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Effects of 17β-estradiol, 4-nonylphenol, and β-sitosterol on the growth hormone-insulin-like growth factor system and seawater adaptation of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Previous studies show that successful adaptation of euryhaline teleost fish to seawater (SW) involves the GH-IGF system. The increasing occurrence, distribution, and concentration of environmental contaminants, including environmental estrogens (EE), in aquatic habit over recent time may compromise the hypoosmoreegulatory ability of fish. In this study, we used rainbow trout (*Oncorhynchus mykiss*) to assess the effects of EE on the GH-IGF system and adaptation to increased salinity. Juvenile trout (ca. 30 g) were exposed to either low (10 μg/l) or high (100 μg/l) concentrations of β-sitosterol, 4-n-nonylphenol (NP), or 17β-estradiol (E2) for 28 days in fresh water (FW); after which, fish were exposed to 20‰ SW. Plasma chloride levels in control fish rose initially, and then declined to initial levels after 48 h. By contrast, plasma chloride levels in all EE-treated groups except β-sitosterol low increased and remained elevated over initial levels after 48 h. Levels GH receptor 1 (GHR 1), GHR 2, insulin-like growth factor-1 (IGF-1), and IGF-2mRNAs in liver of control fish increased 6–12 h after SW exposure. In gill, levels of GHR 1, GHR 2, IGF-1, IGF-2, IGF receptor 1A (IGFR1A), and IGFR1B mRNAs increased in control fish 6–12 h after 20‰ SW exposure. Levels of IGFR1A and IGFR1B mRNAs in white muscle and of IGFR1A mRNA in red muscle increased in control fish 6–12 h after 20‰ SW exposure. Expression of all mRNAs in liver, gill, and red and white muscle declined from peak levels in control fish by 48 h after transfer. Exposure of fish to β-sitosterol, NP, and E2 abolished or attenuated normal salinity-induced changes in the expression of GHR, IGF, and IGFR1 mRNAs in all tissues. These results indicate that EE reduces salinity adaptation by inhibiting components of the GH-IGF system.

1. Introduction

Hypoosmotic regulation of euryhaline fish, including salmonids, is accompanied by an increase in the number and size of seawater (SW)-type chloride cells and by a corresponding increase in the expression and function of Na^+, K^+ ATPases (NKA), Na^+, K^+, $2Cl^-$ cotransporters (NKCC), and Cl^- channels in chloride cells (Evans and Claiborne, 2009). Considerable research has shown that SW adaptability of euryhaline fish is influenced by numerous hormones, including growth hormone (GH), insulin-like growth factor (IGF-1), cortisol, and thyroid hormones (Mancera and McCormick, 1998; McCormick, 2001; Evans, 2002).

Increasing attention has been given to the GH-IGF system and its role in SW readiness and hypoosmoregulatory ability, which appear to be distinct from the actions of the GH-IGF system on growth (Sakamoto and McCormick, 2006; Klein and Sheridan, 2008). Plasma levels of GH and IGF-1 increase following exposure to SW accompanied by increased mRNA expression of the hormones (Sakamoto et al., 1993; McCormick et al., 2000; Agustsson et al., 2001; Shepherd et al., 2005; Nilsen et al., 2008). Heightened sensitivity to GH and IGFs also appears to accompany SW exposure, as expression of GHR and type 1 IGF receptors (IGFR1) increase (Poppinga et al., 2007). In addition, GH and IGF-1 treatment increase salinity tolerance and chloride cell number as well as NKA and NKCC activity/biosynthesis (McCormick et al., 1991; Seidelin et al., 1999; Pelis et al., 2001). The GH-IGF system also appears important for the SW preparatory changes associated with salmonid smoltification. Levels of GH and IGF-1 increase during smoltification in association with increases in chloride cell size/number, NKA, and NKCC activity (Young et al., 1989; McCormick et al., 2002).

The increasing production, use, and disposal of an expanding array of chemicals that enter the environment pose a serious threat to terrestrial and aquatic animals as well as to humans. Of particular concern is a broad spectrum of natural and synthetic compounds that mimic estrogen. Environmental estrogens (EE) include endogenous and synthetic animal estrogens (e.g., 17β-estradiol), phytoestrogens (e.g., β-sitosterol), mycotoxins (e.g., zearalenone), organochlorine pesticides

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(e.g., DDT), polychlorinated biphenyls (PCBs), and alkylphenol polyethoxylates (APEs; e.g., 4-nonylphenol). The impacts of EE are magnified because they accumulate in tissues, have epigenetic effects and affect progeny, and are rapidly transferred through the food web (Hester and Harrison, 1999). Environmental estrogens have been found to disrupt a wide variety of reproductive processes in fish, amphibians, reptiles, and birds, including inhibited testicular growth, reduced sperm production, intersex gonads, reduced egg production, and altered reproductive timing and behavior (Tyler et al., 1998). Notably, 4-nonylphenol (NP) and β-sitosterol increase vitellogenin in the liver of male trout, an action that appears mediated via the estrogen receptor (Jobling and Sumpter, 1993; Tremblay and Van Der Kraak, 1998, 1999). β-sitosterol also decreases plasma levels of sex steroids by reducing gonadal steroidogenesis in goldfish and rainbow trout (MacLatchy and Van Der Kraak, 1995).

Knowledge of the effects of EE on processes other than reproduction is just emerging. For example, 17β-estradiol (E2) and 4-nonylphenol (NP) have been found to reduce salinity tolerance in Atlantic salmon smolts and reduce plasma levels of IGF-1 (Madsen et al., 2004, McCormick et al., 2005; Lerner et al., 2007a). The aims of the present study were to further elucidate the role of the GH-IGF system in adaptation to increased salinity and to determine if the osmodisrupting effects of EE are mediated by alterations in the GH-IGF system.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, and transported to North Dakota State University where they were maintained in 800-l circular tanks supplied with recirculated (100% replacement volume per day) dechlorinated municipal water at 14 °C under a 12L:12D photoperiod. Fish were fed to satiation twice daily with AquaMax Grower (PMI Nutrition International, Inc., Brentwood, MO), except 24–36 h before initiating experimental manipulations.

2.2. Experimental conditions

Fish (ca. 30 g) were anesthetized, measured, weighed, and transferred to 40-l glass aquaria (15 fish per tank) containing fresh water (FW) with or without an EE. Three estrogenic compounds were used: 17β-estradiol (Sigma, St. Louis, MO), 4-*n*-nonylphenol (AlfaAesar, Ward Hill, MA), and β-sitosterol (Calbiochem, San Diego, CA); the treatments groups were as follows: two doses of E2 (10 μg/l and 100 μg/l), two doses of NP (10 μg/l and 100 μg/l), and two doses of β-sitosterol (10 μg/l and 100 μg/l). All compounds were dissolved in ethanol; the final concentration of ethanol did not exceed 0.007%. The vehicle control group received ethanol only. The water was well aerated and the tanks were kept at 14 °C under a 12L: 12D photoperiod. Twenty-four hours after transfer to aquaria, feeding recommenced (1% body weight once per day) and continued throughout the FW exposure period; however, feeding was suspended 24 h prior to salinity challenge. One-half the volume of each tank was removed and replaced with FW containing the appropriate treatment (added so as to maintain the desired final concentration of each test agent) every other day, in a manner similar to that described by Tremblay and Van Der Kraak (1999). Under these conditions, dissolved oxygen ranged from 8–10 mg/l, and ammonia did not exceed 0.25 ppm. Discarded water was filtered through activated charcoal before disposal.

After 28 days, fish were exposed to a salinity challenge. Water was removed from treatment tanks and replaced with Instant Ocean (Aquarium Systems Inc., Mentor, OH) to achieve a final concentration of 20‰ (w/v). The replacement 20‰ SW did not contain EE treatment or vehicle, and the fish were not fed during the challenge period. Fish were sampled 0 h, 6 h, 12 h, and 48 h following 20 ‰ SW exposure. At sampling, fish were anesthetized with 0.05% (v/v) 2-phenoxyethanol, measured, and weighed. Blood was collected with heparinized glass capillary tubes from the severed caudal vessels and centrifuged (5000 *g* for 5 min). Plasma was collected and stored at −80 °C for later analysis. Liver, gill filaments, red muscle, and white muscle samples were taken for mRNA analysis. All samples were immediately placed on dry ice and stored at −80 °C.

2.3. Plasma chloride

Plasma chloride was measured by silver titration with a Buchler–Cotlove Chloridometer and using external standards.

2.4. Quantitative real-time PCR

Frozen tissues were homogenized and total RNA was extracted using TRI reagent® (Molecular Research Center, Cincinnati, OH, USA) as specified by the manufacturer's protocol. RNA pellets were dissolved in 40–100 μl RNase-free deionized water and total RNA was quantified by UV (A_{260}) spectrophotometry. Total RNA was diluted with RNase-free deionized water to 100 ng/μl. RNA was reverse transcribed according to the manufacturer's protocol in a 10-μl reaction using 200 ng total RNA with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA).

Steady-state mRNA levels of GHR 1, GHR 2, IGF-1, IGF-2, IGFR1A, and IGFR1B were determined by quantitative real-time PCR using a Stratagene Mx 3000p detection system (Stratagene, La Jolla, CA) as previously described (Very et al., 2005; Poppinga et al., 2007; Malkuch et al., 2008). Briefly, real-time PCR reactions were carried out for controls, standards, and samples in a 10 μl total volume (1 μl cDNA from reverse transcriptase reactions; 5 μl 2× Brilliant II Master Mix; 1.0 μl of each gene-specific probe, forward primer, and reverse primer at concentrations optimized for each RNA species; 1 μl RNase-free deionized water). Cycling parameters were set as follows: 95 ° C for 10 min, and 50 cycles of 92 ° C for 15 s plus 58 ° C for 1 min. Sample copy number was calculated from the threshold cycle number (C_T), and the C_T was related to a gene-specific standard curve followed by normalization to β-actin. No difference ($p>0.05$) was observed in β-actin among the treatment groups.

2.5. Statistics

Quantitative data are expressed as means ± S.E.M. Statistical differences were analyzed by ANOVA followed by Duncan's multiple range test using SigmaStat (SPSS, Chicago, IL). A probability of 0.05 was used to indicate significance.

3. Results

3.1. Body characteristics

The effects of EE on the body characteristics of rainbow trout following a 28-day exposure in FW are shown in Table 1. Over the course of 28 days, juvenile trout in the control group grew significantly in terms of body length, and although body weight also increased, this change was not significant. Exposure to estrogenic compounds retarded growth to some extent. Although none of the estrogenic compounds affected body length, there was a trend ($p=0.26$ E2 low; $p=0.34$ E2 high) for E2 to repress growth in terms of body weight compared to control-treated fish and a significant ($p<0.05$) depression of body weight observed in fish treated with the high dose of NP. Estrogenic compounds also tended to reduce the condition of fish. This was evidenced by the trend toward reduced condition observed in E2 ($p=0.18$ E2 low; $p=0.30$ E2 high) and low β-sitosterol ($p=0.09$)-

Table 1
Body characteristics of rainbow trout exposed to environmental estrogens for 28 days in fresh water.*

Characteristic	Initial	After 28-day exposure						
		Control	E2 low	E2 high	BS low	BS high	NP low	NP high
Body length (cm)	10.4 ± 1.00^{a}	14.2 ± 0.40^{b}	14.1 ± 0.36^{b}	14.2 ± 0.72^{b}	14.5 ± 0.40^{b}	14.6 ± 0.60^{b}	14.4 ± 0.60^{b}	13.8 ± 0.50^{b}
Body weight (g)	31.7 ± 1.30^{a}	32.7 ± 3.20^{a}	30.1 ± 2.10^{a}	30.5 ± 5.60^{a}	32.3 ± 2.30^{a}	36.4 ± 3.80^{a}	36.1 ± 4.30^{a}	24.3 ± 2.20^{b}
Condition factor†	2.81 ± 0.11^{a}	1.12 ± 0.03^{b}	1.07 ± 0.04^{b}	1.08 ± 0.07^{b}	1.06 ± 0.03^{b}	1.11 ± 0.02^{b}	1.19 ± 0.03^{b}	1.02 ± 0.03^{c}
Hepatosomatic index‡	0.65 ± 0.09^{a}	0.96 ± 0.06^{b}	1.57 ± 0.09^{c}	2.69 ± 0.18^{d}	1.48 ± 0.04^{c}	1.14 ± 0.08^{b}	1.45 ± 0.16^{c}	1.36 ± 0.18^{c}

Fish were immersed in freshwater (control) or freshwater containing 10 μg/l 17β-estradiol (E2 low), 100 μg/l 17β-estradiol (E2 high), 10 μg/l β-sitosterol (BS low), 100 μg/l β-sitosterol (BS high), 10 μg/l 4-n-nonylphenol (NP low), or 100 μg/l 4-n-nonylphenol (NP high).
*Data are presented as means ± SEM (n = 5–7). For a given characteristic, groups with different letters are significantly different ($p<0.05$) from one another.
†Calculated as [body weight/(body length)^3] × 100.
‡Calculated as (liver weight/body length) × 100.

treated fish and the significant ($p<0.05$) decrease in condition observed in fish treated with the high concentration of NP. The HSI of all EE-treated fish, except those treated with the high dose of β-sitosterol, was significantly higher than that of control-treated fish.

3.2. Plasma chloride

The effects of EE on plasma chloride levels of rainbow trout during exposure to 20‰ SW are shown in Fig. 1. Interestingly, high doses of E2 and NP depressed plasma chloride levels over the course of the 28-day exposure period (cf. chloride at time 0 of 20‰ SW challenge versus initial chloride levels before exposure to EE, which were 130 ± 1.2 mM; significant only for E2, $p<0.05$). Chloride levels in control-treated fish increased following 20‰ SW exposure, reaching peak levels at 12 h; after which, chloride levels declined, and by 48 h the levels were similar to those observed at 0 h. By contrast, plasma chloride levels in all of the EE-treated fish, except those treated with the low dose of β-sitosterol, increased following 20‰ SW exposure and remained elevated.

3.3. GH-IGF system

The direct exposure of rainbow trout to 20‰ SW resulted in significant increases in expression of mRNAs encoding GHRs in liver. Two GHR-encoding distinct mRNAs were expressed in the liver of trout. The greatest effect of 20‰ SW exposure was on the expression of GHR 2, which increased 287% 12 h after exposure; afterward, mRNA abundance declined substantially, but remained elevated compared to levels observed at 0 h (Fig. 2B). Exposure to 20‰ SW also increased expression of GHR 1 mRNA, which increased to a maximum extent 6 h after exposure, then declined such that abundance at 48 h was similar to that at 0 h (Fig. 2A). Exposure to 20‰ SW similarly stimulated hepatic expression of IGF-1 and IGF-2. Expression of IGF-1 mRNA increased a maximum of 134% 12 h after 20‰ SW exposure, then declined (Fig. 3A). Expression of IGF-2 increased abruptly 6 h after exposure, then declined (Fig. 3B).

Notably, exposure to some of the EE depressed expression of GHR and IGF mRNAs such that their abundance at 0 h was lower than that observed in control fish at 0 h; such depression was particularly pronounced with E2 and NP treatment (Figs. 2 and 3). Uniformly, exposure to all of the EE, E2, NP, and β-sitosterol, suppressed or

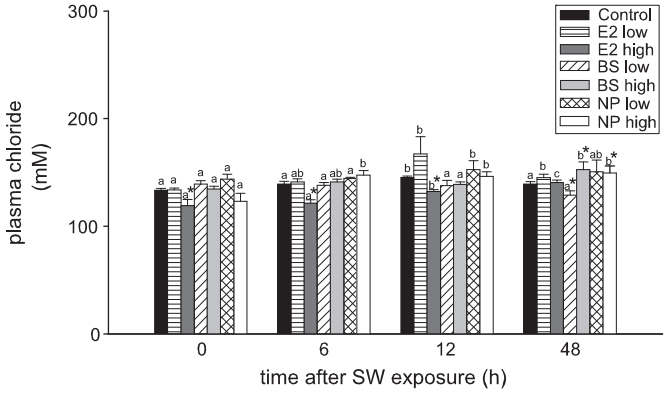

Fig. 1. Effects of environmental estrogens (EE) on plasma chloride concentration in rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

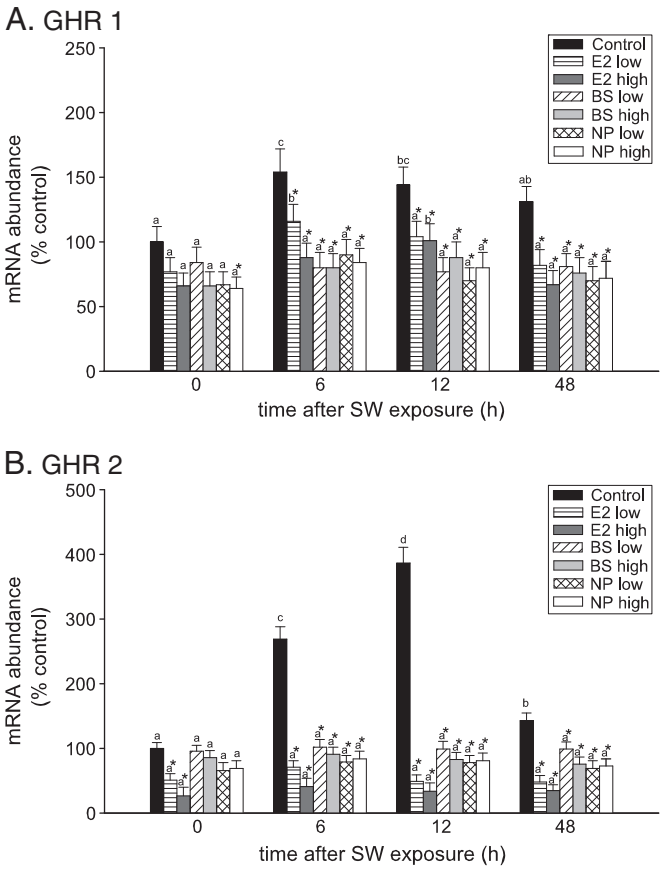

Fig. 2. Effects of environmental estrogens (EE) on the abundance of hepatic (A) growth hormone receptor 1 (GHR 1) and (B) GHR 2 mRNAs in rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

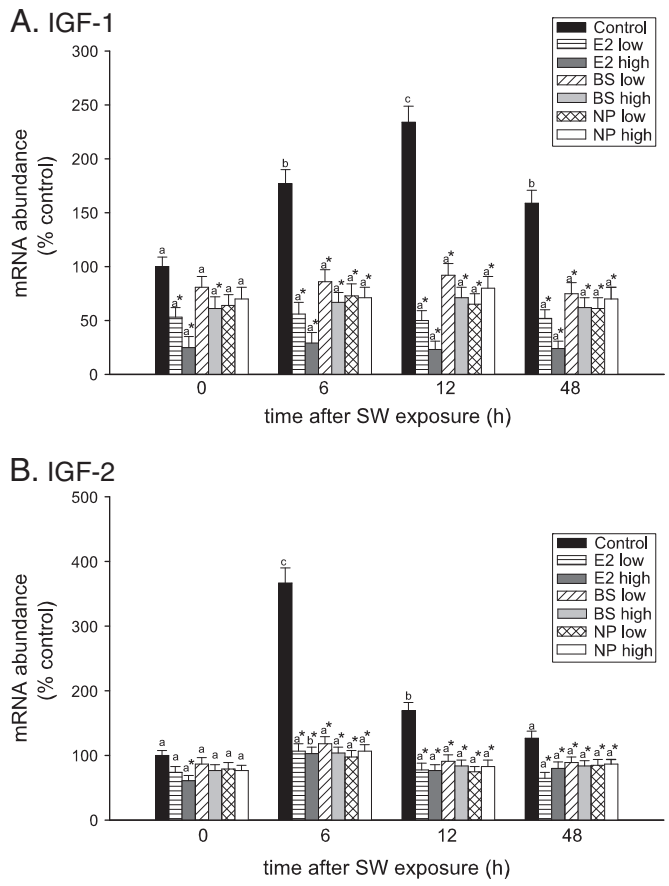

Fig. 3. Effects of environmental estrogens (EE) on the abundance of hepatic (A) insulin-like growth factor-1 (IGF-1) and (B) IGF-2 mRNAs in rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

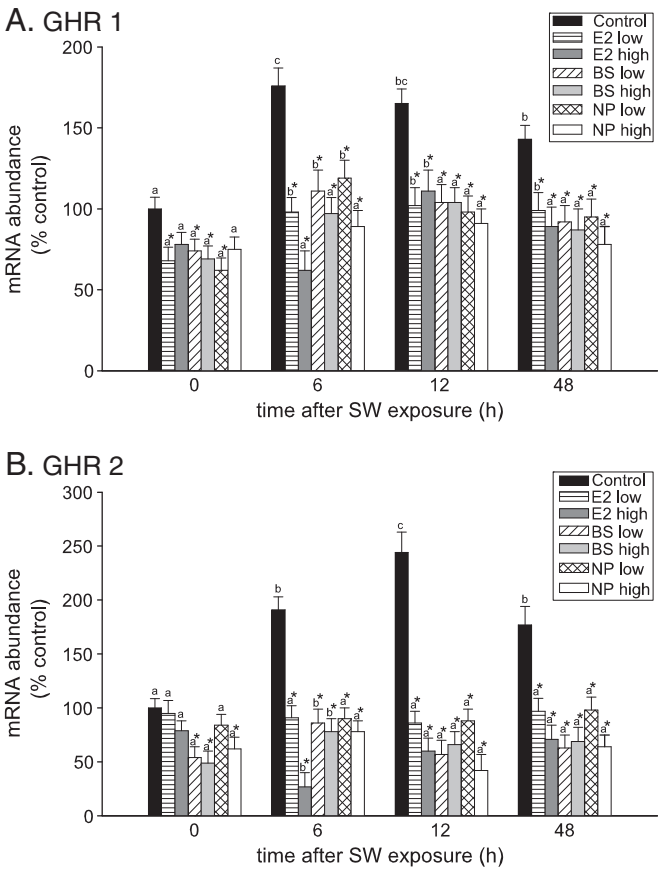

Fig. 4. Effects of environmental estrogens (EE) on the abundance of (A) growth hormone receptor 1 (GHR 1) and (B) GHR 2 mRNAs in gill filaments of rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

abolished the normal salinity-associated increases in the expression of hepatic GHR and IGF mRNAs. In particular, the abundance of GHR 1 mRNA rose following 20‰ SW exposure in E2-treated fish, but the extent of the increase was significantly attenuated compared to that in control fish (Fig. 2A). Salinity-associated increases in expression of GHR 2, IGF-1, and IGF-2 were completely abolished by EE treatment (Figs. 2B and 3).

The patterns of expression of GH-IGF system components in the gill were similar to those observed in the liver. Exposure to 20‰ SW increased the abundance of both GHR mRNAs expressed in gill. GHR 1 mRNA expression increased by 76% after 6 h, whereas GHR 2 mRNA expression increased by 144% after 12 h; the abundance of both GHR mRNAs declined below maximal levels 48 h after exposure (Fig. 4). Exposure to 20‰ SW also stimulated expression of IGF mRNAs in gill. Expression of IGF-1 mRNA increased to peak levels 12 h after 20‰ SW exposure, then declined (Fig. 5A). Expression of IGF-2 mRNA also reached maximum levels 12 h after exposure, increasing 46%; after which, levels subsided (Fig. 5B). Two types 1 IGF receptors, IFGR1A and IGFR1B, were expressed in gill. Expression of both IGFR1 mRNAs increased following 20‰ SW exposure. Expression of IGFR1A mRNA increased a maximum of 65% 12 h after exposure, whereas expression of IGFR1B mRNA increased a maximum of 56% 6 h after exposure (Fig. 6). After 48 h of 20‰ SW exposure, the abundance of both IGFR1 mRNAs declined to values similar to those observed initially at 0 h.

Similar to the situation observed in liver, exposure to EE depressed expression of GH-IGF system components in gill so that mRNA abundance at 0 h was lower than that observed in control fish at 0 h. Such depression was particularly pronounced on the expression of GHR 1, GHR 2, IGF-2, and IGFR1B mRNAs, although there was some variation depending on the specific estrogenic compound (Figs. 4, 5, and 6). Also similar to the case in liver, exposure to all of the EE attenuated or blocked normal salinity-associated increases in GH-IGF system components. Expression of GHR 1 and GHR 2 mRNAs increased following 6–12 h of 20‰ SW exposure in many of the EE-treated fish, but the extent of the increases was significantly attenuated compared to those of control fish (Fig. 4). Notably, GHR 2 mRNA expression in fish treated with the high dose of E2 was lower than that in control fish (Fig. 4B). Salinity-associated increases in the abundance of IGF-1, IGF-2, IGFR1A, and IGFR1B mRNAs were completely blocked by EE treatment (Figs. 5 and 6).

As was the case with gill, two types of IGFR1s, IFGR1A and IGFR1B, were expressed in both red and white muscle. The abundance of both IGFR1s mRNAs increased in white muscle following 20‰ SW exposure. IGFR1A mRNA expression increased a maximum of 47% 6 h after exposure, whereas expression of IGFR1B mRNA increased a maximum of 127% 6 h after exposure (Fig. 7). Expression of IGFR1 mRNAs also increased in red muscle following 20‰ SW exposure; however, only the change in IGFR1A levels was significant, which displayed a maximum increase of 54% 6 h after 20‰ SW exposure (Fig. 8). After 48 h of 20‰ SW exposure, the abundance of both IGFR1 mRNAs in white and red muscle declined to values similar to those observe initially at 0 h.

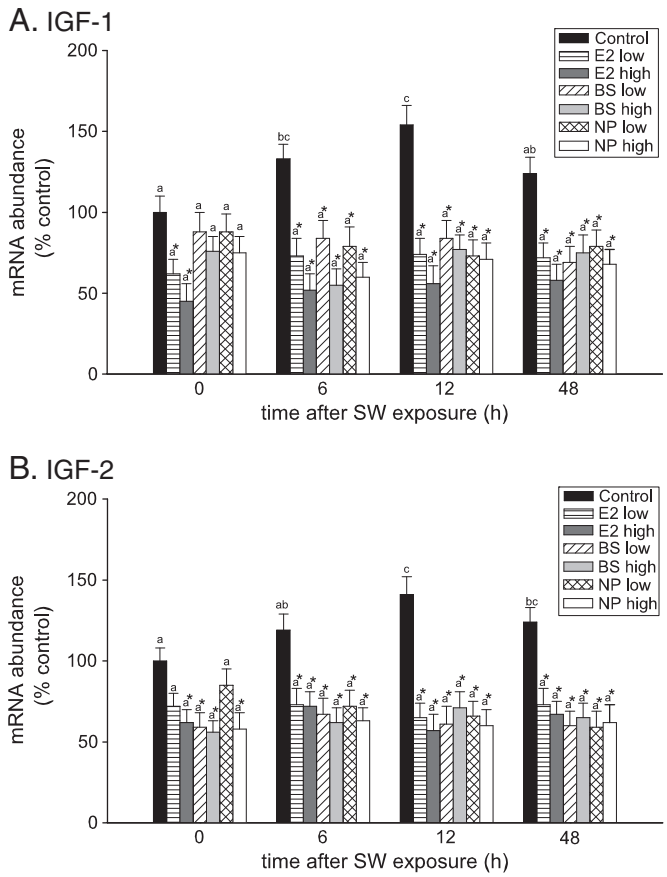

Fig. 5. Effects of environmental estrogens (EE) on the abundance of (A) insulin-like growth factor-1 (IGF-1) and (B) IGF-2 mRNAs in gill filaments of rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

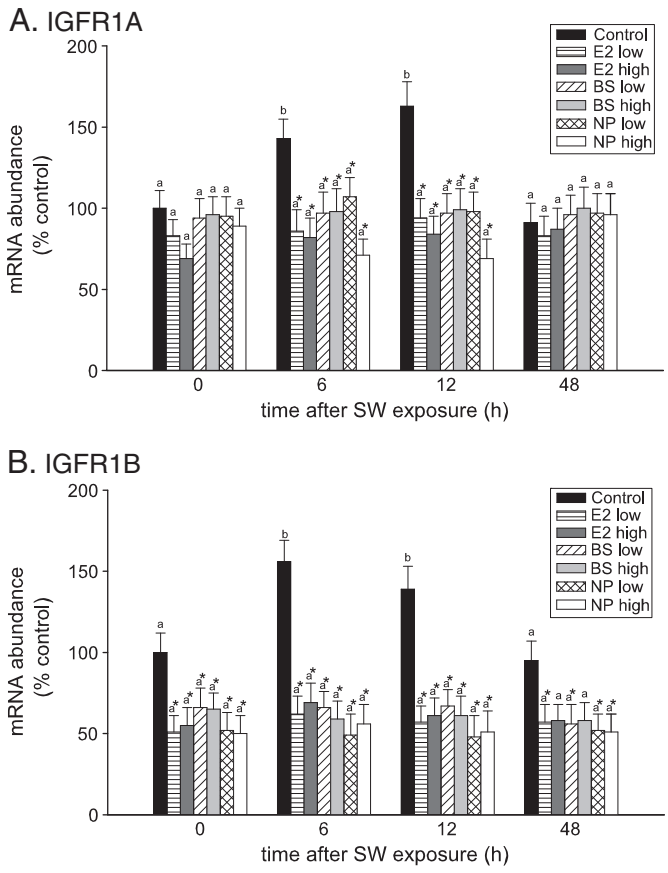

Fig. 6. Effects of environmental estrogens (EE) on the abundance of (A) IGF receptor 1A (IGFR1A) and (B) IGFR1B mRNAs in gill filaments of rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

EE tended to depress the expression IGFR1s in red and white muscle in manner similar to that observed in gill such that mRNA abundance at 0 h was lower than that observed in control fish at 0 h. Such depression was somewhat variable and depended on the specific EE and its concentration (Figs. 7 and 8). Also similar to the observation in gill, exposure to all EE attenuated or blocked normal salinity-associated increases in IGFR1 expression (Figs. 7 and 8).

4. Discussion

The results of this study indicate that acclimation of rainbow trout to 20‰ SW involves transient increases in the expression of GHRs, IGFs (both IGF-1 and IGF-2), and IGFR1s. These findings are consistent with previous observations on GHR, IGF-1, and IGFR1 expression by us (Poppinga et al., 2007) and on IGF-1 expression by others (cf. Sakamoto and McCormick, 2006), and extend our knowledge of the role of the GH-IGF system in salinity adaptation. The observed increase in GHR and IGFR1 expression would lead to heightened sensitivity to GH and IGF in target organs — effects that combined with increased plasma levels of the hormones (Sakamoto et al., 1993; McCormick et al., 2000; Agustsson et al., 2001; Shepherd et al., 2005; Nilsen et al., 2008) would accentuate their action during SW adaptation. Given that GH and IGF-1 enhance salinity tolerance via increases in chloride cell number, NKA and NKCC activity/biosynthesis (McCormick et al., 1991; Seidelin et al., 1999; Pelis et al., 2001), it is reasonable to suggest that accentuated responsiveness to GH and IGF underlies, at least in part, the rapid hypoosmoregulatory ability of euryhaline fish. It is interesting to note that salinity adaption differentially affected the expression of subtypes encoding GHR (in liver and gill) and IGFR1 (in white muscle), but the significance of these observations is unclear. Distinct roles for GHR and IGFR subtypes in fish are suggested by their differential pattern of expression in embryos and adult, and that the patterns of their expression are differentially affected by nutritional state (Very et al., 2005; Norbeck et al., 2007; Malkuch et al., 2008). Recently, we showed that trout GHRs display differential ligand binding and agonist-induced regulation features (Reindl et al., 2009). It is possible, therefore, that the observed salinity-induced changes in GHR and IGFR subtype expression are adaptive for activating hypoosmoregulatory responses.

The results of this study also indicate that exposure of rainbow trout to EE disrupts the GH-IGF system — effects that were elicited at concentrations of E2, NP, and β-sitosterol found in the environment (Blackburn and Waldock, 1995; Cook et al., 1996; Hale et al., 2000). This was evidenced by several observations. First, initial mRNA abundance of several GH-IGF system components (e.g., hepatic GHR 2, hepatic IGF-1, gill GHR 1 and GHR 2, gill IGF-2, gill IGFR1B, red and white muscle IGFR1A, red and white muscle IGFR1B) were depressed by EE, particularly E2 and NP, compared to control-treated animals. Second, exposure to any of the EE used in the study attenuated or abolished the normal salinity-associated increases in the expression of GHR and IGF mRNAs in liver as well as of GHR, IGF, and IGFR1 mRNAs in gill and of IGFR1 mRNAs in red and white muscle. Previous

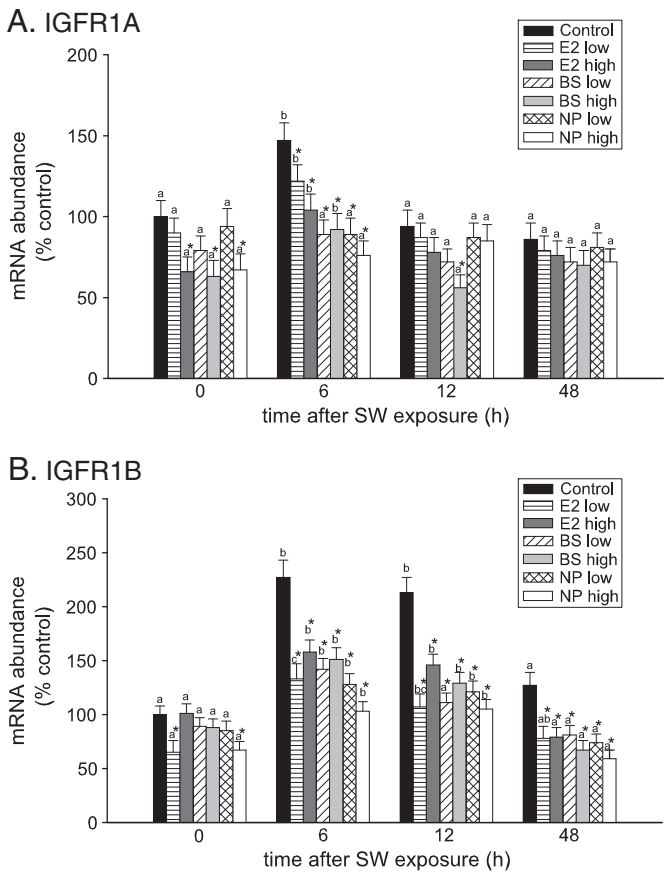

Fig. 7. Effects of environmental estrogens (EE) on the abundance of (A) IGF receptor 1A (IGFR1A) and (B) IGFR1B mRNAs in white muscle of rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

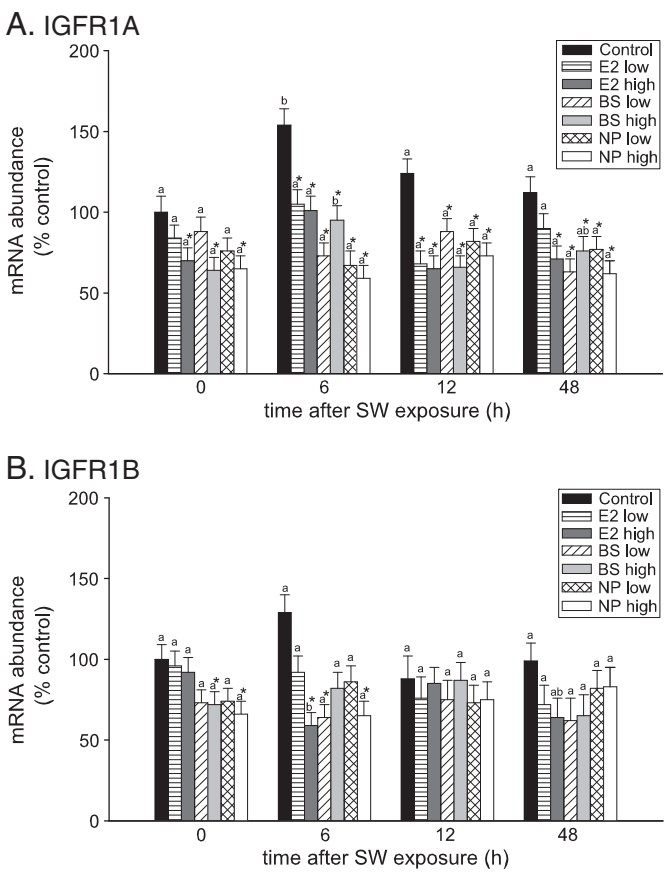

Fig. 8. Effects of environmental estrogens (EE) on the abundance of (A) IGF receptor 1A (IGFR1A) and (B) IGFR1B mRNAs in red muscle of rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 μg/l) or high (100 μg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (BS) in fresh water for 28 days, then sampled at various times after SW exposure. Data are presented as mean ± SEM. (n = 5–7). For a given treatment, groups with different letters are significantly different ($p<0.05$); *indicates a significant difference ($p<0.05$) between the control and EE-treated groups at a given time.

findings in Atlantic salmon showed that E2 and NP had no effect on pituitary GH mRNA and that NP had a variable response (increase at one concentration, 2 μg/g body weight injected intraperitoneally; no effect at lower or higher concentrations) on plasma GH (Yatedie and Male, 2002; McCormick et al., 2005). By contrast, E2 and NP have been shown to decrease plasma IGF-1 levels in Atlantic salmon and rainbow trout (Arsenault et al., 2004; McCormick et al., 2005; Lerner et al., 2007a). Recently, it was reported that in developing tilapia 17α-ethinylestradiol reduced expression of IGF-1 in liver, brain, and gonad as well as of GH in brain (Shved et al., 2008).

Disruption of the GH-IGF system resulted in reduced hypoosmoregulatory ability of rainbow trout. This was evidenced by the general failure of plasma chloride levels in E2-, NP-, and β-sitosterol-treated fish to decline from peak values following exposure to 20‰ SW. It also was notable that plasma chloride values in the initial E2 high group were significantly depressed below those in the initial control group. We recognize that the magnitude of change in plasma chloride levels following 20‰ SW exposure in this study was relatively small, which probably resulted from the use of 20‰ SW rather than full-strength SW, a choice we made to assure survival of small trout exposed to an abrupt change in salinity. The observed decrease in osmoregulatory ability is in agreement with previous studies that found that both NP and E2 reduced salinity tolerance of Atlantic salmon (Madsen et al., 2004; McCormick et al., 2005; Lerner et al., 2007a,b). Carrera et al. (2007) found similar decreased salinity tolerance in gilthead sea bream when treated with NP. However, another study in Atlantic salmon using similar concentrations found that NP did not affect NKA activity unless presented in combination with atrazine (Moore et al., 2003). In light of effects of GH and IGF-1 on gill chloride cell number/size, NKA and NKCC activity discussed above (cf. Sakamoto and McCormick, 2006), it is reasonable to suggest that GH insufficiency (in terms of reduced GH sensitivity in target organs) and IGF insufficiency (in terms of reduced IGF production/release and reduced IGF sensitivity in target organs) underlies, at least in part, the reduced hypoosmoregulatory ability of euryhaline fish exposed to EE.

Disruption of the GH-IGF system also resulted in reduced growth of rainbow trout. This was evidenced by the tendency of E2 to repress body weight as well as by the significant depression of body weight observed in fish treated with the high dose of NP compared to control-treated fish. Estrogenic compounds also tended to reduce the condition of fish, an effect that was most pronounced in NP-treated fish. Previous studies in Atlantic salmon smolts and developing tilapia also found that long-term exposure to E2 and NP reduced growth (Arsenault et al., 2004; Lerner et al., 2007a; Shved et al., 2008). The current study also revealed that E2-, NP-, and β-sitosterol-treated trout display increased HSI, an observation consistent with previous findings in E2-treated gilthead sea bream (Carrera et al., 2007). The observed increase in HSI could be explained by hepatic vitellogenesis, which is stimulated by estrogenic compounds, including E2, NP, and β-sitosterol (Jobling and Sumpter, 1993; Tremblay and Van Der Kraak, 1998, 1999). Taken together, these data suggest that EE reprogram fish to divert energy resources away from growth to reproduction.

Given the involvement of the GH-IGF system in organismal growth (Klein and Sheridan, 2008), it also is reasonable to suggest that the observed insufficiency of GH (in terms of reduced GH sensitivity in target organs) and of IGF (in terms of reduced IGF production and reduced IGF sensitivity in target organs) underlies, at least in part, the means by which EE reprogram the organism and suppress growth.

The cellular mechanism(s) by which EE disrupt the GH-IGF system is (are) not known, although a number of possibilities exist. β-sitosterol, NP, and E2 bind to estrogen receptors (ER) in fish (Tremblay and Van Der Kraak, 1998), forming an active transcription factor that can bind to promoter regions of genes possessing estrogen response elements (ERE) to promote their transcription. The transcription of the vitellogenin gene is stimulated by E2 in this manner, which explains the actions that β-sitosterol and NP have on hepatic vitellogenin synthesis (Tremblay and Van Der Kraak, 1998, 1999). In mammals, it has been shown that E2 can influence GH action by regulating the expression of GHR, the promoter of which possesses an ERE-like motif and an adjacent Ap1 site that differentially interacts with ER subtypes, as well as by interacting with GHR signaling pathways (e.g., JAK-STAT, ERK, and PI3K/Akt) (Leung et al., 2004). Interference with the JAK-STAT pathway, which is important for IGF-1 expression, and/or the ERK and PI3K/Akt pathways, which are important for IGFR action, could explain the observed EE-induced disruption of the GH-IGF system.

In summary, the results of this study indicated that transient activation of GH-IGF system components, including increased expression of GHR 1, GHR 2, IGF-1, IGF-1, IGFR1A, and IGFR1B, accompany salinity adaptation of rainbow trout. The results also indicate that exposure of rainbow trout to EE suppresses the expression of GH-IGF system components and leads to reduced growth and reduced hypoosmoregulatory ability.

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