Labconco, Kansas City, Missouri) with external standards. Total plasma calcium (Ca_T) was measured with an atomic absorption spectrophotometer (AAnalyst 100, Perkin Elmer, Norwalk, CT, USA).

2.5. Growth hormone receptor binding

2.5.1. Tissue preparation

Microsomes were isolated in a manner similar to Gray et al. 1990 [19]. Approximately 0.2–0.4 g of liver or gill tissue were homogenized separately in ice-cold homogenizing solution (250 mM sucrose, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01 TIU/mL aprotinin, pH 7.5). Homogenates were spun at 4 °C for 30 min at 3000*g*; supernatants were then spun for 30 min at 15000*g*. Resulting supernatants were spun at 100,000*g* for 90 min at 4 °C. Membrane pellets were resuspended in 25 mM Tris–HCl (pH 7.5) with 10 mM $MgCl_2$. Protein concentrations of

the membrane preparations were determined by the Bio-Rad dye-binding method (Hercules, CA), using bovine serum albumin (BSA) as a standard. Isolated membrane preparations were stored at −80 °C for 2 days until used in a GH radioreceptor assay as described below.

2.5.2. GH iodination

Salmonid GH (sGH; GroPep, Adelaide, Australia) was iodinated for use in binding assays by a procedure modified from Gray et al. 1990 [19]. Five micrograms of sGH were labeled using lactoperoxidase and 200 μCi of [125I]-NaI (MP Biomedicals, Irvine, CA). Free iodide was separated from 125I-sGH by gel filtration chromatography using a Sephadex G-75 colmum (0.6 × 13 cm) and 0.1 M Tris–HCl, pH 8.5 for elution. Twenty drop fractions were collected and the percentage of label incorporated into the hormone was determined by TCA precipitation. Fractions in which more than 95% of counts were precipitable were saved at 4 °C for later use.

2.5.3. Radioreceptor assay

For liver and gill, 200 and 150 μg of membrane protein, respectively, and 15,000 cpm [125I]-sGH in assay buffer (20 mM Tris–HCl, 0.5% BSA, 0.1% NaN3, with 0.1 TIU/mL aprotinin, pH 7) were incubated in the presence of unlabeled sGH (diluted in incubation Medium), at 0.0, 14.5, 29, 58, 116, 232 and 465 nM. The assay mixture was incubated for 18–20 h at 15 °C. After adding 2 mL ice-cold assay buffer, tubes were centrifuged for 20 min at 1000*g* and the supernatant decanted. Bound radioactivity was quantified with a Beckman Gamma 5500. The percentage of specific binding was calculated as B/T × 100, where B is the difference between total bound and nonspecific bound, and T = the amount of radioactivity originally added to each tube.

2.6. Immunocytochemistry

Monoclonal antisera directed against the α-subunit of chicken Na^+/K^+-ATPase (α5; Iowa Hybridoma Bank Iowa City, IA) and diluted 1:500 was used for immunocytochemical detection of Na^+,K^+-ATPase. Alexa Fluor 546 goat anti-rabbit (Molecular Probes, Eugene, OR, USA) was used as a secondary antibody. Antibody control experiments (primary antibody without secondary antibody, and secondary antibody without primary antibody) showed no specific staining and low background.

Fixed gill tissue was rinsed in 10 μmol^{-1} phosphate-buffered saline (PBS), placed in PBS with 30% (w/v) sucrose for one hour and then frozen in embedding medium. Five μm sections were cut in a cryostat at −23 °C in two orientations. Sagittal sections were cut parallel to the long axis of primary filaments and perpendicular to the attachment of secondary lamellae. Cross sections were cut perpendicular to the long axis of primary filaments. The tissue was placed on poly-L-lysine-coated slides, dried, rinsed with PBS and then incubated in 2% normal goat serum in PBS for 0.5 h at room temperature. Slides were exposed to primary antibody in antibody dilution buffer (0.01% NaN_3, 0.1% bovine serum albumin, 2% normal goat serum and 0.02% keyhole limpet hemocyanin in PBS) and incubated overnight at 4 °C. After incubation, the slides were rinsed several times with PBS, exposed to secondary antibody at room temperature for 2 h and then rinsed several times with PBS. The tissue was covered by a cover slip and examined with a Nikon inverted fluorescent microscope with a mercury lamp. Images were taken within 4 h of completion of staining for subsequent counting and morphometric analysis.

From each fish, immunoreactive ionocytes on the primary filament were counted from cross sections of gill filament and expressed per mm of primary filament. Ionocytes on the secondary lamellae were counted from sagittal sections of gill filament and expressed per secondary lamellae. Mean numbers of ionocytes for each group were obtained using the means calculated from at least 8 sections from each fish. Cell size (staining area; μm^2 cell $^{-1}$) was obtained from ionocytes using MetaMorph 4.1.2 (Universal Imaging Corporation, West Chester, PA, USA). At least 50 individual ionocytes from several different tissue sections were measured from at least six fish per group.

2.7. Statistics

All values 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. All statistics were analyzed using Statistica (Version 7; Statsoft Inc, Tulsa, OK, USA).

3. Results

There were no mortalities over the course of the study. E2 and NP treatment elevated plasma Ca_T of FW smolts 57% and 30%, respectively compared to controls (Table 1). E2 had a similar effect on SW-acclimated fish, increasing Ca_T 58% (Table 1). Compared to control values, HSI was also increased by E2 and NP 2.2 and 1.5-fold in FW smolts, respectively and 2.7-fold by E2 in SW fish (Table 1).

Scatchard plots revealed that dissociation constants (K_d) of sGH for GHR were equivalent between hepatic and branchial membranes from FW and SW fish ($p = 0.10$ and $p = 0.62$, respectively; Fig. 1). Binding capacity (B_{max}) was 3 to 4-fold greater in hepatic membranes, regardless of salinity (Fig. 1). Treatment did not alter the K_d of sGH for GHR in hepatic (FW E2, $p = 0.86$; FW NP $p = 0.83$; SW E2 $p = 0.64$) or branchial membranes (FW E2, $p = 0.99$; FW NP $p = 0.335$ SW E2 $p = 0.824$) from FW or SW fish (Fig. 1). E2 and NP reduced B_{max} 50% and 57%, respectively in hepatic membranes of FW smolts, and 58% and 53%, respectively in branchial membranes of FW smolts compared to controls (Fig. 1). In SW fish, E2 treatment resulted in a reduction in B_{max} of 29% and 36% in hepatic and branchial membranes respectively, relative to controls (Fig. 1).

In FW smolts, E2 and NP treatment decreased plasma levels of GH 4.5-fold and 2.3-fold, respectively (Fig. 2). In contrast, plasma GH in SW fish was increased 3.5-fold after administration of exogenous E2 (Fig. 2). Fresh water smolts exhibited 2.7-fold reductions in plasma levels of IGF-I following E2 and NP treatment, while E2 reduced plasma IGF-I in SW fish 3.5-fold (Fig. 2).

Gill Na^+/K^+-ATPase activity was unaffected by treatment of FW smolts with either E2 or NP ($p = 0.81$; $p = 0.61$, respectively; Fig. 3). In contrast, SW fish treated with E2 exhibited 22% lower gill Na^+/K^+-ATPase activity than controls (Fig. 3). Compared to controls, plasma chloride concentration was decreased 5.5 mM by treatment with E2 and unaffected by NP ($p = 0.30$; Fig. 3). In SW, E2 increased plasma chloride concentration 10.5 mM over controls

Table 1
Mean plasma total calcium levels (Ca_T), and hepatosomatic index (HSI) of FW and SW-acclimated Atlantic salmon treated with E2, NP, or vehicle control.

	Ca_T (mM)	HSI
FW		
Control	1.9 ± 0.03	0.97 ± 0.06
E2	2.9 ± 0.04*	2.10 ± 0.16*
NP	2.5 ± 0.03*	1.43 ± 0.10*
SW		
Control	1.9 ± 0.04	1.11 ± 0.03
E2	3.0 ± 0.06*	3.04 ± 0.06*

Asterisks represent significant differences from controls. Values are means ± standard error; FW, $n = 12$ and SW $n = 10$.

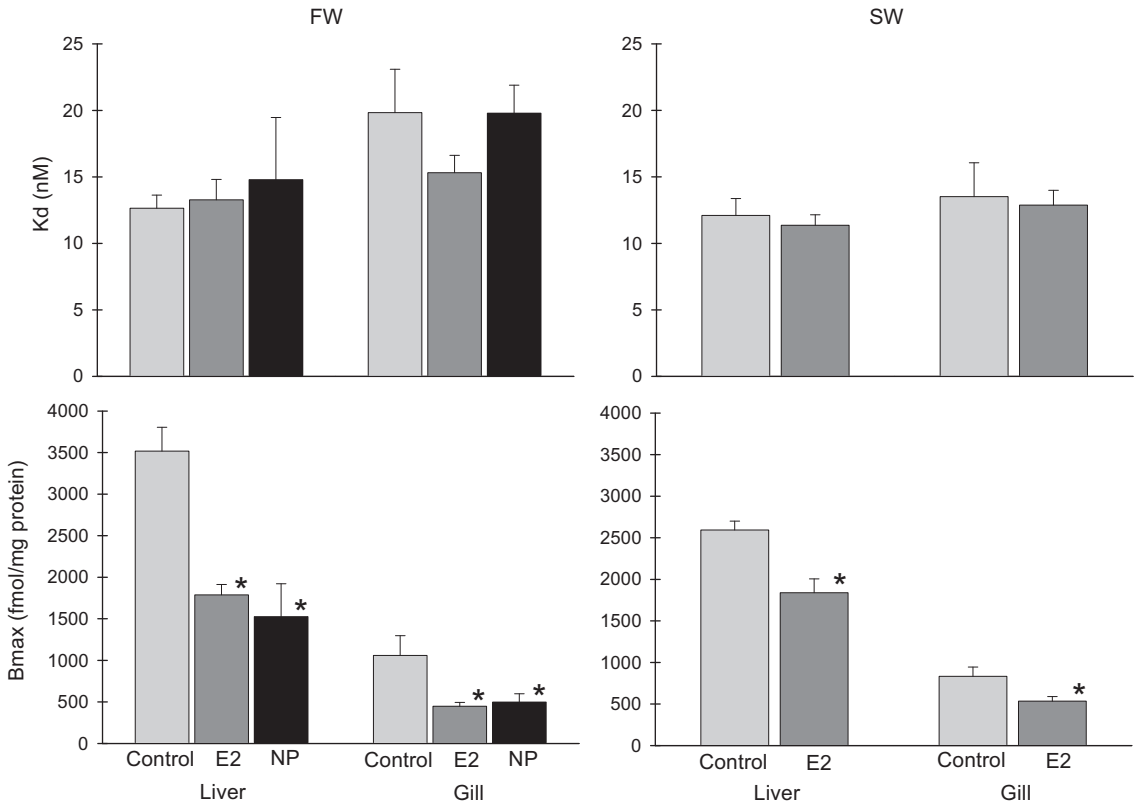

Fig. 1. The effects of E2 and NP on the dissociation constant (K_d; upper left) and maximum binding capacity (B_{max}; lower left) of sGH in FW Atlantic salmon smolts and the effects of E2 on the dissociation constant (K_d; upper right) and maximum binding capacity (B_{max}; lower right of sGH in seawater-acclimated Atlantic salmon. Data are presented as mean values (±S.E.M.; FW, n = 5–7; SW, n = 12). Bars with an asterisk are significantly different from control (P < 0.05).

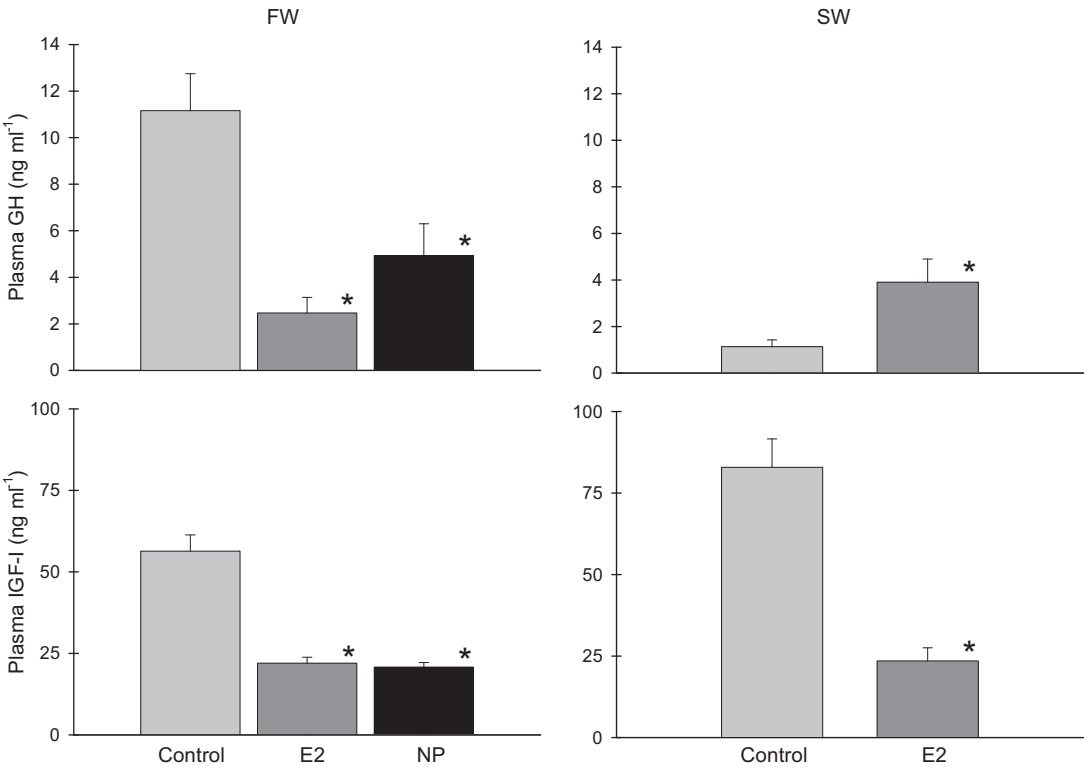

Fig. 2. The effects of E2 and NP on plasma GH levels (upper left) and plasma IGF-I levels (lower left) in FW Atlantic salmon smolts and the effects of E2 on plasma GH levels (upper right) and IGF-I levels (lower right) in seawater-acclimated Atlantic salmon. Data are presented as mean values (±S.E.M.; FW, n = 12; SW, n = 10). Bars with an asterisk are significantly different from control (P < 0.05).

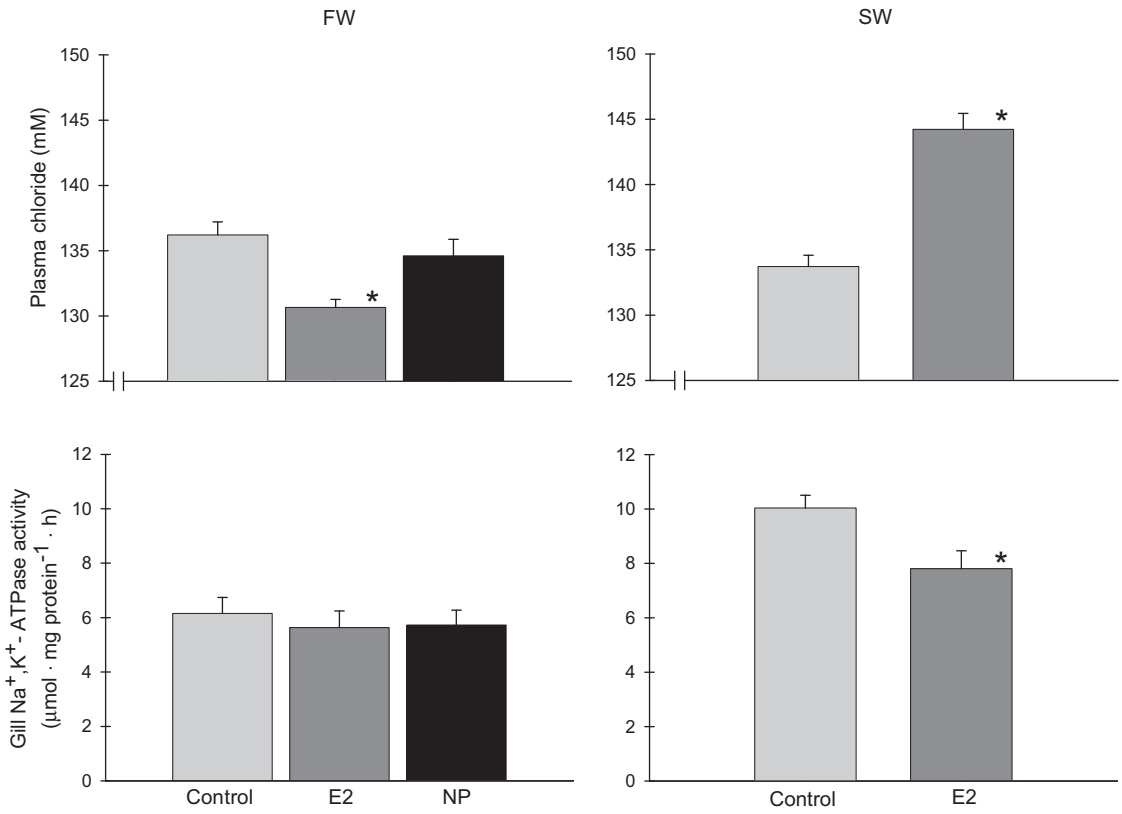

Fig. 3. The effects of E2 and NP on plasma chloride concentration (upper left) and gill Na^+,K^+-ATPase activity (lower left) in FW Atlantic salmon smolts and the effects of E2 on plasma chloride concentration (upper right) and gill Na^+,K^+-ATPase activity (lower right) in seawater-acclimated Atlantic salmon. Data are presented as mean values (±S.E.M.; FW, $n = 12$; SW, $n = 10$). Bars with an asterisk are significantly different from control ($P < 0.05$).

(Fig. 3). Treatment of SW fish with E2 reduced the number and size of ionocytes on the primary gill filament 51–53% (Fig. 4 and Table 2) and increased the number of ionocytes on the secondary lamellae 6.5-fold (Fig. 4 and Table 2).

4. Discussion

Our results indicate that E2 and NP can downregulate GHR abundance in the gill and liver and thus alter the GH/IGF-I axis of Atlantic salmon smolts. The decline in GHR abundance in the liver was associated with and likely causal to the observed decreases in circulating IGF-I following injection of E2 or NP. Recent studies confirm the role of estrogenic compounds in decreasing circulating IGF-I during smolt development [1,45]. We also observed impacts of estrogenic compounds on hypoosmoregulatory ability which are likely due to alterations in the GH/IGF-I axis.

Hepatic GHR is abundant in euryhaline salmonids [17,19,58] and increases during seawater acclimation of rainbow trout [58]. Exogenous GH stimulates both hepatic and branchial IGF-I gene expression in coho salmon [9]. Circulating levels of GH increase during smolt development in Atlantic salmon [43] and exogenous GH treatment increases gill Na^+/K^+-ATPase activity and salinity tolerance in salmonids [7,8,24,32]. Similarly, circulating levels of IGF-I increase prior to smolting in Atlantic salmon [43]. IGF-I mRNA increases in the gill and kidney of rainbow trout following exposure to seawater [59], and administration of exogenous IGF-I treatment increases salinity tolerance of rainbow trout and Atlantic salmon [40,46]. These data support the role of the GH/IGF-I axis in promoting salinity tolerance of anadromous salmonids and suggest that the negative impacts of E2 on gill Na^+/K^+-ATPase activity and salinity tolerance are through reductions in circulating IGF-I.

There is also evidence that local GH/IGF-I signaling is involved in control of osmoregulation. GH receptors are found in several tissues other than liver, including in osmoregulatory tissues and are especially abundant in the gill [17,19,58]. *In vitro* treatment of gill lamellae with GH does not increase gill Na^+/K^+-ATPase activity [42], but prior *in vivo* treatment with GH increases the *in vitro* capacity of IGF-I to increase Na^+/K^+-ATPase activity [34]. Insulin-like growth factor I mRNA increases in the gill during SW acclimation of rainbow trout [59]. Increased transcription of branchial GHR, IGF-I and IGF-I receptor has been observed during smolt development of anadromous Atlantic salmon, and is less pronounced in landlocked strains that do not develop salinity tolerance in spring [49]) GHR and IGF-I mRNA are expressed in the gills of the euryhaline tilapia [5,6,57], and the presence of IGF-I in gill ionocytes has been detected using immunocytochemistry [57]. These data are not sufficient to determine the role of GH and its receptor, but suggest that local production of IGF-I in the gill may be important for hypoosmoregulatory ability. Thus, the decrease in GHR in gill tissue following NP and E2 treatment may also play a role in alteration in osmoregulation of juvenile Atlantic salmon.

In the present study, plasma levels of GH were decreased by treatment of FW fish with E2 and NP and increased by treatment of SW fish with E2. McCormick et al. 2005 [45] found variation in the GH response to dose and timing of administration of NP ranging from 0.5 to 150 $\mu g\, g^{-1}$ and 7 and 14 days, respectively. One possible explanation of the difference in E2 impact in different salinities is that GH sensitivity to negative feedback by IGF-I is altered by environmental salinity. Differences in dose and/or developmental timing may have also played a role.

The exact mechanism (s) of impact of E2 on the GHR in fish is not currently known. Estrogen may reduce GHR availability by

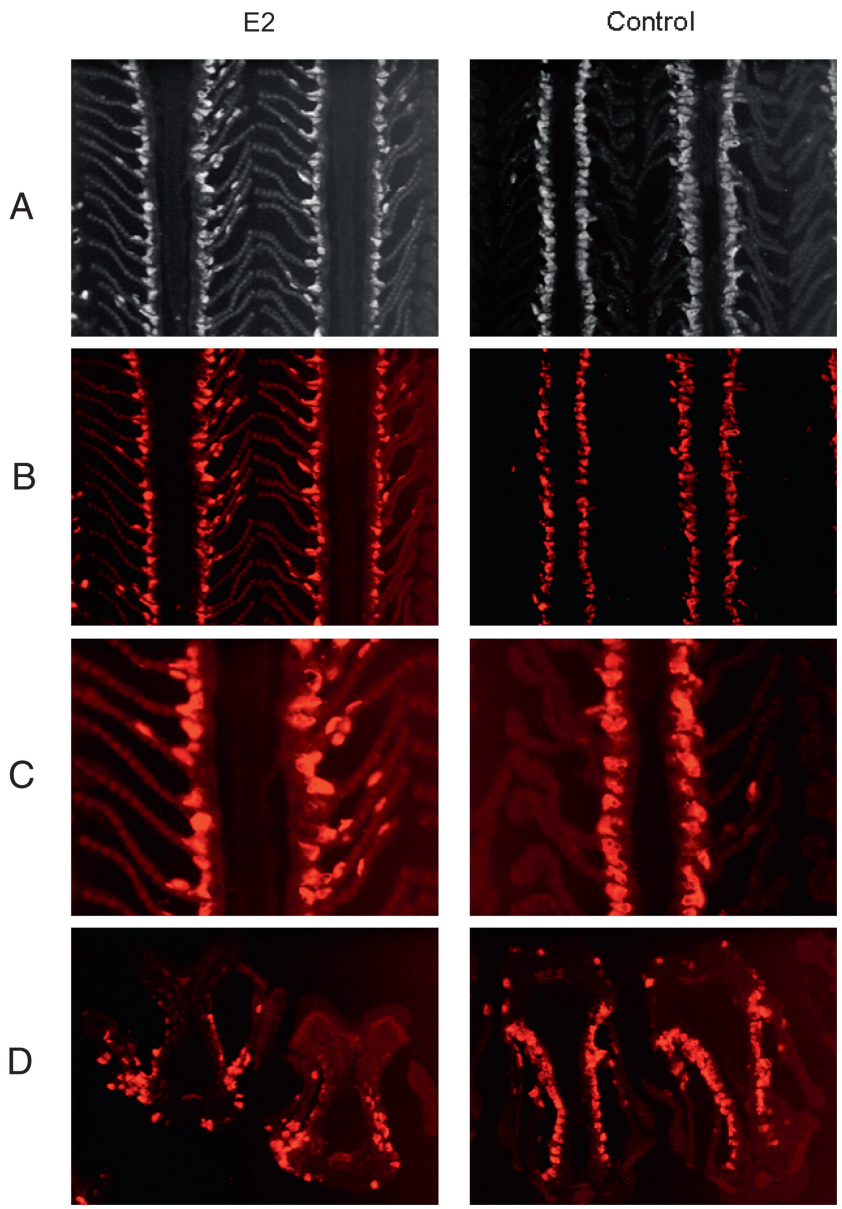

Fig. 4. Representative images of the effects of E2 on ionocyte distribution and abundance in seawater-acclimated Atlantic salmon. Shown are sagittal sections without staining (A), sagittal sections with staining indicating E2-induced increase in proliferation of ionocytes on secondary lamellae (B), 2× magnification of images in B (C), and cross sections of primary filaments showing reduced number and size of ionocytes after treatment with E2 (D). The width of images in A, B and D is 820 μm. The width of the images in C is 410 μm.

Table 2
Quantification of the effects of E2 on ionocyte abundance and distribution in seawater-acclimated Atlantic salmon.

	Cell density filament (cells mm^{-1})	Lamellae (cells mm^{-1})	Cell size filament (μm^2)
Control	61.4 ± 5.1	0.18 ± 0.02	247.1 ± 7.4
E2	30.8 ± 1.7*	1.34 ± 0.15*	115.5 ± 3.6*

Presence of ionocytes was determined by immunoreactive localization of Na^+/K^+-ATPase. Data are presented as mean values ±SEM. At least 50 individual ionocytes from several different tissue sections were measured from at least 6 fish per group. Asterisks represent significant differences from controls.

down-regulating receptor expression, or it may suppress GH signaling, or both. The observed effects of estrogenic compounds on GHR is supported by evidence indicating that transcription of hepatic GHR is decreased following E2 treatment of tilapia [11]. Previous work on human breast cancer cells indicates that E2 attenuates the metabolic action of GH in humans [30]. This earlier work demonstrated that E2 suppressed JAK2 phosphorylation, a component of the GHR-induced JAK/STAT second messenger pathway in part by increasing the mRNA abundance of a suppressor of cytokine signaling (SOCS-2). A variety of ligands and their receptors stimulate the JAK/STAT pathway including GH. Intracellular activation occurs when ligand binding induces the multimerization of receptor subunits. For some ligands, such as erythropoietin and GH, the receptor subunits are bound as homodimers while, for others, such as interferons and interleukins, the receptor subunits are heteromultimers. In either case signal propagation is dependent on the association of the cytoplasmic domains of two receptor subunits with phosphorylated JAK tyrosine kinases, and thus the demonstrated suppression by E2 attenuates the downstream products of GH signal propagation.

Although we have interpreted our results of NP and E2 on osmoregulation as occurring primarily through their impact on the GH/IGF-I axis, we cannot rule out the possibility that there are other pathways through which these estrogenic compounds may be acting. It is possible that E2 has direct effects on the gill and other osmoregulatory organs. E2 receptors (ER) are abundant in the liver, present in small amounts in scales, but are undetectable in the gill and skin of rainbow trout [54]. However, recent studies indicate that ERα mRNA increases in the gill of sockeye salmon in spring and is decreased by treatment with NP [31]. Further studies are necessary to determine whether a physiologically active E2 receptor is present in the gill and whether it is involved in regulation of ion transport.

We found that E2 reduces gill Na^+/K^+-ATPase activity and increases plasma chloride in seawater-acclimated Atlantic salmon smolts. These results are consistent with research on smolts exposed to SW for 24 h after exposure to E2 and NP in freshwater [28]. Additional studies have shown that these impacts also have negative effects on behavioral preference for seawater [29]. The concentration of E2 used in SW-acclimated salmon in the present study has been demonstrated to result in physiological levels of E2, similar to those occurring during maturation of female salmon [15,50,63]., Plasma Ca_T and HSI are elevated during reproductive maturation and production of vitellogenin [18,35], and increase following E2 or NP treatment in the present and previous studies [3,10,45].

Similar to the present study, Madsen et al. demonstrated that E2 reduced the number of gill ionocytes in freshwater smolts [35] and delayed downstream migration [37]. In the present study, E2 treatment reduced the size and number of ionocytes on the primary filament and increased the number of ionocytes on the secondary lamellae of seawater-acclimated Atlantic salmon smolts. Previous work has localized Na^+/K^+-ATPase to the tubular system of ionocytes [23], therefore, positively stained cells were identified as ionocytes based on the localization of Na^+/K^+-ATPase, size, morphology, and location within the gill. Ionocytes on the primary filament have previously been shown to increase in number and size in response to seawater acclimation of many teleost fish [25,40]. In addition, ionocytes on the secondary lamellae are abundant in Atlantic salmon smolts in freshwater, but decline in number or are completely absent in seawater-acclimated smolts as demonstrated in the current study and others [52,56]. These data suggest that E2 directs the cellular morphology of the gills of seawater-acclimated Atlantic salmon away from ion secretion and toward ion absorption.

The effect of E2 to increase ionocytes in the secondary lamellae of Atlantic salmon in seawater may be related to a role of E2 in ion uptake. There is speculation that there is a division of labor between ionocytes on the primary filament and on the secondary lamellae, the latter being involved in ion uptake [55]. For example, the number of ionocytes on the secondary lamellae is high in freshwater American shad (*Alosa sapidissima*) and nearly absent in seawater fish of this species [67]. In addition, several other teleosts exhibit increased numbers of ionocytes on secondary lamellae after exposure to low ion water [53]. Over the course of development during smolting and seawater acclimation in Atlantic salmon, the number of ionocytes on the primary filaments and secondary lamellae are inversely related, with the greatest number of ionocytes present on the latter during freshwater stages [52]. In contrast, there are few to no ionocytes present on the secondary lamellae of striped bass (*Morone saxatilis*), regardless of environmental salinity [36]. Numerous ionocytes are present on the primary filament and secondary lamellae in the Hawaiian goby (*Stenogobius hawaiiensis*), and the number and density of ionocytes on the secondary lamellae were unaffected by seawater exposure [47]. These data point to species specific differences in the function of ionocytes on the secondary lamellae of euryhaline fish. Nonetheless, the shift in size and number of ionocytes following E2 treatment indicates that this hormone may be involved in osmoregulation during normal migration from seawater into freshwater.

Given the clear effect of E2 treatment in freshwater on ionocytes, and previous research demonstrating an effect of estrogenic compounds on gill Na^+/K^+-ATPase activity in smolts, it is somewhat surprising that an effect of E2 and NP on gill Na^+/K^+-ATPase activity was not observed in the present study. Previous research has indicated that the stage at which treatment begins can affect the relative potency of treatment with estrogenic compounds [29]. Treatment in the present study occurred relatively early in smolt development as indicated by the moderate levels of gill Na^+/K^+-ATPase in control fish. The impact of E2 on plasma chloride in the absence of any impact on gill Na^+/K^+-ATPase activity indicates that other mechanisms than this transport enzyme are involved in regulating the set point for plasma ion levels in freshwater.

The present results demonstrate that exposure to E2 or NP compromises the normal morphological and physiological changes that occur during smolting and seawater acclimation. This change is brought about by reducing GHR abundance and activity thereby inducing a shift from ion secretion necessary for seawater acclimation, toward an increased capacity for ion-uptake necessary for freshwater osmoregulatory ability. This inhibitory effect on seawater tolerance may be related to the anadromous life history of salmon in which endogenous sex steroids are extremely low during development until maturation, and then increase in association with reproductive maturation and movement back into freshwater. These results indicate a dual role for E2 during maturation, providing an osmoregulatory function in addition to its reproductive role. Estrogens and androgens may play a critical role in freshwater-acclimation and migratory behavior of adults returning to their natal streams. As juveniles, compounds mimicking the actions of E2 in the environment may compromise smolt development thereby reducing salmon survival and the success of salmon populations. The ability of estrogenic compounds to decrease growth hormone receptor in the liver and circulating levels of IGF-I suggest the possibility that estrogenic compounds could also influence the growth of salmonids and perhaps other teleost fish.

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References

[1] J.T.M. Arsenault, W.L. Fairchild, D.L. MacLatchy, L. Burtidge, K. Haya, S.B. Brown, Effects of water-borne 4-nonylphenol and 17 beta-estradiol exposures during parr-smolt transformation on growth and plasma IGF-I of Atlantic salmon (*Salmo salar* L.), Aquat. Toxicol. 66 (2004) 255–265.

[2] P.A. Bennett, A. Levy, D.F. Carmignac, I.C.A.F. Robinson, S.L. Lightman, Differential regulation of the growth hormone receptor gene: effects of dexamethasone and estradiol, Endocrinology 137 (1996) 3891–3896.

[3] B.Th. Björnsson, C. Haux, H.A. Bern, L.J. Deftos, 17B-Estradiol increases plasma calcitonin levels in salmonid fish, Endocrinology 125 (1989) 1754–1760.

[4] B.Th. Björnsson, G.L. Taranger, T. Hansen, S.O. Stefansson, C. Haux, The interrelation between photoperiod, growth hormone, and sexual maturation of adult atlantic salmon (*Salmo salar*), Gen. Comp. Endocrinol. 93 (1994) 70–81.
[5] J.P. Breves, B.K. Fox, A.L. Pierce, T. Hirano, E.G. Grau, Gene expression of growth hormone family and glucocorticoid receptors, osmosensors, and ion transporters in the gill during seawater acclimation of Mozambique tilapia, *Oreochromis mossambicus*, J. Exp. Zool. A Ecol. Genet. Physiol. 313 (7) (2010) 432–441.
[6] J.P. Breves, A.P. Seale, R.E. Helms, C.K. Tipsmark, T. Hirano, E.G. Grau, Dynamic gene expression of GH/PRL-family hormone receptors in gill and kidney during freshwater-acclimation of Mozambique tilapia, Comp. Biochem. Physiol. A Mol. Integr. Physiol. 158 (2) (2011) 194–200.
[7] G. Boeuf, P. Prunet, P.-Y. Le Bail, Is growth hormone treatment able to stimulate the smoltification in the Atlantic salmon?, Compt Rend. Acad. Sci. 310 (1990) 75–80.
[8] J.P. Bolton, N.L. Collie, H. Kawauchi, T. Hirano, Osmoregulatory actions of growth hormone in rainbow trout (*Salmo gairdneri*), J. Endocr. 112 (1987) 63–68.
[9] Q.-P. Cao, S. Duguay, E. Plisetskaya, D.F. Steiner, S.J. Chan, Nucleotide sequence and growth hormone-regulated expression of salmon insulin-like growth factor I RNA, Mol. Endocrinol. 3 (1990) 2005–2010.
[10] J.F. Carragher, J.P. Sumpter, The mobilization of calcium from calcified tissues of rainbow trout (*Oncorhynchus mykiss*) induced to synthesize vitellogenin, Comp. Biochem. Physiol. A (99) (1991) 169–172.
[11] L.K. Davis, A.L. Pierce, N. Hiramatsu, C.V. Sullivan, T. Hirano, E.G. Grau, Gender-specific expression of multiple estrogen receptors, growth hormone receptors, insulin-like growth factors and vitellogenins, and effects of 17 beta-estradiol in the male tilapia (*Oreochromis mossambicus*), Gen. Comp. Endocrinol. 156 (3) (2008) 544–551.
[12] H.M. Domene, G. Marin, J. Sztein, Y.M. Yu, J. Baron, F.G. Cassorla, Estradiol inhibits growth hormone receptor gene expression in rabbit liver, Mol. Cell. Endocrinol. 103 (1994) 81–87.
[13] C.M. Duan, Nutritional and developmental regulation of insulin-like growth factors in fish, J. Nutr. 128 (1998) 306–314.
[14] F.H. Epstein, A.I. Katz, G.E. Pickford, Sodium- and potassium-activated adenosine triphosphatase of gills: role in adaptation of teleosts to salt water, Science 156 (1967) 1245–1247.
[15] F. Estay, R. Neira, N.F. Diaz, L. Valladares, A. Torres, Gametogenesis and sex steroid profiles in cultured coho salmon (*Oncorhynchus kisutch*, Walbaum), J. Exp. Zool. 280 (1998) 429–438.
[16] W.L. Fairchild, E.O. Swansburg, J.T. Arsenault, S.B. Brown, Does an association between pesticide use and subsequent declines in catch of Atlantic salmon (*Salmo salar*) represent a case of endocrine disruption?, Environ Health Perspect. 107 (1999) 349–357.
[17] J.N. Fryer, H.A. Bern, Growth hormone binding to tissues of normal and stunted juvenile coho salmon, *Oncorhynchus kisutch*, J. Fish Biol. 15 (1979) 527–533.
[18] J.M. Gomez, B. Mourot, A. Fostier, G.F. Le, Growth hormone receptors in ovary and liver during gametogenesis in female rainbow trout (*Oncorhynchus mykiss*), J. Reprod. Fertil. 115 (1999) 275–285.
[19] E.S. Gray, G. Young, H.A. Bern, Radioreceptor assay for growth hormone in coho salmon (*Oncorhynchus kisutch*) and its application to the study of stunting, J. Exp. Zool. 256 (1990) 290–296.
[20] W.S. Hoar, The physiology of smolting salmonids, in: W.S. Hoar, D. Randall (Ed.), Fish Physiology, vol. XIB, first ed., Academic Press, New York, pp. 275–343.
[21] K. Ikuta, K. Aida, N. Okumoto, I. Hanyu, Effects of sex steroids on the smoltification of masu salmon, *Oncorhynchus masou*, Gen. Comp. Endocrinol. 65 (1987) 99–110.
[22] M. Iwata, K. Yamauchi, R.S. Nishioka, R. Lin, H.A. Bern, Effects of thyroxine, growth hormone and cortisol on salinity preference of juvenile coho salmon (*Oncorhynchus kisutch*), Mar. Behav. Physiol. 17 (1990) 191–201.
[23] K.J. Karnaky, L.B. Kinter, W.B. Kinter, C.E. Stirling, Teleost chloride cell II. Autoradiographic localization of gill Na, K-ATPase in killifish *Fundulus heteroclitus* adapted to low and high salinity environments, J. Cell Biol. 70 (1976) 157–177.
[24] M.P. Komourdjian, R.L. Saunders, J.C. Fenwick, Evidence for the role of growth hormone as a part of a 'light-pituitary axis' in growth and smoltification of Atlantic salmon (*Salmo salar*), Can. J. Zool. 54 (1976) 544–551.
[25] P. Laurent, S.F. Perry, Environmental effects on fish gill morphology, Physiol. Zool. 64 (1991) 4–25.
[26] D.T. Lerner, L.K. Davis, T. Hirano, E.G. Grau, S.D. McCormick, Effects of 4-nonylphenol and 17β-estradiol on osmoregulatory homeostasis and endocrine responses of Mozambique tilapia, *Oreochromis mossambicus*, Int. Comp. Biol. 45 (2005) 1158.
[27] D.T. Lerner, S.D. McCormick, Physiological and behavioral effects of nonylphenol and 17β-estradiol on larval survival and smolt development of Atlantic salmon, *Salmo salar*, Int. Comp. Biol. 44 (2004) 591.
[28] D.T. Lerner, B.Th. Björnsson, S.D. McCormick, Larval exposure to 4-nonylphenol and 17β-estradiol affects physiological and behavioral development of seawater adaptation in Atlantic salmon smolts, Environ. Sci. Technol. 41 (12) (2007) 4479–4485.
[29] D.T. Lerner, B.Th. Björnsson, S.D. McCormick, Aqueous exposure to 4-nonylphenol and 17β-estradiol increases stress sensitivity and disrupts ion regulatory ability of juvenile Atlantic salmon, Environ. Toxicol. Chem. 26 (7) (2007) 1433–1440.
[30] K.C. Leung, N. Doyle, M. Ballesteros, K. Sjogren, C.K.W. Watts, T.H. Low, G.M. Leong, R.J.M. Ross, K.K.Y. Ho, Estrogen inhibits GH signaling by suppressing GH-induced JAK2 phosphorylation, an effect mediated by SOCS-2, Proc. Nat. Acad. Sci. USA 100 (2003) 1016–1021.
[31] Q. Luo, M. Ban, H. Ando, T. Kitahashi, R.k. Bhandari, S.D. McCormick, C. Urano, Distinct effects of 4-nonylphenol and estrogen-17 beta on expression of estrogen receptor alpha gene in smolting sockeye salmon, Comp. Biochem. Physiol. C-Toxicol. Pharmacol. 140 (1) (2005) 123–130.
[32] S.S. Madsen, The role of cortisol and growth hormone in seawater adaptation and development of hypoosmoregulatory mechanisms in sea trout parr (*Salmo trutta trutta*), Gen. Comp. Endocrinol. 79 (1990) 1–11.
[33] S.S. Madsen, B. Korsgaard, Time-course effects of repetitive oestradiol-17B and thyroxine injections on the natural spring smolting of Atlantic salmon, *Salmo salar*, L. J. Fish Biol. 35 (1989) 119–128.
[34] S.S. Madsen, H.A. Bern, In vitro effects of insulin-like growth factor-I on gill Na+, K+- ATPase in coho salmon, *Oncorhynchus kisutch*, J. Endocrinol. 138 (1993) 23–30.
[35] S.S. Madsen, A.B. Mathiesen, B. Korsgaard, Effects of 17 beta-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*), Fish Physiol. Biochem. 17 (1997) 303–312.
[36] S.S. Madsen, S.D. McCormick, G. Young, J.S. Endersen, R.S. Nishioka, H.A. Bern, Physiology of seawater acclimation in the striped bass, *Morone saxatilis* (Walbaum), Fish Physiol. Biochem. 13 (1994) 1–11.
[37] S.S. Madsen, S. Skovbolling, C. Nielsen, B. Korsgaard, 17-beta estradiol and 4-nonylphenol delay smolt development and downstream migration in Atlantic salmon, *Salmo salar*, Aquat. Toxicol. 68 (2004) 109–120.
[38] S.D. McCormick, Fluorescent labelling of Na+, K+-ATPase in intact cells by use of a fluorescent derivative of ouabain: Salinity and teleost chloride cells, Cell Tissue Res. 260 (1990) 529–533.
[39] S.D. McCormick, Methods for non-lethal gill biopsy and measurement of Na+, K+-ATPase activity, Can. J. Fish. Aquat. Sci. 50 (1993) 656–658.
[40] S.D. McCormick, Hormonal control of gill Na+, K+-ATPase and chloride cells function, in: C.M. Wood, T.J. Shuttleworth (Eds.), Fish Physiology, Ionoregulation: Cellular and Molecular Approaches, vol. XIV, Academic Press, New York, 1995, pp. 285–315.
[41] S.D. McCormick, Endocrine control of osmoregulation in teleost fish, Am. Zool. 41 (2001) 781–794.
[42] S.D. McCormick, H.A. Bern, In vitro stimulation of Na+, K+-ATPase activity and ouabain binding by cortisol in coho salmon gill, Am. J. Physiol. 256 (1989) R707–R715.
[43] S.D. McCormick, S. Moriyama, B.T. Björnsson, Low temperature limits photoperiod control of smolting in Atlantic salmon through endocrine mechanisms, Am. J. Physiol. -Reg. Int. Comp. Physiol 278 (2000) R1352–R1361.
[44] S.D. McCormick, R.J. Naiman, Hypoosmoregulation in an anadromous teleost: influence of sex and maturation, J. Exp. Zool. 234 (1985) 193–198.
[45] S.D. McCormick, M.F. O'Dea, A.M. Moeckel, D.T. Lerner, B.T. Bjornsson, Endocrine disruption of parr-smolt transformation and seawater tolerance of Atlantic salmon by 4-nonylphenol and 17 beta-estradiol, Gen. Comp. Endocrinol. 142 (2005) 280–288.
[46] S.D. McCormick, T. Sakamoto, S. Hasegawa, T. Hirano, Osmoregulatory actions of insulin-like growth factor-I in rainbow-trout (*Oncorhynchus mykiss*), J. Endocrinol. 130 (1991) 87–92.
[47] S.D. McCormick, K. Sundell, B.T. Bjornsson, C.L. Brown, J. Hiroi, Influence of salinity on the localization of Na+/K+-ATPase, Na+/K+/2Cl(-)cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*), J. Exp. Biol. 206 (2003) 4575–4583.
[48] S. Moriyama, P. Swanson, M. Nishii, A. Takahashi, H. Kawauchi, W.W. Dickhoff, E.M. Plisetskaya, Development of a homologous radioimmunoassay for coho salmon insulin-like growth factor-I, Gen. Comp. Endocrinol. 96 (1994) 149–161.
[49] T.O. Nilsen, O.E. Ebbesson, B.T. Kiilerich, B. Th. Bjornsson, S.S. Madsen, S.D. McCormick, S.O. Stefansson, Endocrine systems in juvenile anadromous and landlocked Atlantic salmon (*Salmo salar*): seasonal development and seawater acclimation, Gen. Comp. Endocrinol. 155 (3) (2008) 762–772.
[50] N.W. Pankhurst, N.E. Stacey, R.E. Peter, An evaluation of techniques for the administration of 17B-estradiol to teleosts, Aquaculture 52 (1986) 145–155.
[51] R. Patiño, C.B. Schreck, Sexual dimorphism of plasma sex steroid-levels in juvenile coho salmon, *Oncorhynchus kisutch*, during smoltification, Gen. Comp. Endocrinol. 61 (1986) 127–133.
[52] R.M. Pelis, J. Zydlewski, S.D. McCormick, Gill Na+-K+-2Cl(-) cotransporter abundance and location in Atlantic salmon: effects of seawater and smolting, Am. J. Physiol. -Reg. Int. Comp. Physiol. 280 (2001) R1844–R1852.
[53] S.F. Perry, The chloride cell: structure and function in the gills of freshwater fishes [review], Ann. Rev. Physiol. 59 (1997) 325–347.
[54] P. Persson, J.M. Shrimpton, S.D. McCormick, B.T. Bjornsson, The presence of high-affinity, low-capacity estradiol-17 beta binding in rainbow trout scale indicates a possible endocrine route for the regulation of scale resorption, Gen. Comp. Endocrinol. 120 (1) (2000) 35–43.
[55] M. Pisam, A. Caroff, A. Rambourg, Two types of chloride cells in the gill epithelium of a freshwater-adapted euryhaline fish: *Lebistes reticulatus*; their modifications during adaptation to saltwater, Am. J. Anat. 179 (1987) 40–50.
[56] M. Pisam, P. Prunet, G. Boeuf, A. Rambourg, Ultrastructural features of chloride cells in the gill epithelium of the Atlantic salmon, *Salmo salar*, and their modifications during smoltification, Am. J. Anat. 183 (1988) 235–244.
[57] M. Reinecke, A. Schmid, R. Ermatinger, D. Loffingcueni, Insulin-like growth factor I in the teleost *Oreochromis mossambicus*, the tilapia: gene sequence,

tissue expression, and cellular localization, Endocrinology 138 (1997) 3613–3619.
[58] T. Sakamoto, T. Hirano, Growth hormone receptors in the liver and osmoregulatory organs of rainbow trout: characterization and dynamics during adaptation to seawater, J. Endocrinol. 130 (1991) 425–433.
[59] T. Sakamoto, T. Hirano, Expression of insulin-like growth factor-I gene in osmoregulatory organs during seawater adaptation of the salmonid fish: possible mode of osmoregulatory action of growth hormone, Proc. Natl. Acad. Sci. USA 90 (1993) 1912–1916.
[60] T. Sakamoto, S.D. McCormick, T. Hirano, Osmoregulatory actions of growth hormone and its mode of action in salmonids: a review, Fish Physiol. Biochem. 11 (1993) 155–164.
[61] R.L. Saunders, E.B. Henderson, Changes in gill atpase activity and smolt status of Atlantic salmon (*Salmo salar*), J. Fish. Res. Board Can. 35 (1978) 1542–1546.
[62] M. Schmitz, I. Mayer, Effect of androgens on seawater adaptation in Arctic char, *Salvelinus alpinus*, Fish. Physiol. Biochem. 12 (1993) 11–20.
[63] G. VanDerKraak, H.M. Dye, E.M. Donaldson, Effects of Lh-Rh and Des-Gly10[D-Ala6]Lh-Rh-Ethylamide on plasma sex steroid profiles in adult female coho salmon (*Oncorhynchus kisutch*), Gen. Comp. Endocrinol. 55 (1984) 36–45.
[64] G.A. Wedemeyer, R.L. Saunders, W.C. Clarke, Environmental factors affecting smoltification and early marine survival of anadromous salmonids, Mar. Fish. Rev. 42 (1980) 1–14.
[65] G. Young, B.T. Björnsson, P. Prunet, R.J. Lin, H.A. Bern, Smoltification and seawater adaptation in coho salmon (*Oncorhynchus kisutch*) – plasma prolactin, growth-hormone, thyroid-hormones, and cortisol, Gen. Comp. Endocrinol. 74 (1989) 335–345.
[66] W.S. Zaugg, L.R. McLain, Adenosine triphosphatase activity in gills of salmonids: seasonal variations and salt water influences in coho salmon, *Oncorhynchus kisutch*, Comp. Biochem. Physiol. 35 (1970) 587–596.
[67] J. Zydlewski, S.D. McCormick, Developmental and environmental regulation of chloride cells in young American shad, *Alosa sapidissima*, J. Exp. Zool. 290 (2001) 73–87.