EFFECTS OF ENVIRONMENTAL ESTROGENS ON THE GROWTH HORMONE-INSULIN-LIKE GROWTH FACTOR SYSTEM IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

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. Fish in aquatic habitats are exposed to increasing concentrations of environmental contaminants, including environmental estrogens (EE). In this work, rainbow trout were used to assess the effects of EE on the growth hormone (GH)-insulin-like growth factor (IGF) system, specifically focusing on osmoregulation, organismal growth, and growth at the molecular level. Juvenile trout were exposed to varying concentrations of 17β-estradiol (E2), β-sitosterol (βS), and 4-*n*-nonylphenol (NP) *in vivo* and *in vitro*. Real-time quantitative-PCR was used to measure levels of mRNA expression (GH receptor 1 (GHR1), GHR2, IGF-1, IGF-2, IGF receptor 1A (IGFR1A), and IGFR1B) in multiple tissues, including liver, gill, and muscle. Western blotting was used to elucidate signaling pathways affected by EE-treatment (e.g., JAK-STAT, MAPK, PI3K). Environmental estrogen-treated fish displayed depressed growth in terms of body mass and body length. The observed effects on organismal growth appeared to be due to a decrease in food conversion, as food consumption was not significantly different between treatment groups. Hepatic, gill, and muscle levels of mRNAs encoding GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B decreased in a concentration-, time-, and compound-dependent manner *in vivo* and *in vitro*. Furthermore, EE-treated fish displayed decreased osmoregulatory function when subjected to a salt water challenge, as evaluated by measuring plasma chloride levels and mRNA expression of GHRs, IGFs, and IGFRs. The suppression of mRNA expression of components of the GH-IGF system by EE was linked to suppressed phosphorylation of JAK-STAT, MAPK, and PI3K-Akt in a concentration- and time-dependent manner in hepatocytes and gill filaments, an effect that was ER-dependent. Classically, the ER has been thought to function as a nuclear

receptor; however, the observed results support the notion that the ER (and thus EE) may have nongenomic effects as well. The results of this dissertation indicate that EE suppress growth at the organismal and molecular level via inhibition of growth-related signaling cascades and repression of gene expression elements of the GH-IGF system.

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ABBREVIATIONS

BSA……Bovine Serum Albumin

βS…….....β-sitosterol

cDNA…….Complementary Deoxyribonucleic Acid

CF……...Condition Factor

E2……..17β-estradiol

EE……Environmental Estrogen

FW……..Fresh Water

GH…….Growth Hormone

GHRH……...Growth Hormone Releasing Hormone

GHR……..…….Growth Hormone Receptor

HSI……...Hepatosomatic Index

IGF……..Insulin-like Growth Factor

IGFR……Insulin-like Growth Factor Receptor

mRNA……..Messenger Ribonucleic Acid

NP……...…….4-*n*-nonylphenol

PCR……...….Polymerase Chain Reaction

RT…….....…Reverse Transcription

RT-QPCR…….Real-time Quantitative Polymerase Chain Reaction

SW……...Seawater

TBS-T……...Tris-Buffered Saline with Tween20

GENERAL INTRODUCTION

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. Fish in aquatic habitats are exposed to increasing concentrations of environmental contaminants, including endocrine disrupting compounds (EDCs). EDCs are introduced to the environment via the air, water, and soil, and once present, may persist at low levels. Chronic exposure to these compounds may cause a host of detrimental effects. While much of the research to date has focused on the effects of EDCs on the reproductive system, less is known about how these compounds affect growth. In this work, rainbow trout were used to assess the effects of EDCs, specifically environmental estrogens (EE), on the growth hormone (GH)-insulin-like growth factor (IGF) system. Our specific aim was to elucidate the effects of EE on osmoregulation, organismal growth, growth at the molecular level both *in vitro* and *in vivo*, and the signaling pathways responsible for the observed effects on growth.

Endocrine Disruption

Endocrine Disrupting Compounds

Rapid technological development over the last 60+ years has been accompanied by increased production, use, and disposal of an expanding array of chemicals. As a result, chemicals are introduced into the environment through the air, soil, and water. Increasing research shows the deleterious effects of environmental contamination on microbes, aquatic and terrestrial plants, invertebrates, fish, amphibians, birds, and mammals, including humans (Hester and Harrison, 1999; Diamani-Kandarakis et al., 2009), primarily due to the ability of these compounds to bioaccumulate in tissues. Alarming to the universal health of organisms is the notorious ability of

these compounds to affect the endocrine system, thus leading to the popular term endocrine disrupting compounds (EDCs).

Endocrine disruptors are defined by the Environmental Protection Agency as exogenous chemical substances or mixtures that cause adverse effects to an organism, its progeny, populations, or subpopulations through altering the structure or function of the endocrine system. Endocrine disrupting compounds can act through a variety of mechanisms, including direct interaction with hormone receptors (i.e., agonistic or antagonistic). Endocrine disrupting compounds also can indirectly interfere with the endocrine system by affecting the concentration of hormones (i.e., hormone metabolism, hormone synthesis, storage, and release) and the concentration of hormone receptors. As a result of interfering with the endocrine system, these compounds can subsequently affect a number of physiological processes including development, osmoregulation, male and female reproduction, neuroendocrinology, obesity, growth, and disease states such as breast and prostate cancer (Diamani-Kandarakis et al., 2009). A wide range of EDCs can be found in the environment, which has led to great concern about how these compounds affect wildlife and human health alike.

There are several classes of EDCs, including pesticides, industrial chemicals, plastics, fuels and natural hormones (reviewed in Lintelmann et al., 2003; Diamani-Kandarakis et al., 2009). EDCs enter soil and water through agricultural (including farm animal production) and veterinary (e.g., diethylstilbestrol, estradiol cypionte; widely used in dogs, cats, and horses) application, manufacturing, natural hydrological processes, and watershed/waste water management practices. 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 (β-sitosterol), mycotoxins (e.g.,

zearalenone), organochlorine pesticides (e.g., DDT), polychlorinated biphenyls (PCBs), and alkylphenol polyethoxylates (APEs; e.g., 4-nonylphenol) (Turner, 1999; Birkett, 2010).

Once in the environment, these compounds can persist at low levels (resistant to chemical, biological, and photolytic degradation), thus disrupting aquatic and terrestrial habitats at multiple levels. For example, some organic pesticides, such as DDT, DDE, and methoxychlor, are particularly persistent and can be found in areas where they have never been used or produced, such as the Arctic (Vallack et al., 1998). Aquatic organisms have been shown to act as valuable biological indicators of EDC exposure and studies on the effects of these compounds on teleosts have proven to be invaluable in studying the effects of EDCs on terrestrial vertebrates, including humans.

Endogenous and synthetic estrogens have been detected in the effluent of publicly owned sewage treatment plants and surface waters on every continent (Anderson et al., 2012). In the environment the concentrations of several contaminants have been well-documented and appear to contrast greatly depending largely upon location. For example, β-sitosterol is a highly lipophilic compound found in plant oils, legumes, and wood that has been found in effluent from paper mills at significant concentrations. Cook et al. (1996) found that total sterol concentrations in effluent from pulp and paper mills in the U.S. ranged from 71 to 535 µg/l, while Fernandez et al. (2007) found that levels reached only 7.2 µg/L in a Canadian testing site. A breakdown product of the APEs (industrial chemicals), 4-*n*-nonylphenol (NP), common in a variety of products including detergents, commercial and household cleaning products, and plastics (Nimrod and Benson, 1996, Thiele et al., 1997), has been measured in lakes and rivers around the world. In rivers in England, concentrations of alkylphenols have reached levels up to 336 µg/l (Blackburn and Wadlock, 1995). Conversely, APE concentrations were shown to vary

between 3 to 11.5 µg/L depending on seasonal temperature in a river in the United States (Lozano et al., 2012). In Europe, models have predicted that sewage discharge concentrations of ethinylestradiol are higher than the proposed environmental quality standard (0.035 ng/L) for up to a third of the total lengths of the rivers (Johnson et al., 2013), while in Canada ethinylestradiol wasn't detected at measurable levels in wastewater effluent (Metcalfe et al., 2013). While much of the contamination has been localized to streams and rivers, Scott et al. (2006) hypothesized that even aquatic vertebrates in the open ocean may be experiencing similar endocrine disrupting effects as vertebrates exposed via more direct routes due to effects on the food chain. Despite the fact that many EE are found at levels below the legal limit, the potential long-term impact of chronic exposure to low levels is not clear, especially given the persistence of the compounds, the ability to be rapidly transferred through the food web and accumulate in tissues, and the capacity to elicit epigenetic effects that may affect progeny (Lech et al., 1996; Phillips and Harrison, 1999; Uguz et al., 2003; Diamani-Kandarakis et al., 2009).

The detrimental effects of EDCs can be observed at the organismal and molecular level and include developmental and reproductive effects (e.g., sex reversal, sterility, altered reproductive timing and behavior) that result in reduced fecundity in teleosts and mammals (Kime et al., 1998; Cabaton et al., 2011). The reproductive effects of EDCs have been particularly well-studied in vertebrates (reviewed in Dickerson and Gore, 2007 and Kloas et al., 2008). For example, NP (at low concentrations) has been shown to inhibit testicular growth in rainbow trout (Jobling et al., 1996), while phytosterols have been linked to reproductive dysfunction in both rainbow trout and goldfish (MacLatchy and Van der Kraak, 1995; Tremblay and Van der Kraak, 1998). Similarly, in mice long-term EDC exposure (specifically BPA) lead to uterine abnormalities including adenomyosis, leiomyomas, stromal polyps, and hyperplasia (Newbold et al., 2007).

In addition to reproductive effects, a host of other disease states/physiological disruptions may arise following EDC exposure. Diethylstilbestrol (DES) has been implicated as both an estrogen mimetic and as a carcinogen. Exposure of neonatal mice to DES has been shown to promote the development of uterine carcinomas, presumably via epigenetic mechanisms (i.e., alteration of chromatin modifying proteins) (Newbold et al., 1990; Jefferson et al., 2013). Furthermore, EDCs have been observed to alter behavior (Filby et al., 2012; Wolstenholme et al., 2012; Brodin et al., 2013). For example, altered behavior (i.e., more active fish, less social fish, and bolder fish) has been observed in European perch following exposure to benzodiazepines, a group of persistent psychotherapeutic drugs (Brodin et al., 2013). Other endpoints of EDC exposure (e.g., metabolism and osmoregulation) will be further discussed later.

Growth Effects of EDCs

While several studies have focused on the reproductive aspects of endocrine disruption, fewer studies have focused on how these compounds affect growth. In order to understand the effects that EDCs have on growth, it is important to understand the major hormonal mechanisms regulating growth in vertebrates. Organismal growth integrates a host of biological processes that lead to a fertilized egg developing into an adult and to the maintenance and modulation of adult structures appropriate to their function. Animal growth is influenced by genetic, environmental, and nutritional factors. Extrinsic factors are particularly important in the growth of poikilothermic vertebrates such as teleost fish, which rely on temperature, photoperiod, and food availability to trigger developmental processes such as hatching, metamorphosis, smoltification, sexual maturation, and spawning (Bjornsson, 1997; Duan, 1998; Mommsen, 2001). Integration

of external and internal cues leads to the coordination of animal growth via the interplay of numerous hormones (Very and Sheridan, 2004).

Organismal Growth

A primary growth promoting factor in vertebrates is growth hormone (GH), which has been isolated from the pituitary gland from representatives in every extant class of vertebrate (Harvey, 1993). GH results in increased amino acid uptake, increased RNA synthesis, increased protein synthesis, increased muscle growth, and increased cartilage synthesis (Tannenbaum and Epelbaum, 1999; Butler and LeRoith, 2001). GH is produced primarily in the brain, gill, and heart, and to some extent in ovary, kidney, liver, and pyloric caeca in rainbow trout (Yang et al., 1999). GH elicits its physiological effects by binding to GHRs, members of the class I cytokine receptor family. In rainbow trout there are two receptor isoforms, GHR1 and GHR2 (Agellon et al., 1988; Reindl and Sheridan, 2012). These two receptors are differentially expressed and found in the liver (primary location), muscle, adipose tissue, mammary gland, bone, kidney, and other tissues (Kopchick and Andry, 2000). Like most components of the GH-IGF system, expression of GHRs is regulated by development, hormone availability, and nutritional status. Growth hormone binding protein (GHBP) is a protein typically found in serum and membranes in both mammals and teleosts and is responsible for regulating the availability of GH in the organism (Baumann et al., 1988). In rats and mice, it appears that GHBP and GHR are products of the differential splicing of a single gene (Talamantes and Ortiz, 2002), whereas in humans, GHBPs appear to be produced via proteolytic cleaving of the membrane-bound GHR (Forsyth and Wallis, 2002).

Many of the growth-promoting actions of GH in fish and other animals are mediated by insulin-like growth factor-1 (IGF-1) (Moriyama et al., 2000; LeRoith et al., 2001; Mommsen,

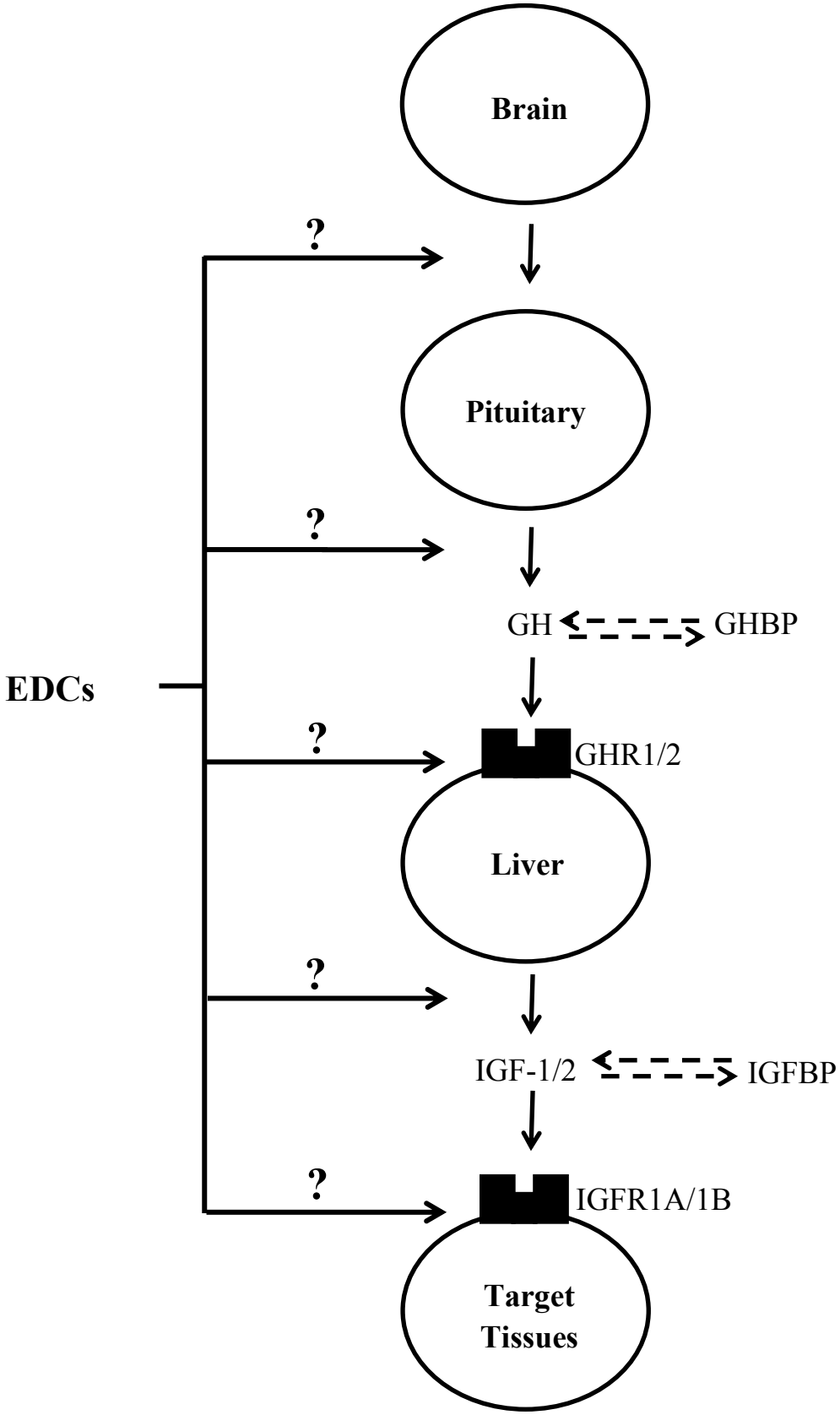
2001; Reindl and Sheridan, 2012). Following stimulation of the release of GH by the pituitary, circulating GH binds to its cytokine receptor and subsequently activates signaling pathways such as JAK-STAT, which leads to the synthesis and secretion of IGF-1 from liver and other sites (e.g., gill, muscle, etc.). IGF-1, in turn, binds to IGF-1 receptors (IGFRs), which are encoded by two different genes in rainbow trout (Greene and Chen, 1999; Reindl and Sheridan, 2012). These receptors are expressed in teleost brain, ovary, gill, muscle, and heart (Duan, 1998; Greene and Chen, 1999). Following binding to IGFRs on target tissues, pathways such as MAPK and PI3K/Akt are activated, ultimately resulting in the growth promoting and other biological actions (e.g., energy metabolism, osmoregulation) of IGF-1 (Fig. 1) (Kelley et al., 2000; Butler and LeRoith, 2001; Wood et al., 2005). Subsequently, IGF-1 inhibits the synthesis and secretion of GH via a negative feedback mechanism. The availability and actions of IGF-1 are influenced by IGF-1 binding proteins (IGFBPs) (Duan, 2002; Duan and Xu, 2005; Wood et al., 2005). IGFBPs also have direct, IGF-1-independent effects on growth (Butler and LeRoith, 2001; Duan and Xu, 2005; Wood et al., 2005). Additionally, IGF-1 may have growth-modulating effects that are independent of GH (Duan and Xu, 2005). While imperative for growth, the GH-IGF system has also been shown to play an important role in other physiological processes including energy metabolism, reproduction, and osmoregulation.

Much of the research on the regulation of animal growth has focused on the production and release of GH from the pituitary (Harvey, 1993). In most species studied, including mammals and fish, pituitary GH release is under dual antagonistic control from the hypothalamus as well as from systemic hormones. The hypothalamic hormone growth hormone-releasing hormone (GHRH) acts to promote GH transcription (Kopchick and Andry, 2000). In teleosts, hormones such as ghrelin, E2, and dopamine have been shown to lead to increased plasma GH levels (Zou

et al., 1997). Major inhibitors of GH release are somatostatins, a structurally and functionally diverse family of peptides derived from multiple genes with wide-spread anatomical distribution (Nelson and Sheridan, 2005; Klein and Sheridan, 2008). Dopamine, GHRH, gonadotropin releasing hormone (GnRH), thyrotropin releasing hormone (TRH), cholecystokinin (CCK), and ghrelin, on the other hand, stimulate GH release (Canosa et al., 2007). Given the possibility of both a direct and indirect mode of action of GH and the complexity of the GH-IGF-1 system, which in teleost fish, such as rainbow trout, includes multiple subtypes of GHRs and IGFRs, it is becoming increasingly clear that the regulation of growth may occur at many levels outside the pituitary (Talamantes and Ortiz, 2000; Bjornsson et al., 2004; Reinecke et al., 2005; Klein and Sheridan, 2008).

While the endogenous endocrine regulation of growth appears to be fairly well-understood, studies have just begun to emerge over the past few years indicating how exogenous compounds such as EDCs may alter the GH-IGF system (Fig. 1). As growth is a multifaceted process, it is important to examine the effects of EDCs not only from the viewpoint of level of growth regulation (e.g., GH sensitivity, IGF-1 synthesis), but also from the aspect of organismal life history. Fish are an interesting species to study because they display indeterminate growth, and thus are continuously allocating energy resources towards growth. From a life history perspective, juveniles typically allocate energy resources towards growth prior to undergoing sexual maturation. Treatment at this critical life stage with a sex steroid such as estrogen, or a compound mimicking estrogen (e.g., β-sitosterol, NP), may reprogram the fish to divert energy resources towards reproduction rather than growth. Additionally, the effects of exposure of an organism to an EDC may not be manifested until later in life, thus adding another degree of complexity to the physiological effects of these compounds. Evidence of alterations to the GH-

Figure 1. Schematic representation of the growth hormone-insulin-like growth factor system in vertebrates and possible targets of endocrine disrupting compounds on this axis. growth hormone, GH; growth hormone binding protein, GHBP; growth hormone receptor, GHR; insulin-like growth factor, IGF; insulin-like growth factor binding protein; insulin-like growth factor receptor, IGFBP; endocrine disrupting compounds, EDCs


Brain
?
Pituitary
?
GH
GHBP
EDCs
?
GHR1/2
Liver
?
IGF-1/2
IGFBP
?
IGFR1A/1B
Target
Tissues

IGF system at these different levels and different life history stages (larval, juvenile, etc.) is subsequently presented.

EDCs have been observed to target growth at multiple levels, including overall organismal growth in several teleost species. At the embryonic/larval stage, BPA, NP, malathion, and perfluorinated chemicals have been shown to depress growth in terms of body weight and body length in medaka, Atlantic salmon, zebrafish (Cook et al., 2005; Lee et al., 2012; Spachmo and Arukwe, 2012). Zebrafish, stickleback, fathead minnow, and tilapia exposed to ethinylestradiol at multiple stages of life (e.g., larval, fry, juvenile) also exhibited retarded growth (Bell, 2004; Schafers et al., 2007; Johns et al., 2011; Shved et al., 2008). Comparable results have been observed in other juvenile teleosts where aqueous exposure of rainbow trout, Atlantic salmon, Arctic charr, swordtails, and platyfish to NP, methoxychlor, Arochlor, or PCBs has been shown to reduce organismal growth when compared to control fish (Magliulo et al., 2002; Arsenault et al., 2004; Jorgensen et al., 2004). Similarly, sublethal exposure to the pesticides malathion and dimethoate was shown to decrease final body weight and standard growth rate in juvenile Nile tilapia (Sweilum, 2006). Acute aqueous exposure of juvenile black-striped pipefish to ethinylestradiol caused diminished juvenile growth, whereas exposure to the androgenic compound, tributyltin (TBT) lead to an increase in growth. Interestingly, when the juveniles were exposed to a mixture of ethinylestradiol and TBT, growth was depressed, thus suggesting a more potent effect of ethinylestradiol on juvenile growth (Sarria et al., 2011). Conversely, cadmium exposure in juvenile tilapia (*O. mossambicus*) increased growth in length and weight and caused early maturation in tilapia fries (Amutha and Subramanian, 2013). While many studies have focused on aqueous exposure, dietary exposure of yellow perch to E2 also lead to an increase in length and weight of juveniles compared to control animals (Goetz et al., 2009).

While the period of development may be a critical window of exposure, adult fish are not immune to the effects of EDCs. BPA has been shown to retard growth in adult rainbow trout (Jobling, 1993; Warhurst, 1994). Adult zebrafish and fathead minnow exposed to ethinylestradiol at also exhibited retarded growth (Bell, 2004; Schafers et al., 2007). Dietary exposure to DES caused diminished growth rates in European catfish, whereas no effects on growth were observed with dietary exposure to E2 (Krol et al., 2013). While growth in adult zebrafish were subjected to endosulfan treatment was not affected after a 21 d exposure, the HSI of male fish was elevated (Han et al., 2011), possibly indicating the diversion of energy resources towards reproduction. Taken together, these studies indicate that EDCs of numerous classes are capable of eliciting growth-disrupting effects in an organism at distinct life stages, with the most critical period appearing to occur before sexual maturity. The differential effects of EE on organismal growth could be partially attributed to the exposure of teleosts during the juvenile life stage, a stage in which the fish are typically undergoing rapid growth (Mommsen, 2001).

Many of these studies examined the effects of EDC exposure on growth following continuous exposure; however, chronic exposure of fish to low levels of many EE may cause deleterious growth effects for an extended period of time post-exposure. For example, Ashfield et al. (1998) found that APEs can cause a decrease in body weight and length up to one year following a one month exposure in female rainbow trout. Similarly, Nye et al. (2007) exposed female killifish to contaminated soil (high in several types of pollutants) and then switched these fish back to a reference site (low in pollutants) and discovered that offspring born to these females were smaller and had lower growth rates than the groups that remained in a constant environment. The authors (Nye et al., 2007) suggested that these results may be due to a fitness tradeoff that occurs due to contaminant resistance. The aforementioned studies support the notion that both the age of

the organism, as well as the duration of the exposure, may have immediate and lasting effects on growth.

Endocrine disrupting compounds have been shown to be able to regulate growth at the level of the pituitary in many teleost species, as well as regulate growth via extrapituitary effects on the growth axis. The perfluorinated chemical, PFOS, was found to produce a time-dependent decrease in GH expression in both the head and body region of Atlantic salmon larvae and embryos (Spachmo and Arukwe, 2012). Similarly, pituitary GH mRNA expression in tilapia was suppressed in both females (75 days post fertilization) and males (165 days post fertilization) following dietary exposure to ethinylestradiol (Shved et al., 2007).

Studies on peripheral organs in teleosts have demonstrated negative effects on growth hormone sensitivity as well. Treatment with E2 or ethinylestradiol has been shown to lead to decreased hepatic GHR mRNA expression in Atlantic salmon, tilapia, sea bream, rainbow trout, and fathead minnow (McCormick et al., 2005; Davis et al., 2007; Filby et al., 2007; Davis et al., 2008; Jiao and Chang, 2010; Norbeck et al., 2011). In agreement with the GH mRNA expression data, synthesis of GH was also suppressed in several studies. For example, circulating levels of GH were significantly depressed in catfish and Arctic char exposed to malathion and Arochlor, respectively (Jorgensen et al., 2004; Lal et al., 2013). This suppression of GH appeared to be in part due to the down-regulation of GHR expression, as was found in seabream hepatocytes exposed to BPA *in vitro* (Jiao and Chang, 2010). Moreover, functional expression of GHRs was diminished following aqueous exposure of Atlantic salmon to E2 and NP in an osmoregulatory context (Lerner et al., 2012).

A number of studies have observed a depression in GH or GHR mRNA levels, but other studies have yielded conflicting results at the level of both the pituitary and peripheral organs.

For example, Yatedie and Male (2002) found no difference in pituitary GH mRNA expression following exposure of Atlantic salmon to E2 or NP. Similarly, Filby et al. (2006) exposed fathead minnows to ethinylestradiol for 14 days and observed no change in either liver or brain GH expression, whereas gonadal GH mRNA expression was up-regulated. Pituitary GH secretion in goldfish was significantly increased in E2-treated fish, however GH mRNA levels remained unchanged (Zou et al., 1997). Conversely, in cultured rainbow trout pituitary cells, TCDD, DDT, or E2 increased GH mRNA levels (Elango et al., 2006). This is in agreement with a study by Holloway and Leatherland (1997) that found that GH plasma levels increased in juvenile rainbow trout following E2 administration. Interestingly, the differences in GH and GHR mRNA and protein expression in these studies do not appear to be age-dependent, thus it is possible that differences in GH sensitivity, synthesis, etc., expression are due to treatment with different EDCs (e.g., physical and chemical nature of EDC), different EDC treatment concentrations or routes of administration (aqueous versus injected versus *in vitro* in above studies), different exposure time (24 hours to 21 days), species-specific differences (e.g., different isoforms of GHRs, tissue-specific distribution of GHRs), or overall health of the organism (i.e., ability to metabolize EDCs).

While many studies have examined the direct effects of EDCs on GH, some studies have also examined the possibility of indirect effects on this axis. Somatostatin is well-known as a growth inhibitor in rainbow trout (Very et al., 2005). Insulin and GH stimulate SSTR expression in rainbow trout, suggesting that regulation of SSTRs may be important for the coordination of growth, development, and metabolism of vertebrates (Nelson et al., 2006). Canosa et al. (2002) demonstrated that E2 increased expression of somatostatin (SS) in goldfish brain. Similarly, BPA can modulate the level of SS in the diencephalic region of the brain in Mediterranean rainbow

wrasse (Alo et al., 2005). In both cases, E2 or E2 mimetics may indirectly suppress hypothalamic GH synthesis.

Examining further levels of the GH-IGF system such as IGF synthesis has shown to be equally challenging. The majority of the studies looking at IGF expression following EDC exposure have looked at the effects of EDCs, particularly estrogen mimetics, at the level of the liver and target tissues (e.g., gill, ovaries, etc.). For example, dietary ethinylestradiol treatment was observed to down-regulate hepatic IGF-1mRNA expression in developing tilapia *in vivo* (Shved et al., 2007). Similar decreases were observed in sexually mature sea bream (Carnevali et al., 2005), tilapia (Riley et al., 2004), fathead minnow (Filby et al., 2006), and rainbow trout (Norbeck et al., 2011) exposed to E2 (*in vitro* and *in vivo*) and sexually mature sea bream exposed to BPA (Jaio and Chang, 2010). In Atlantic salmon larvae and embryos, PFOS was found to produce a decrease in IGF-1 expression (Spachmo and Arukwe, 2012). The routes of administration for these studies included dietary, aqueous, intraperitoneal, and cell culture. In agreement with observed effects on mRNA expression levels, developing tilapia treated with E2 or ethinylestradiol had reduced serum IGF-1 levels up to a month post-exposure (Shved et al., 2007; Davis et al., 2007; Davis et al., 2008). Similarly, aqueous exposure of Atlantic salmon to NP also lead to a decrease in plasma IGF-1 levels after an extended period of time (Arsenault et al., 2004; McCormick et al., 2005). Malathion and Arochlor suppressed IGF-1 serum levels in catfish (Lal et al., 2013) and Arctic charr (Jorgensen et al., 2004), respectively.

In contrast to the previous studies, juvenile perch fed E2 demonstrated an increase in hepatic IGF-1 (Goetz et al., 2009). Similarly, IGF-1 mRNA expression was induced in the brain and pituitary of males and female fathead minnows following exposure to ethinylestradiol (Filby et al., 2006). However, target tissues such as gill and liver showed depressed IGF-1 mRNA levels

(Filby et al., 2006; Filby et al., 2007), which was in agreement with the above studies. As previously mentioned, these contradictory results may arise from differences in length of exposure, health of the organism, type of exposure, or concentration of EDC. From a toxicological standpoint, concentration effects are often inconsistent, as EE may have unpredictable effects at low doses which are not anticipated based on the effects observed at higher doses (Vandenberg et al., 2012). The variation in growth effects of EE has been difficult to generalize and no clear patterns between studies have emerged to date.

While less is known about the effects of EDCs on IGF sensitivity, a decrease in IGFR mRNA was observed in target tissues (e.g., gill, red and white muscle, etc.) of sexually maturing fathead minnow treated with E2 (Filby et al., 2006), and a functional decrease in expression of IGFRs was observed in juvenile rainbow trout exposed to E2 (Norbeck et al., 2011). Conversely, aqueous exposure of salmon larvae to PFOA displayed fluctuating IGFR mRNA expression levels across the time course in body tissue, while PFOS treatment was associated with increased levels of IGFRs in the head region (Spachmo and Arukwe, 2012). In this case, contradictory results may correspond to the age of the organism as IGFRs play a vital role in transducing the developmental signals of IGF-2 in many vertebrates.

As EE affect central elements of the GH-IGF system, it is not surprising that E2 has been associated with alterations in GHBP and IGFBP in teleosts and mammals. E2 has been linked to changes in plasma levels of GHBP. Co-treatment of mouse hepatocytes with GH and E2 increased expression of GHBP (Contreras and Talamantes, 1999) and increased plasma levels of GHBP in rats (Carmignac et al., 1993). Similarly, E2 has been shown to stimulate the release of IGFBP (25 kDa and 30 kDa) from hepatocytes in tilapia in a sex-dependent manner (Riley et al., 2004). In humans, E2 has also been observed to increase circulating levels of IGFBP (Paassilta et

al., 2000). It is plausible that EE may alter GHBP and IGFBP expression, and thus the bioavailability of GH and IGF. Taken together, these data support the possibility that EE may in part elicit their negative effects on the GH-IGF system through interactions with the binding proteins.

Some of the observed effects of EDCs on growth are particularly alarming due to recent evidence that the epigenetic effects of EDCs are heritable across multiple generations, thus potentially leading to a host of diseases states (Anway et al., 2005; Anway and Skinner, 2006). A classic example of epigenetic modification by an EDC is that of DES offspring. Pregnant women who took DES were observed to have an abnormally high rate of reproductive tract anomalies and rare cervical cancers. These effects appear to be transmitted to subsequent generations via modifications to DNA methylation patterns (Li et al., 1997; Skinner et al., 2005). In addition to DES, a host of EDCs including phthalates, BPA, DDT, vinclozolin, and methoxychlor have also been observed to be capable of epigenetic modifications (e.g., histone modifications, DNA methylation, DNA acetylation, etc.) (Anway et al., 2005; Dolinoy et al., 2007; Kang and Lee, 2005; Nilsson et al., 2008; Shutoh et al., 2009).

Although the epigenetic effects of EDCs in regards to growth are not well known, there have been studies showing that exposure of oocytes to BPA prior to fertilization leads to retarded growth in the adult trout (Aluru et al., 2010). Additionally, the results of this study found that embryonic GH levels and GHR transcript levels were altered, IGF mRNA and IGFR mRNA expression levels were suppressed in embryogenesis, and plasma glucose and plasma cortisol levels were subsequently altered in the adult trout (Aluru et al., 2010). In mammals, further evidence of epigenetic modification to somatotropic genes was observed in a study by Ren et al. (2001) in which the IGFR gene appeared to be modified by the phytoestrogen daidzein in piglets.

These epigenetic effects may also be indirectly related to effects of EDCs on IGF levels, as IGF-1 has been shown to promote the acetylation of histone H3 and H4 (Sun and D'Ercole, 2006) and regulate methionine synthase (thus regulating the availability of methyl donors) (Waly et al., 2004). While these studies were completed in neural tissue (i.e., cortex, hippocampus, neuroblastoma cell line), the ability of IGF-1 to promote both acetylation and methylation of the genome, and therefore affect expression of the functional IGF-1 protein, may indicate that similar effects in other tissues, specifically those known to play an integral role in growth (e.g., liver), are possible.

There is a delicate balance that exists between the gonadotropic and somatotropic axis when an organism is growing and developing. Generally, hepatic pathways play an important role in regulating the balance between allocating energy towards gonadal versus somatic growth. For example, in several fish species, energy may be diverted from somatic growth to development as evidenced by increased vitellogenin (an egg yolk precursor protein generally only produced by female fish in teleosts) synthesis following exposure to environmental estrogens (Pelissero et al., 1993; Mellanen et al., 1996; Tremblay and Van der Kraak, 1998; Kime et al., 1999; Bowman et al., 2002; Kidd et al., 2007). The differential effects of EDCs on somatotropic genes appear to depend on a host of variables. As the action of GH in the liver is ultimately mediated by IGF-1, 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 actions of EDCs, which are not only acting at numerous levels in the GH-IGF system in teleosts, but also are triggering downstream effects in the peripheral organs to decrease the growth-promoting actions of GH.

Other Vertebrates

While teleosts make an excellent model organism for examining the detrimental effects of EDCs, several studies have used other systems and found comparable results, likely due to the highly conserved nature of the GH-IGF axis among vertebrates. In terms of overall organismal growth, exposure of guppies to the phthalate, DEHP (di-ethyl-hexyl-phthalate) was associated with a decrease in both weight and length compared to the control counterparts (Zanotelli et al., 2009). Following aqueous exposure to coal combustion waste, green frog tadpole and wood frog tadpole growth was not only delayed, but treatment groups were significantly smaller than the control tadpoles at metamorphosis (Snodgrass et al., 2004).

Examining the GH-IGF system at the molecular level in other vertebrates exhibited variable results. Cows fed daidzein had offspring with an increased birth weight and up-regulated IGFR mRNA levels in muscle tissue (Ren et al., 2001). Chicken eggs injected with Arochlor showed increased levels of GH mRNA in the pituitary, whereas no effect was observed on plasma GH and IGF (Gould et al., 1997). In combination, GH and E2 increased GHR mRNA levels in mouse hepatocytes, while no effects were observed with either singular treatment, thus suggesting the two hormones act synergistically to elicit the observed physiological effects (Contreras and Talamantes, 1999). Conversely, BPA has been shown to be able to suppress GHRH-stimulated and basal levels of GH (Katoh et al., 2004). This suppression of GH appears to be in part due to the down-regulation of GHR expression, as was found in seabream hepatocytes (Jiao and Cheng, 2010) and ovine pituitary cells (Katoh et al., 2004) exposed to BPA. Dietary exposure to the phytoestrogen genistein was observed to reduce IGF levels in a dose-dependent manner in transgenic mice (Lamarthiniere et al., 2002). *In vivo* and *in vitro* studies with DDT and its derivatives have shown that DDE can increase the expression of IGF in rats and humans

(Holloway et al., 2007). In addition, DDE may to be linked to diminished IGF-1 levels in boys (Zumbado et al., 2010). As was the case in teleosts, these effects were highly variable and could depend on a number of factors including the sex and age of the organism, the mechanisms responsible for growth control in a particular organism, the route of exposure to the EDC, and the compound and tissue examined.

Indirect growth effects, such as those observed in EDC-exposed progeny have been documented as well. Female rats given octylphenol produced offspring with significantly suppressed growth compared to control rats (Sharpe et al., 1995). Similarly, in humans, PCB and dioxin concentrations in pregnant women's bodies were observed to be negatively correlated to the birth weight of the offspring (Fein et al., 1984). Maternal exposure of ducks to daidzein led to a significant decrease in the mass of ducklings at hatching. At the molecular level, reduced GH mRNA expression in the pituitary, and decreased IGF1R mRNA expression in the liver was observed in the ducklings (Zhao et al., 2004). Paralleling the results in teleosts, effects on overall growth in an organism or its progeny tend to be variable. For example, American alligators hatching in Lake Apopka, a lake in Florida known for its elevated pesticide and agricultural pollution, were observed to have a lower body mass and condition index compared to hatchlings at a clean reference site. However, the alligators from Lake Apopka grew faster and were larger than the controls by 5 months of age (Moore et al., 2012). Additionally, rats exposed to BPA or genistein and mice exposed to DES had offspring that were greater in body weight than the control counterparts (Rubin et al., 2001; Newbold et al., 2004; Newbold et al., 2007).

Additional Endocrine Effects

Several other hormones are well-known for interacting with the GH-IGF system (e.g., cortisol, thyroid hormone, insulin, somatostatin), and thus the effects of EDCs on other hormonal

systems should not be overlooked. Arukwe et al. (2000) showed that NP can penetrate the blood-brain barrier in Atlantic salmon, indicating a possible effect on the hypothalamus-pituitary axis. E2 has been shown to reduce plasma T3 levels in teleosts (Mercure et al., 2001; McCormick et al., 2005), thus it is not surprising that EDCs, in particular estrogen mimetics, may affect the thyroid system. In fact, the effects of EDCs on the thyroid system are well-documented (reviewed in Brown et al., 2004 and Boas et al., 2012) and a few examples are subsequently presented. Arochlor and malathion exposure in Arctic charr and catfish, respectively, has been shown to decrease plasma levels of T4/T3 (Jorgensen et al., 2004; Lal et al., 2013). PCB exposure in the San Francisco Bay area was linked to depressed T4 concentrations in surfperch and sculpin (Brar et al., 2010). Similarly, PCBs were able to reduce plasma thyroid levels in rats (Gauger et al., 2004; Martin and Klassen, 2010), while DDT was able to produce the same effect in sparrows (Scollon et al., 2004). Additionally, in humans urinary phthalate concentrations were negatively associated with thyroid hormones, IGF-1, and childhood growth rates (Boas et al., 2010).

The reproductive system has been well studied in regards to EDCs. For example, rainbow trout treated with NP showed decreased semen quantity and egg survival (Lahnsteiner et al., 2005) and guppies treated with octylphenol had decreased testes growth (Toft and Baatrup, 2001). At the molecular level, the relationship between the GH-IGF system and reproduction in complicated. Fathead minnows exposed to ethinylestradiol showed an increase in gonadal GH expression (Filby et al., 2006). E2 treatment during a pre-reproductive period additionally inhibited IGF-1 and increased expression of IGFBP2 in sea bream ovary. Conversely, during the reproductive stage of life, E2 stimulated IGF-1, IGF-2, and IGFR expression (Gioacchini et al., 2005). These results indicate a possible effect on gametogenesis that is further supported at the

neuroendocrine level. Ethinylestradiol and NP were shown to increase the number of GnRH neurons in zebrafish embryos (Vosges et al., 2010). Additionally, BPA, NP, and E2 decreased GnRH3 neurons in medaka embryos (Lee et al., 2012). As IGF has been observed to regulate GnRH release and expression (Hiney et al., 2004), it is possible that the disruptive effects on the reproductive and growth systems is additive in nature.

While the majority of studies on EDCs and other endocrine systems have focused on reproductive and the thyroid hormones, some research has examined neuroendocrine responses. For example, BPA has been shown to affect NPY expression (Masuo et al., 2004). Additionally, TCDD exposure in rats has been shown to increase NPY, POMC, and CART mRNA (Fetissov et al., 2004). Fetissov et al. (2004) explained that these results may be due to an energy depletion status being activated by TCDD. As appetite and growth are intricately connected, the physiological effects of EDCs on both of these systems may be expected.

Endocrine disrupting compounds have likewise been suggested to affect metabolism, which is closely linked to growth. For example, BPA and DES treatment in pancreatic cells *in vitro* and *in vivo* lead to altered glucose and lipid metabolism (Alonso-Magdalena et al., 2005; Alonso-Magdalena et al., 2006), while malathion has been shown to induce lipolysis in catfish (Lal et al., 2013). Endocrine disrupting compounds such as BPA, DDT, and the organotin TBT (tributyltin chloride) can interfere with metabolism (e.g., adipogenesis) *in vivo* and *in vitro* thus contributing to metabolic diseases such as obesity. The effects of plasticizers, pesticides, pthlatates, dioxins and surfactants on metabolism have been shown to be regulated via interaction with nuclear receptor, peroxisome proliferator-activated receptor (PPAR), activity (Ward et al., 1998; Palut et al., 2001; Baillie-Hamilton, 2002; Grun et al., 2006; Takeuchi et al., 2006; McAllister et al., 2009).

Signaling Mechanisms

The actions of EDCs may occur via alteration to a variety of genomic and nongenomic signaling mechanisms, many of which are poorly understood. Here we will focus on those mechanisms that may be related to xenoestrogens because they are the most studied type of EDC and because several classes of EDCs fall into the xenoestrogen category, thus providing us with a general picture of the biological mechanisms at work. To understand the signaling cascades activated or repressed, we must first understand the types of receptors thought to be responsible for the physiological actions of estrogen.

The estrogen receptor is a type I nuclear receptor that is found in the cytosol of the cell. The ER possesses six functional domains, A through F. The A and B domains have ligand-independent transactivation function AF-1. The C domain is the DNA binding-domain and is involved in dimerization of the ER and its subsequent binding to the ERE. The D domain is the hinge domain, and the E domain is the ligand binding-domain. The function of the F domain remains unclear. ERs have multiple isoforms that are species-dependent. For example, rainbow trout possess both ERα and ERβ, each of which have two different isoforms distributed in a tissue-specific manner, with the highest distribution in tissues such as the liver and testes (Nagler et al., 2007). Generally, upon ligand binding, the ER dissociates from a heat shock protein, forms a homodimer or a heterodimer, translocates to the nucleus, and then binds to the ERE to subsequently activate or repress transcription (e.g., interaction of basal transcriptional machinery, chromatin modification, etc.) of estrogen-responsive genes. Additionally, the E2-ER complex may activate transcription factors such as activator protein-1 (Ap-1), which may then alter transcription (Kushner et al., 2000). Different subtypes and combinations of dimers, as well as

the tissue-specific nature of ERs, may account for the pleiotropic physiological effects of estrogen and certain EDCs.

Endocrine disrupting compounds including PCBS, phthalates, APEs, BPA, and phytoestrogens can bind to the estrogen receptor in mammals and teleosts, albeit with a much lower affinity (Sumpter and Jobling, 1995; Tremblay and Van der Kraak, 1998; Knudsen and Pottinger, 1999; Bonefeld-Jorgensen et al., 2001). For example, ethinylestradiol, zearanolone, genistein, DES, OP, NP, and BPA all induced enzyme activity in a luciferase reporter assay of rainbow trout ER, indicating the binding of these compounds to the receptor and subsequent transcriptional effects (Cosnefroy et al., 2009). While EDCs can bind to the ER, the elucidation of signaling mechanisms is further complicated by the structure of an EDC, as it alone cannot predict if a compound will bind to the ER. Additionally, while the parent EDC may not bind to the ER, metabolites of the parental compound may be capable of directly binding to the ER.

In addition to traditional nuclear responses, EDCs may elicit nongenomic responses in order to induce or inhibit signaling pathways and modulate gene expression (Fig. 2). Interference with the JAK-STAT pathway, which plays a critical role in IGF-1 expression, and/or the Erk and PI3K/Akt pathways, which are necessary for IGFR action, could lend support to the observed effects of EDCs on organismal growth. Nongenomic responses of EDCs may include the inhibition or activation of kinases such as Erk1/Erk2, PI3K, PKC, and PKA (Li et al., 2006). Membrane-bound ER may also activate various signaling cascades and second messengers (Nadal et al., 2000; Watters et al., 2000; Qin et al., 2004; Alonso-Magdalena et al., 2005). For example, coumestrol, NP, endosulfan, DDE, and dieldrin stimulated a rapid membrane-initiated cascade in a prolactinoma cell line known to possess abundant mER (Bulayeva and Watson,

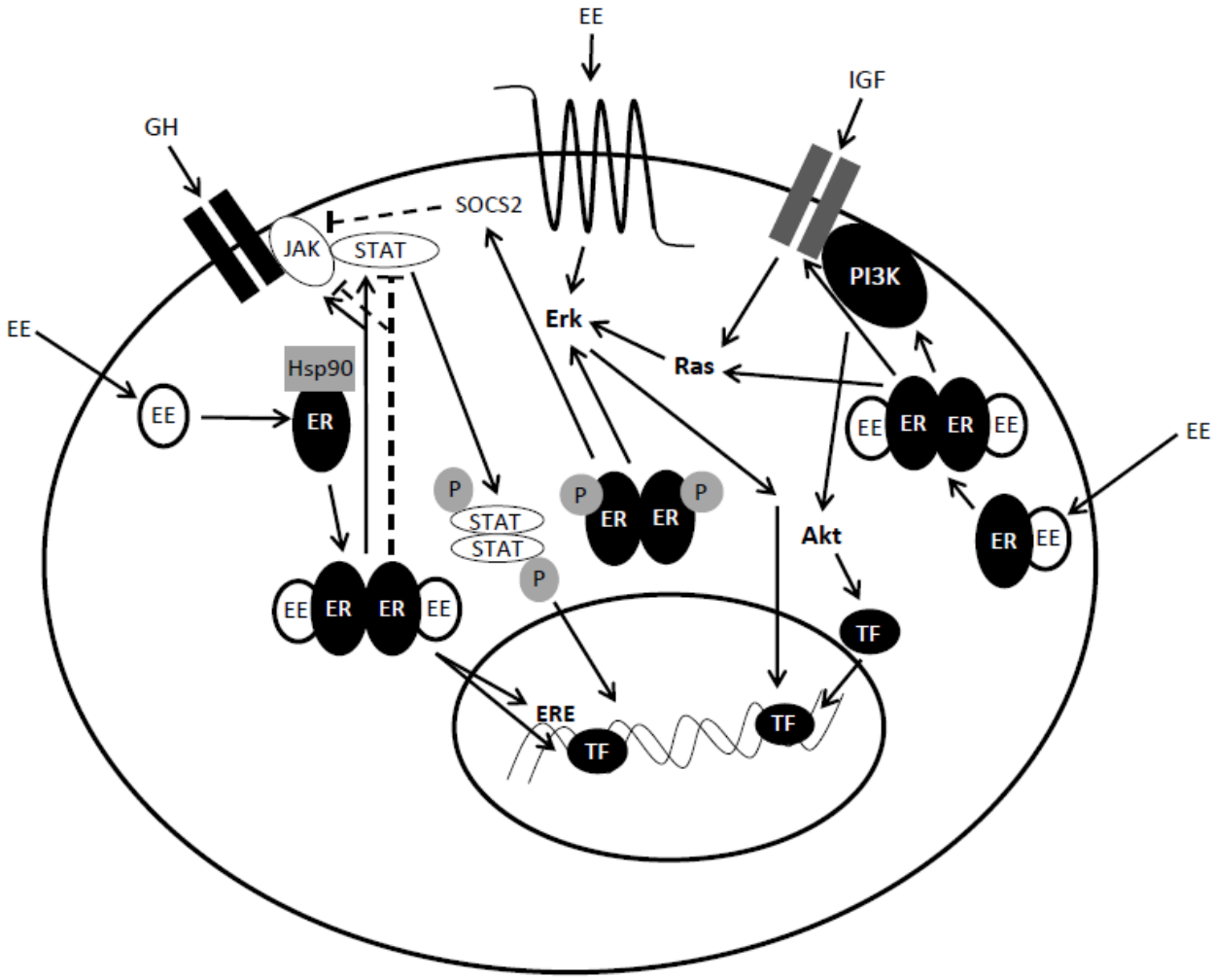


Figure 2. Schematic diagram of possible environmental estrogen signaling mechanisms. As several endocrine disrupting compounds mimic estrogen, these compounds may work via similar mechanisms. Environmental estrogens represented include coumestrol, endosulfan, dieldrin, nonylphenol (NP), octylphenol (OP), bisphenol A (BPA), dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyltrichloroethane (DDT), and polychlorinated biphenyls (PCBs). Environmental estrogens, EE; heat shock protein 90, Hsp90; estrogen receptor, ER; phosphorylated, P; transcription factor, TF; estrogen response element, ERE; growth hormone, GH; insulin-like growth factor, IGF.

2004). While these actions occurred via a mER, EDCs have also been shown to elicit physiological effects (e.g., growth, proliferation) via GPR30 (an orphan G protein-coupled receptor) (Filardo et al., 2000). BPA was observed to stimulate proliferation, growth, and migration of breast cancer cells via the activation of GPR30 (Pupo et al., 2012). Additionally, BPA has been shown to promote cellular proliferation via G-protein coupled receptors and the PKG pathway (Bouskine et al., 2009).

Endocrine disrupting compounds alter signaling cascades in a seemingly time-, concentration-, and compound-dependent manner. Endpoint effects may include reduced cellular proliferation (via reduced aromatase activity), such as was observed in MCF-7 breast cancer cell lines treated with genistein (Brooks and Thompson, 2005). Specific examples of EDCs activating or inhibiting kinases include Erk1/Erk2 phosphorylation, which was activated by NP, but unable to be phosphorylated by treatment with BPA (Bulayeva and Watson, 2004). However, BPA, OP, NP, and E2 rapidly (within 30 minutes) activated ERK1/2 and PI3K in rat pituitary cells (Dang et al., 2009). E2, DES, coumestrol, endosulfan, and BPA can also lead to calcium influx and activation of MAPK (Watson et al., 2007). Additionally, DDT exposure has been observed to increase protein kinase activity (Enan et al., 1998). While results (time and concentration, activation and inhibition) may be variable, it is evident that EDCs can disrupt signaling pathways, thus affecting physiological endpoints such as cellular growth.

The interplay of EDCs and growth-specific signaling pathways is complex. Cytokine receptors, such as GHR, elicit their physiological effects (e.g., growth control, development, etc.) largely in part via the JAK-STAT pathway. DDT and PCBs have been shown to activate receptor tyrosine kinase pathways such as JAK-STAT (Shen and Novak, 1997; Enan et al., 1998). Conversely, E2 was observed to inhibit GH signaling via interactions with JAK2 (Leung et al.,

2004). Similarly, normal GH-induced JAK-STAT activation was inhibited by E2 in a dose-dependent manner in ovariectomized hypophysectomized rats. This appeared to be the result of suppression of GH-induced JAK2 phosphorylation and subsequent reduction in the transcriptional activity of STAT3 and STAT5 in hepatocytes (Murphy and Friesen, 1988).

In order to regulate GH signaling, the suppressors of cytokine signaling (SOCS) family may regulate cytokine receptor action. In mammals, there are eight SOCS family members, SOCS1-7 and cytokine-inducible SH2-containing protein (CISH). The three most studied SOCS family members are SOCS-1, SOCS-2, and SOCS-3. SOCS-1 is responsible for regulating IFN-γ signaling, SOCS-2 regulates growth hormone signaling, and SOCS-3 is important for trophoblast differentiation and fetal erythropoiesis (Ilangumaran et al., 2004). Of interest, SOCS-2 has been implicated as a negative regulator of GHR signaling via binding to tyrosines on GHRs. GH has been observed to up-regulate the expression of SOCS-2 (Tollet-Egnell et al., 1999; Paul et al., 2000; Rico-Bautista et al., 2004). Although the SOCS proteins appear to primarily inhibit cytokine receptor signaling, certain positive regulatory roles have also been demonstrated. For example, SOCS-2 can boost GHR signaling depending on the dose of GH administered (Favre et al., 1999). SOCS-2 may also play a role in IGF-1 signaling, as SOCS-2 has also been shown to bind to IGFRs (Dey et al., 1998).

The previously mentioned inhibition of the JAK-STAT signaling cascade may, in part, be due to E2 stimulation of SOCS-2 expression in hepatocytes (Jelinsky et al., 2003; Leung et al., 2003; Leong et al., 2004). Through the inhibition of JAK2 (Leung et al., 2003), multiple effector pathways may be affected downstream of the GHR. Additionally, E2 may suppress GHR signaling (i.e., JAK2/STAT5) via the activation of PLC (Fernandez et al., 1998). E2 may activate $G_{\alpha q}$ and $G_{\beta\gamma}$ via a mER or GPR30. These subunits subsequently induce PLC activation (Filardo,

2002; Razandi et al., 2003). As estrogen is able to up-regulate SOCS-2, it is reasonable to suggest that EDCs, specifically those mimicking estrogen, may be able to elicit the same response.

Cross-talk with the GH-IGF system may be explained by multiple observations. First, the trout GH gene possesses a putative ERE in its promoter region (Melamed et al., 1998), thus presenting a target for transcriptional regulation. Additionally, direct interaction of the ER with signaling pathways has been evidenced by the co-immunoprecipitation of ERα with different components of the MAPK pathway in MCF-7 cells, thus indicating direct action on Ras/Erk kinases (Migliaccio, et al., 1996). IGF-1 can also phosphorylate ERα, thus activating the receptor and allowing the ER complex to elicit its physiological effects (Lanzino et al., 2008). In mammalian cell lines, cross-talk has been observed between estrogen receptors, IGF, and IGFR (Kahlert et al., 2000; Klotz et al., 2002; Mendez et al., 2006), thus eliciting downstream signaling effects and regulating the expression of proteins involved in IGF-1 signal transduction (Bernard et al., 2006). Further evidence supporting the cross-talk between EDCs and growth pathways was provided by Yu et al. (2012), as E2 was able to rapidly stimulate MAPK signaling (essential for IGF signaling) and induced the phosphorylation of ERα in cells with a functional IGFR versus in those without a functional IGFR.

Other transcription factors may play a role in the genomic effects of EDCs on growth. The E2-ER complex can interact with AP-1 signaling. Jun and Fos family proteins dimerize to bind AP-1sites, then recruit coactivators such as p300/CBP to bind to the ER. AP-1 activity can be affected by EDCs including arochlor and DDT (Shekhar et al., 1997; Frigo et al., 2002), thus leading to potential down-stream effects on coactivators, corepressors, and transcription. Other transcription factors such as AhR, Kruppel-like factor-6, Sp1, Sp3, MSY-1, p53, and various

STATs may also be involved in the interplay of EDC and growth signaling (Schwartzbauer et al., 1998; Schwartzbauer and Menon, 1998; Wang and Jiang, 2005; Yu et al., 1999; Wood et al., 2005; Elango et al., 2006; Sarfstein et al., 2006), as the promoter regions of genes encoding GHR, IGF, and IGFR have been shown to be capable of binding to a variety of transcription factors.

As many of the signaling pathways involved in growth and estrogen signaling converge, the effects of genomic and nongenomic signaling may be additive. Further complicating the elucidation of the cross-talk between these pathways is that many of these studies have investigated effects of EDCs using immortal cancer cell lines, which undergo growth and proliferation at an accelerated rate, and thus may not accurately portray the mechanisms at work. Additional problems arise in generalizing the response(s) of cells to EDCS, as many of these response(s) appear to follow a non-traditional dose-response curve (Watson et al., 2007) and seem to be both tissue- and compound-dependent.

Environmental Factors

While internal factors play a critical role in the response of an organism to EDCs, there are other environmental factors that may influence the response of an organism to low-level, chronic exposure to EDCs in terms of growth. In teleosts, GH levels are affected by external factors such as fasting, water temperature, and day length. Generally, increased temperature and extended photoperiod both tend to elevate growth in teleosts. Estrogen-responsive genes (e.g., ERα, ERβ, vitellogenin) are also sensitive to temperature and photoperiod. Specifically, Jin et al., (2011) found that exposure to ethinylestradiol and NP at different temperatures and photoperiods led to a significantly altered response in estrogen-responsive genes in medaka.

In addition, processes that rely on environmental conditions, such as osmoregulation, may be affected by EDCs. During osmoregulation, mRNA expression levels and plasma levels of GH and IGF-1 increase following salt water exposure (Sakamoto et al., 1993; McCormick et al., 2001; Agustsson et al., 2001; Shepherd et al., 2005; Nilsson et al., 2008). Additionally, heightened sensitivity to GH and IGF has been observed (Poppinga et al., 2007). The suggestion that EDCs may affect osmoregulation is not surprising given the close relationship between the GH-IGF system and its role in salt water readiness and hypoosmoregulatory ability (Sakamoto and McCormick, 2006). Previous studies have found that both NP and E2 reduced salinity tolerance of Atlantic salmon, (Madsen et al., 2004, McCormick et al., 2005; Lerner et al., 2007; Lerner et al., 2012), and sea bream (Carrera et al., 2007; Mancera et al., 2007) via disruption of the GH-IGF system (e.g., depressed IGF-1 mRNA, reduced GH binding capacity).

Conclusion

Endocrine disrupting compounds can affect growth at multiple levels and generally appear to act in a tissue-and compound-dependent manner. While the effects on GH and IGF tend to parallel each other, these effects also seem to be highly variable. Moreover, signaling mechanisms evoked tend to be variable in response perhaps due to the diversity of ERs (i.e., mER, GPR30, ERα, ERβ). Further complicating matters is that responses appear to be related to stage of life, sex, environmental factors, etc. Age of an organism may also make a major difference in the effects of EDCs, as liver detoxification, DNA repair mechanisms, the immune system, and the blood brain barrier are not fully developed at the fetal/neonatal stage of life (Bern, 1992). Additionally, exposure to combinations of EDCs may dramatically affect cellular function as these mixtures may be 1000 times more potent than singularly acting EDCs (Arnold et al., 1996; Benachour et al., 2007). Even though regulatory agencies may restrict or ban the use

of these compounds, many are persistent in the ecosystem at chronically low levels. Expanded research of EDCs is critical in order to fully assess the threat posed to all organisms.

Objectives

In a world with rapid technological advancements, we not only reap the benefits of these advancements, but are also faced with the consequences of our actions in terms of by-products and waste. Global production of EDCs has reached a staggering 400 million tons per year. While much of the research to date has focused on the reproductive and developmental consequences of EDCs, less attention has been directed towards how these compounds affect growth. There has been considerable research on the maintenance and modulation of growth and increasing evidence suggests that the regulation of growth occurs at several levels. The hypothesis of this dissertation is that EE modulate growth via the GH-IGF axis. Specifically, we will examine the following: 1) assess the actions of EE on osmoregulatory ability 2) examine the effects of EE on growth at the organismal and molecular level *in vivo* 2) assess the actions of EE on GH production, IGF-1 production, and IGF-1 responsiveness *in vitro,* and 4) elucidate the influence of EE on growth-related intracellular signaling pathways. The long-term impact of EE on population and community structure via epigenetic and transgenerational effects is not well understood. Moreover, virtually nothing has been reported regarding the effects of EE on organismal growth. In this project, rainbow trout will be used as a model system to evaluate the impact of EE on animal health as assessed by organismal growth. The findings of this project will establish whether or not these agents act as endocrine disruptors of growth in a wide-ranging aquatic species. While this work pertains specifically to rainbow trout, because the elements of the GH-IGF-1 system are highly conserved among vertebrates, the findings will have broad relevance to understanding the impact of EE in other aquatic and terrestrial species. Ultimately,

this work will inform policies and practices aimed at reducing the impact of EE on aquatic habitats and on the health of wildlife and humans.

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CHAPTER 1: 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*)

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 habitat over recent time may compromise hypoosmoregulatory ability of fish. In this study, we used rainbow trout (*Oncorhynchus mykiss)* to assess the effects of EE on the GH-IGF system and seawater adaptation. Juvenile trout (ca. 30 g) were exposed to either low (10 μg/l) or high (100 μg/l) concentrations of β-sitosterol (βS), 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, then declined to initial levels after 48 h. By contrast, plasma chloride levels in all EE-treated groups except βS low increased and remained elevated over initial levels after 48 h. Levels GH receptor 1(GHR1), GHR2, insulin-like growth factor-1 (IGF-1), and IGF-2 mRNAs in liver of control fish increased 6-12 h after SW exposure. In gill, levels of GHR1, GHR2, IGF-1, IGF-2, IGF receptor 1A (IGFR1A) and IGFR1B mRNAs increased in control fish 6-12 h after 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 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 βS, NP, and E2 abolished or attenuated normal salinity-

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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.

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 Clairborne, 2009). Considerable research has shown that SW adaptability of euryhaline fish is influenced by numerous hormones, including growth hormone (GH), insulin-like growth factor-1 (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 GH receptors (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 (β-sitosterol), mycotoxins (e.g., zearalenone), organochlorine pesticides (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 (βS) 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 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.

Materials and Methods

Experimental 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 ºC 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.

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 17β-estradiol (10 μg/l and 100 μg/l), two doses of 4-nonylphenol (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 was recommenced (1% body weight once per day) and continued throughout the FW exposure period; however; feeding was suspended 24 h prior to SW 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 SW (Instant Ocean, Aquarium Systems Inc., Mentor, OH) to achieve a final concentration of 20 ‰ (w/v). The replacement 20 ‰ SW did not contain estrogen 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 ºC 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 ºC.

Plasma Chloride

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

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 GHR1, GHR2, 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 2X 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). Primer and probe sets for each of the genes are listed in Table 1. Cycling parameters were set as follows: 95° C for 10 min, and 50 cycles of 92° C for 15 s plus 58° C for1 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.

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.

Results

Body Characteristics

The effects of EE on the body characteristics of rainbow trout following a 28-day exposure in FW are shown in Table 2. 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 βS ($p = 0.09$)-treated fish and the significant ($p < 0.05$) decrease in condition observed in NP-treated fish. The hepatosomatic index (HSI) of all environmental estrogen-treated fish, except those treated with the high dose of βS, was significantly higher than that of control-treated fish.

Plasma Chloride

The effects of EE on plasma chloride levels of rainbow trout during exposure to 20‰ SW are shown in Fig. 3. 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 SW challenge versus initial chloride levels before exposure to estrogenic compounds, which were 130±1.2 mM; significant only for E2, $p < 0.05$). Chloride levels in control-treated fish increased following 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 estrogen-treated fish, except those treated with the low dose of βS, increased following 20 ‰ SW exposure and remained elevated.

GH-IGF System

The direct exposure of rainbow trout to 20‰ SW resulted in significant increases in steady-state levels of mRNA encoding GHRs in liver. Two GHR-encoding distinct mRNAs were expressed in the liver of trout. The greatest effect of SW exposure was on the abundance of GHR1 mRNAs, which increased 287% 12 h after exposure; afterward, mRNA abundance declined substantially, but remained elevated compared to levels observed at 0 h (Fig. 4B).

Table 1. Primer and probe sets for real-time PCR

Transcript	Primer/Probe	Sequence
GHR1	Forward Primer	5'-TGAACGTTTTTGGTTGTGGTCTA-3'
	Reverse Primer	5'-CGCTCGTCTCGGCTGAAG-3'
	Probe	5'^{FAM}-CAAATGCAAGGATTCC-^{MGBNFQ}3'
GHR2	Forward Primer	5'-CATGGCAACTTCCCACATTCT-3'
	Reverse Primer	5'-GCTCCTGCGACACAACTGTTAG-3'
	Probe	5'^{FAM}-TTCATTTGCCTTCTCC-^{MGBNFQ}3
IGF-1	Forward Primer	5'-GTGGACACGCTGCAGTTTGT-3'
	Reverse Primer	5'-CATACCCCGTTGGTTTACTGAAA-3'
	Probe	5'^{FAM}-AAAGCCTCTCTCTCCA-^{MGBNFQ}3
IGF-2	Forward Primer	5'-ACGTGTCGGCCACCTCTCTA-3'
	Reverse Primer	5'-TGGGACATCCTGTTTGATTGTG-3'
	Probe	5'^{FAM}-AGATCATTCCCATGGTGC-^{MGBNFQ}3'
IGFR1A	Forward Primer	5'-AGAGAACACATCCAGCCAGGTT-3'
	Reverse Primer	5'-TCCTGCCATCTGGATCATCTT-3'
	Probe	5'^{FAM}-TGCCCCCGCTGAA-^{MGBNFQ}3'
IGFR1B	Forward Primer	5'-CCTGAGGTCACTACGGGCTAAA-3'
	Reverse Primer	5'-TCAGAGGAGGGAGGTTGAGACT-3'
	Probe	5'^{FAM}-ATCCGTCCCAGTCCT-^{MGBNFQ}3'
β-actin	Forward Primer	5'-GGCTTCTCTCTCTCCACCTTCCA-3'
	Reverse Primer	5'-AGGGACCAGACTCGTCGTAACTC-3'
	Probe	5'^{VIC}-TGCTTGCTGATCCACAT-^{MGBNFQ}3'

Abbreviations: GHR, growth hormone receptor; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor

Table 2. Body characteristics of rainbow trout exposed to environmental estrogens for 28 days in fresh water.*

		After 28-day exposure						
Characteristic	Initial	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 ± 3.20^{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 ± 0.04^{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] x 100

‡Calculated as (liver weight/body length) x 100

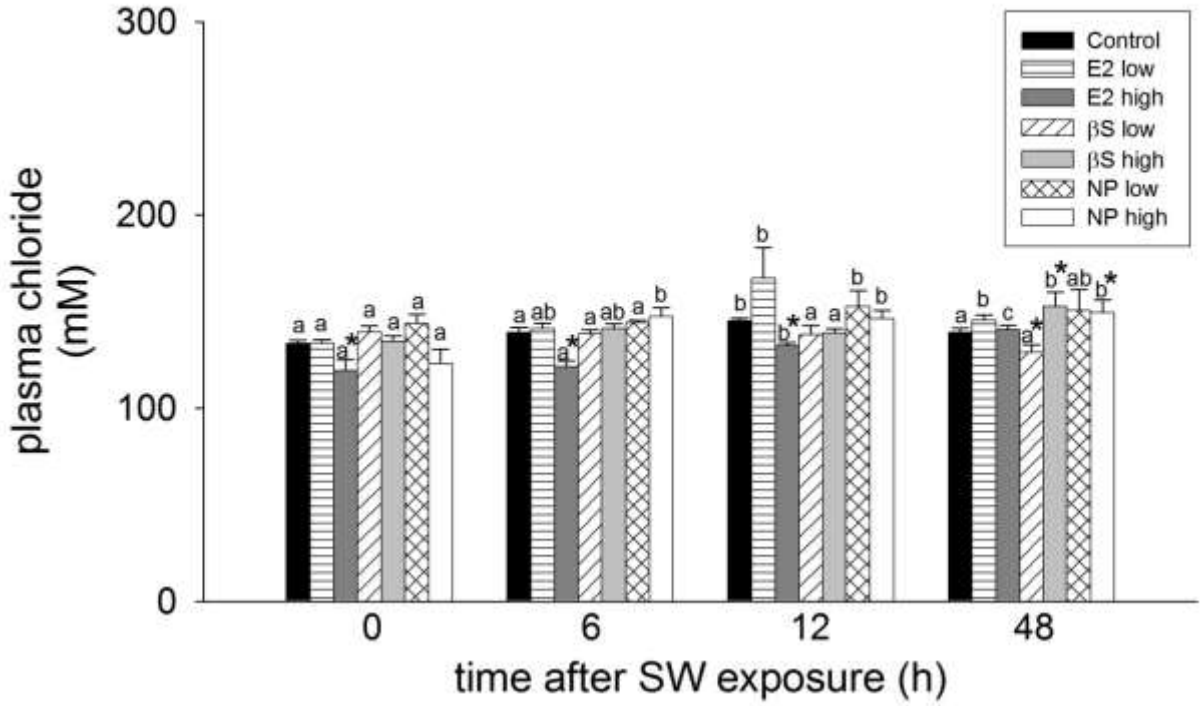

Figure 3. Effects of environmental estrogens (EE) on plasma chloride concentration in rainbow trout during acclimation to 20‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) in fresh water for 28 days, 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.

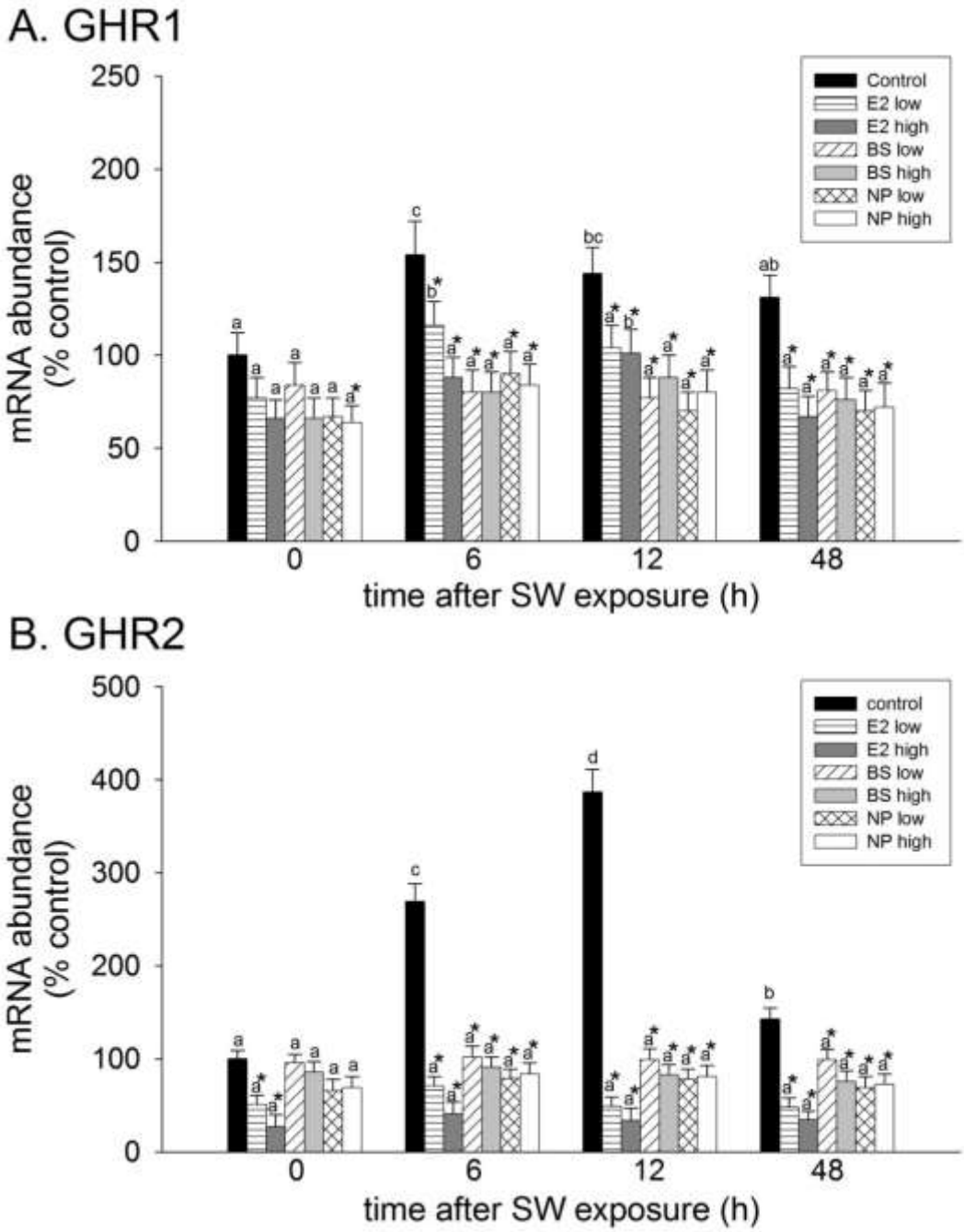

Figure 4. Effects of environmental estrogens (EE) on the abundance of hepatic (A) growth hormone receptor 1 (GHR1) and (B) GHR2 mRNAs in rainbow trout during acclimation to 20 ‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) 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.

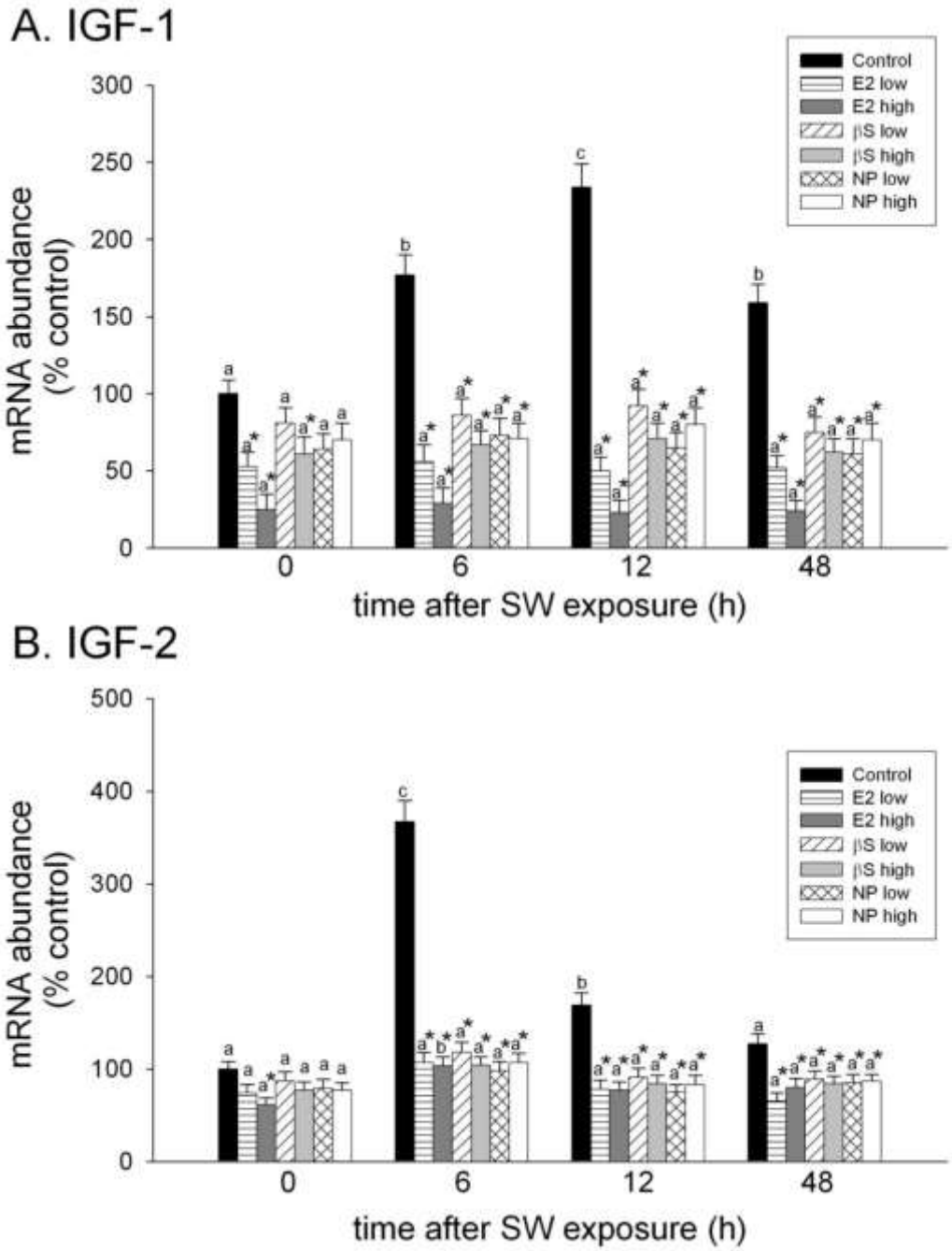

Figure 5. Effects of environmental estrogens (EE) on the abundance of hepatic (A) insulin-like growth factor-1 (IGF-1) and (B) IGF-2 mRNAs in rainbow trout during acclimation to 20 ‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) 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.

Seawater exposure also increased expression of GHR2 mRNA, which increased to maximum levels 6 h after exposure, then declined such that levels at 48 h were similar to those at 0 h (Fig. 4A). Exposure to 20 ‰ SW similarly stimulated hepatic mRNA expression of IGF-1 and IGF-2. Expression of IGF-1 mRNA increased a maximum of 134% 12 h after SW exposure, then declined (Fig. 5A). Expression of IGF-2 mRNAs increased abruptly 6 h after exposure, then levels declined (Fig. 5B).

Notably, exposure to some of the EE depressed expression of GHR and IGF mRNAs such that their levels at 0 h were lower than those observed in control fish at 0 h; such depression was particularly pronounced with E2 and NP treatment (Fig. 4 and Fig. 5). Uniformly, exposure to all of the EE, E2, NP and βS, suppressed or abolished the normal SW-associated increases in the expression of hepatic GHR and IGF mRNAs. In particular, GHR1 mRNA levels rose following SW exposure in E2-treated fish, but the extent of the increases was significantly attenuated compared to those control fish (Fig 4A). Salinity-associated increases in levels of GHR2, IGF-1 and IGF-2 were completely abolished by estrogen treatment (Fig. 4B and Fig. 5).

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. GHR1 mRNA expression increased by 76% after 6 h, whereas GHR2 mRNA expression increased by 144% after 12 h; levels of both GHR mRNAs declined below maximal levels 48 h after exposure (Fig. 6). Exposure to 20 ‰ SW exposure also stimulated expression of IGF mRNAs in gill. IGF-1 mRNA increased to peak levels 12 h after SW exposure, then declined (Fig. 7A). Expression of IGF-2 mRNA also reached maximum levels 12 h after exposure, increasing 46%; after which, levels subsided (Fig. 7B). Two types 1 IGF receptors,

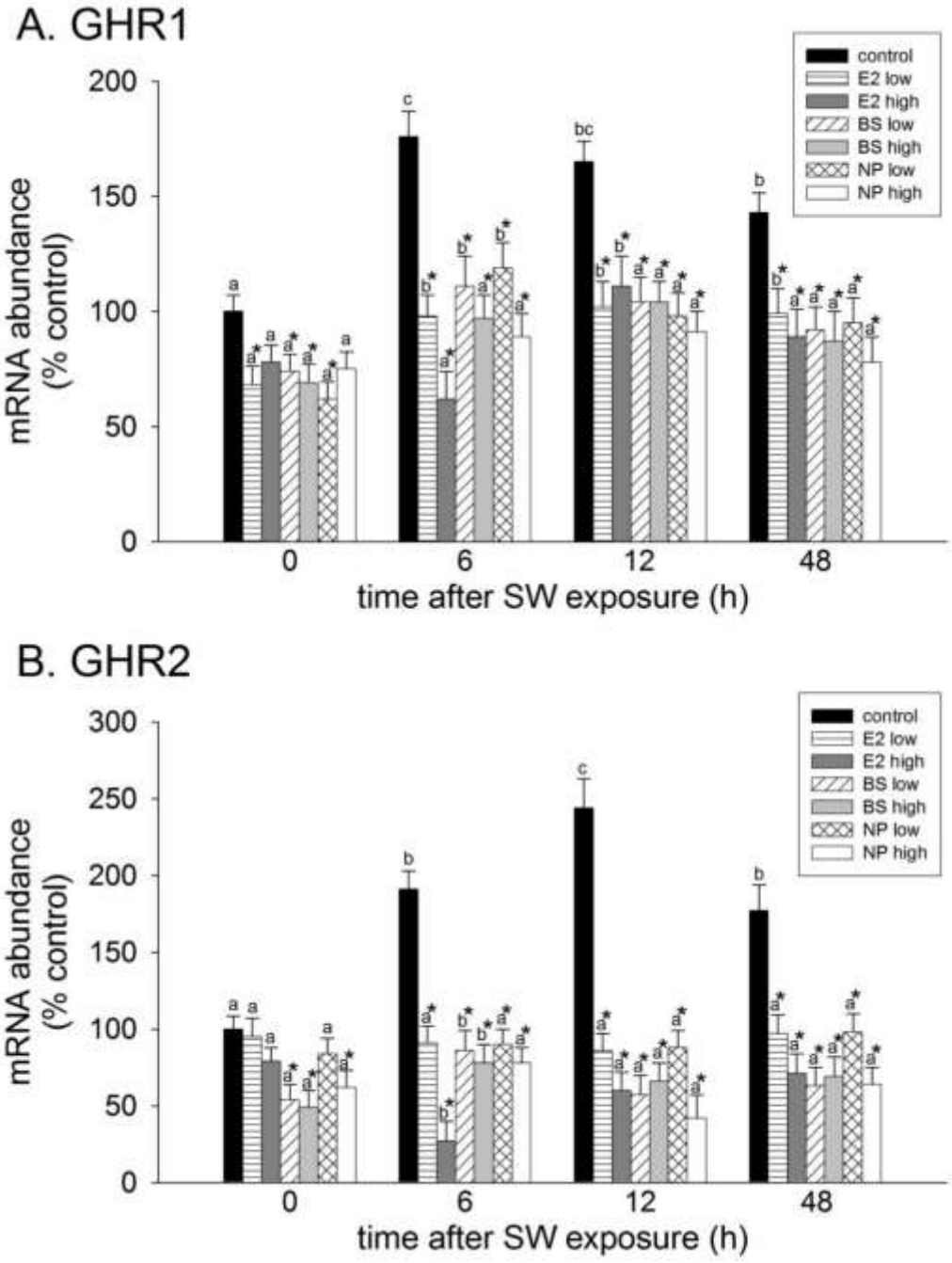

Figure 6. Effects of environmental estrogens (EE) on the abundance of (A) growth hormone receptor 1 (GHR1) and (B) GHR2 mRNAs in gill filaments of rainbow trout during acclimation to 20 ‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) 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.

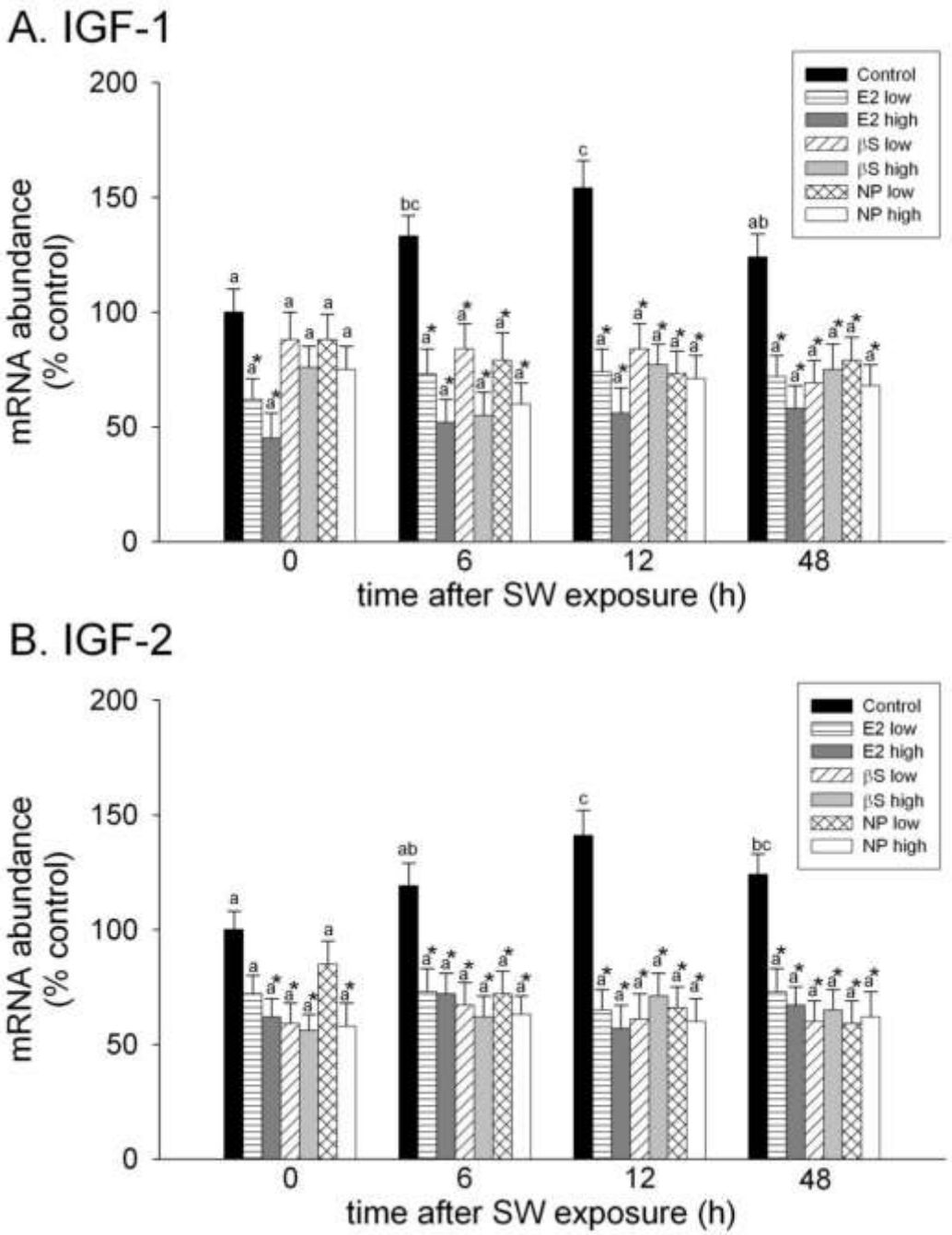

Figure 7. Effects of environmental estrogens (EE) on the abundance of (A) insulin-like growth factor-1 (IGF-1) and (B) IGF-2 mRNAs in gill filaments of rainbow trout during acclimation to 20 ‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) 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.

IFGR1A and IGFR1B, were expressed in gill. mRNA levels of both IGFR1s increased following SW exposure. Expression of IGFR1A mRNA increased a maximum of 65% 12 h after exposure, whereas levels of IGFR1B increased a maximum of 56% 6 h after exposure (Fig. 8). After 48 h of SW exposure, levels of both IGFR1 mRNAs declined to values similar to those observe 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 levels at 0 h were lower than those observed in control fish at 0 h. Such depression was particularly pronounced on the expression of GHR1, GHR2, IGF-2, and IGFR1B mRNAs, although there was some variation depending on the specific estrogenic compound (Fig. 6, Fig. 7, and Fig. 8). 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 GHR1 and GHR2 mRNAs increased following 6-12 h of SW exposure in many of the estrogen-treated fish, but the extent of the increases was significantly attenuated compared to those of control fish (Fig. 6). Notably, GHR2 expression in fish treated with the high dose of E2 was lower than that in control fish (Fig. 6B). Salinity-associated increases in levels of IGF-1, IGF-2, IGFR1A, and IGFR1B mRNAs were completely blocked by estrogen treatment (Fig. 7 and Fig. 8).

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 IGFR1 mRNAs increased in white muscle following SW exposure. IGFR1A mRNA expression levels increased a maximum of 47% 6 h after exposure, whereas expression of IGFR1B mRNA increased a maximum of 127% 6 h after exposure (Fig. 9). Expression of IGFR1 mRNAs also increased in red muscle following 20 ‰

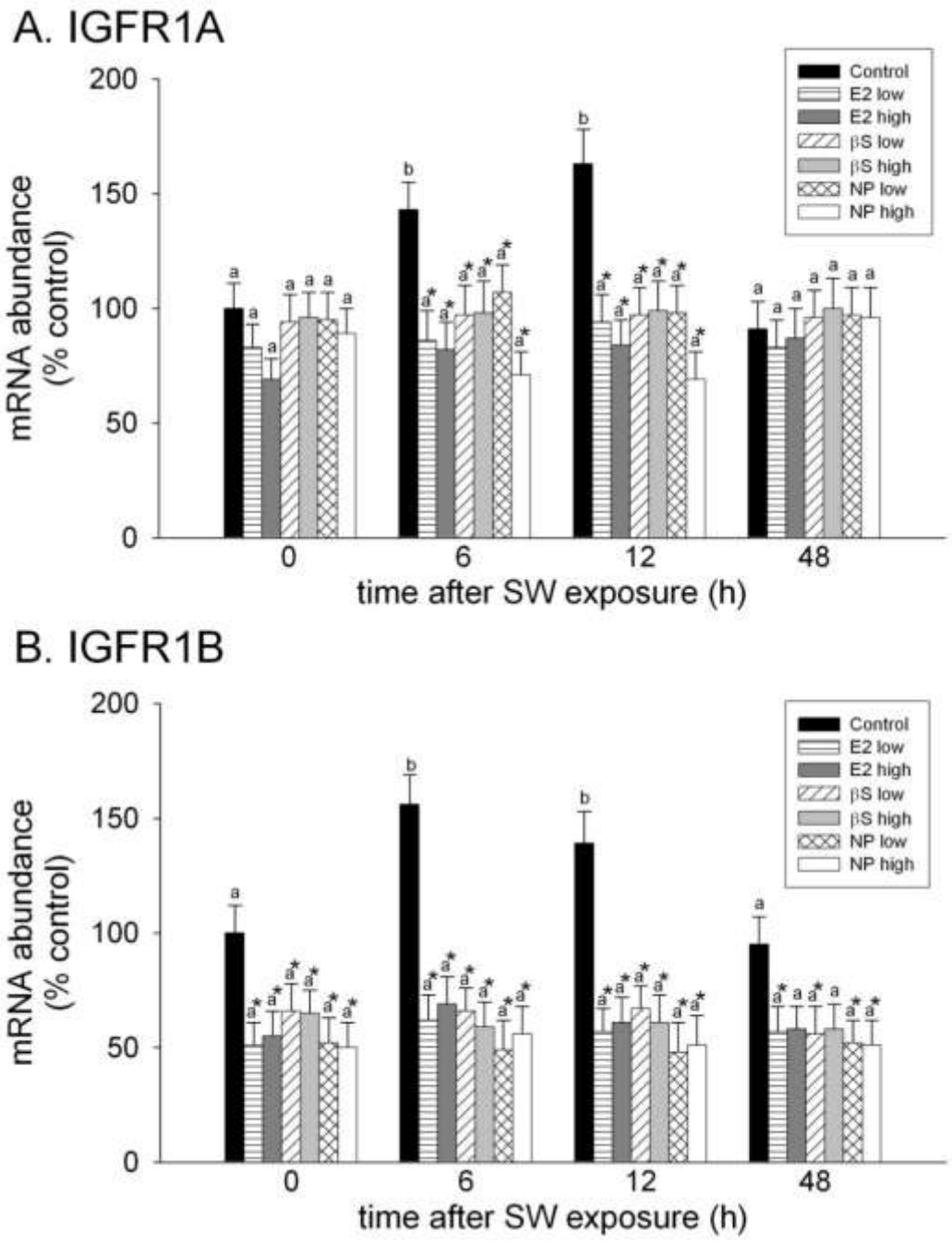

Figure 8. Effects of environmental estrogens (EE) on the abundance of (A) IGF receptor 1A (IGFR1A) and (B) IGFR1B mRNAs in gill filaments of rainbow trout during acclimation to 20 ‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) 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.

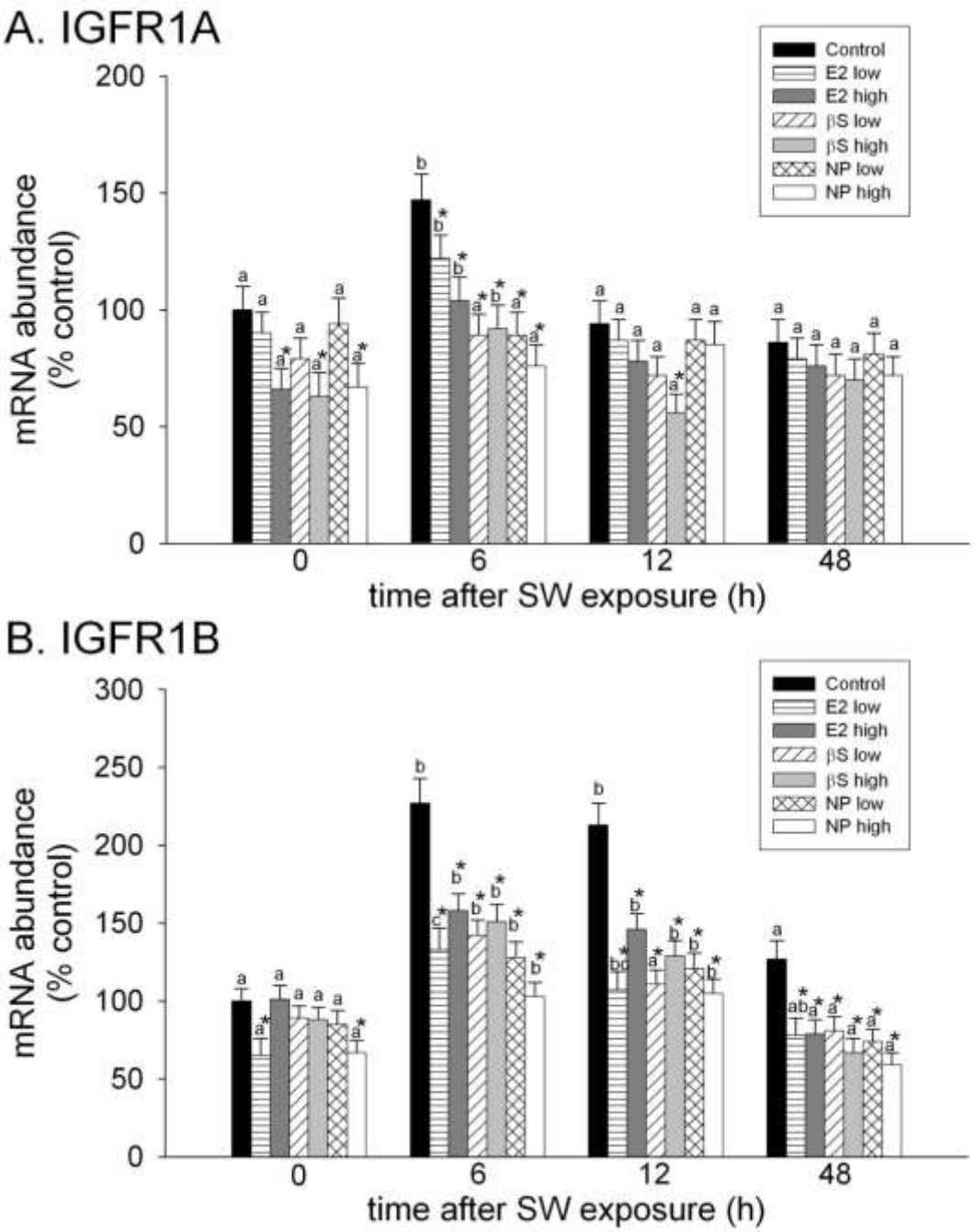

Figure 9. Effects of environmental estrogens (EE) on the abundance of (A) IGF receptor 1A (IGFR1A) and (B) IGFR1B mRNAs in white muscle of rainbow trout during acclimation to 20 ‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) 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.

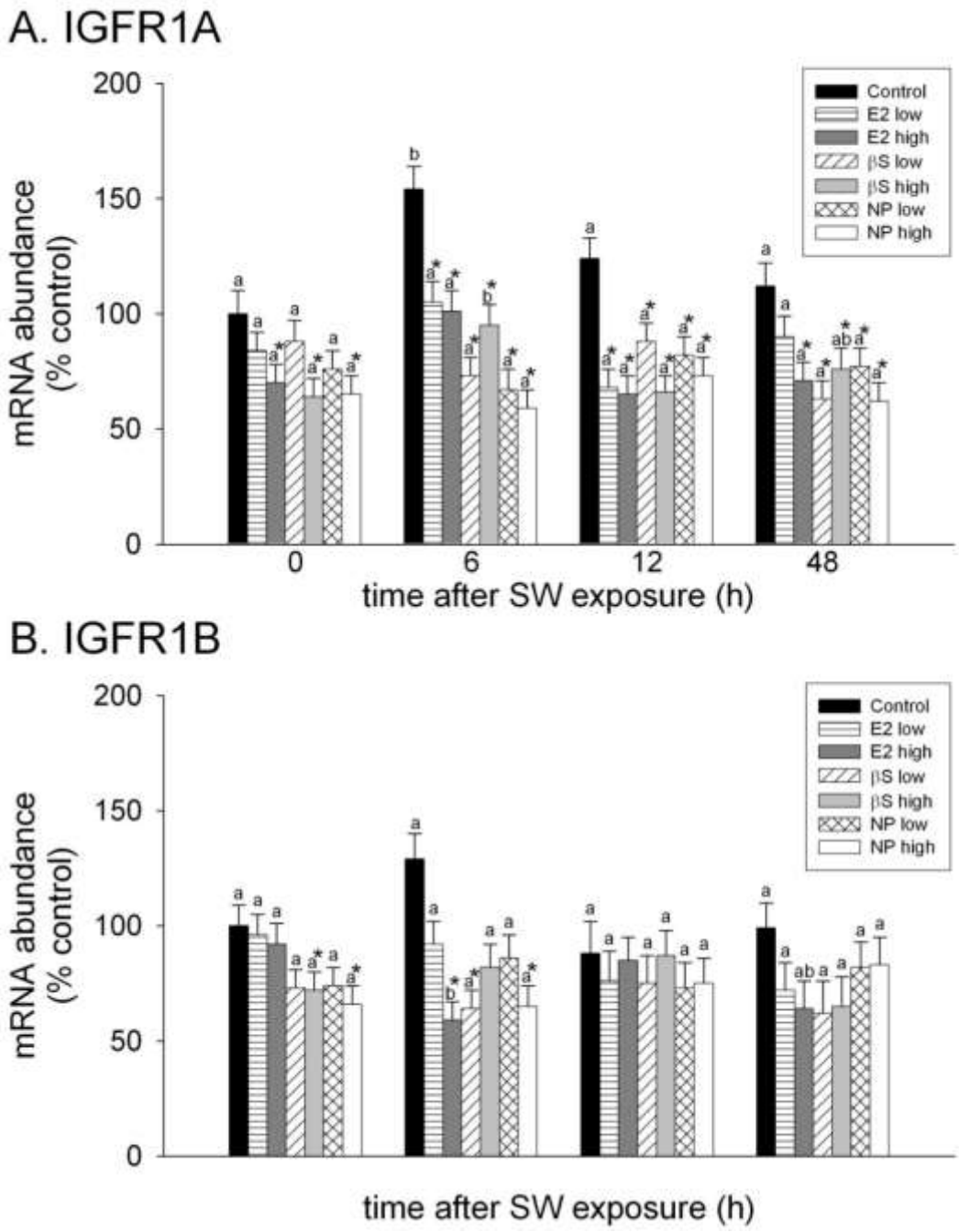

Figure 10. Effects of environmental estrogens (EE) on the abundance of (A) IGF receptor 1A (IGFR1A) and (B) IGFR1B mRNAs in red muscle of rainbow trout during acclimation to 20 ‰ seawater (SW). Fish were exposed to either a low (10 µg/l) or high (100 µg/l) dose of 17β-estradiol (E2), 4-nonylphenol (NP), or β-sitosterol (βS) 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.

SW exposure; however, only the change in IGFR1A levels was significant, which displayed a maximum increase of 54% 6 h after SW exposure (Fig. 10). 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.

Environmental estrogens tended to depress the mRNA expression of IGFR1s in red and white muscle in manner similar to that observed in gill such that mRNA levels at 0 h were lower than those observed in control fish at 0 h. Such depression was somewhat variable and depended on the specific estrogenic compound and its concentration (Fig. 9 and Fig. 10). Also similar to the observation in gill, exposure to all of EE attenuated or blocked normal salinity-associated increases in IGFR1 expression (Fig. 9 and Fig. 10).

Discussion

The results of this study indicate that SW acclimation of rainbow trout involves transient increases in the expression of GHRs, IGFs (both IGF-1 and IGF-2), and type I IGFRs. 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 SW 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 observation is unclear. Distinct roles for GHR and IGFR subtypes in fish are suggested by their differential pattern of expression in embryos and adults, 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 βS found in the environment (Blackburn and Wadlock, 1995; Cook et al., 1996; Hale et al., 2000). This was evidenced by several observations. First, initial mRNA levels of several GH-IGF system components (e.g., hepatic GHR2, hepatic IGF-1, gill GHR1 and GHR2, 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 all of the EE used in the study attenuated or abolished the normal SW-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 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, 2μg/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 βS-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 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; 291 Lerner et al., 2007a, b). 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). Carrera et al. (2007) found similar decreased salinity tolerance in gilthead sea bream when treated with NP. 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 toward 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 306 al., 2004; Lerner et al., 2007a; Shved et al., 2008). The current study also revealed that E2-, NP-,and βS-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, Tremblay and Van Der Kraak, 1999). Taken together, these data indicate 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 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. E2, NP, and βS bind to estrogens 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 β-sitosterol and NP 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 327 pathways (e.g., JAK-STAT, ERK, 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 GHR1, GHR2, IGF-1, IGF-1, IGFR1A, and IGFR1B, accompany adaptation of rainbow trout to SW. The results also indicate that exposure of rainbow trout to environmental estrogen suppresses expression of GH-IGF systems components and leads to reduced growth and reduced hypoosmoregulatory ability.

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CHAPTER 2: ENVIRONMENTAL ESTROGENS INHIBIT GROWTH OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) BY MODULATING THE GROWTH HORMONE INSULIN-LIKE GROWTH FACTOR SYSTEM *IN VIVO*

Abstract

The increasing production, use, and disposal of an expanding array of chemicals that enter the environment and mimic the action of estrogen pose a serious threat to terrestrial and aquatic animals as well as to humans. Although such compounds, referred to as environmental estrogens (EE), have been found to disrupt a wide variety of developmental and reproductive processes in vertebrates, there is a paucity of information concerning their effects on organismal growth, particularly postembryonic growth. In this study, we exposed juvenile rainbow trout (*Oncorhynchus mykiss*) *in vivo* to 17β-estradiol (E2), β-sitosterol (βS), or 4-*n*-nonylphenol (NP) to assess the effects of EE on overall organismal growth and on the growth hormone-insulin-like-growth factor (GH-IGF) system. EE treatment significantly reduced food conversion, body condition, and body growth. EE-inhibited growth resulted from alterations in peripheral elements of the GH-IGF system, which includes multiple GH receptors (GHRs), IGFs, and IGF receptors (IGFRs). In general, E2, βS, and NP reduced the expression of GHRs, IGFs, and IGFRs; however, the effects varied in an EE-, tissue-, element type-specific manner. These findings indicate that EEs disrupt post-embryonic growth by reducing GH sensitivity, IGF production, and IGF sensitivity.

Introduction

Over the past few decades, the increased production, use, and disposal of an expanding array of chemicals has led to increased introduction of these compounds into the environment through

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the air, soil, and water. Increasing research shows the deleterious effects of environmental contamination on aquatic and terrestrial plants, fish, amphibians, birds, and mammals (Hester & Harrison, 1998), including disruption of the endocrine system. Many endocrine disrupting compounds (EDCs) have been found to mimic the effects of endogenous estrogen. These compounds are typically called environmental estrogens (EEs) and include endogenous and synthetic animal estrogens (e.g., 17β-estradiol), phytoestrogens (β-sitosterol), mycotoxins (e.g., zearalenone), organochlorine pesticides (e.g., DDT), polychlorinated biphenyls (PCBs), and alkylphenol polyethoxylates (APEs; e.g., 4-*n*-nonylphenol).

The detrimental effects of EDCs can be observed at the organismal and molecular level and include developmental and reproductive effects (e.g., sex reversal, sterility, altered reproductive timing and behavior) that result in reduced fecundity (Kime et al., 1998). Several studies have demonstrated increased vitellogenin (an egg yolk precursor protein generally only produced by female fish in teleosts) synthesis in both males and females exposed to EEs, including 17β-estradiol, wood-derived estrogens, and ethinylestradiol (Kidd et al., 2007; Mellanen et al., 1996; Tremblay and Van der Kraak, 1998). Similarly, 4-*n*-nonylphenol (at low concentrations) has been shown to inhibit testicular growth in rainbow trout (Jobling et al., 1996), while phytosterols have been linked as potential causative agents in reproductive dysfunction in both rainbow trout and goldfish (MacLatchy and Van der Kraak, 1995; Tremblay and Van der Kraak, 1998).

Other physiological effects of EDCs include effects on behavior, metabolism, and osmoregulation. For example, benzodiazepines, a group of persistent psychotherapeutic drugs, have been exhibited to alter the behavior of European perch, leading to more active, less social, and bolder fish (Brodin et al., 2013). It has been hypothesized that EDCs such as bisphenol A and DDT can interfere with metabolism (e.g., adipogenesis) thus contributing to metabolic

diseases such as obesity (Baillie-Hamilton, 2002; McAllister et al., 2009). Additionally, several studies have demonstrated that 4-*n*-nonylphenol exposure can reduce salinity tolerance of Atlantic salmon, rainbow trout, and gilthead sea bream (Carrera et al., 2007; Hanson et al., 2012; Lerner et al., 2007; Madsen et al., 2004; McCormick et al., 2005). Despite the fact that many EDCs are found at levels below the legal limit, the potential long-term impact of chronic exposure to low levels is not clear, especially given that EE are rapidly transferred through the food web, accumulate in tissues, and can have epigenetic effects (Baccarelli and Bollati, 2009; Phillips and Harrison, 1999; Skinner et al., 2010).

The maintenance and modulation of animal growth involves the coordination of external and internal cues that are transduced into chemical mediation that result in a host of physiological responses. The growth hormone (GH)-insulin-like growth factor-1 (IGF-1) system plays an integral role in the control of growth in vertebrates. GH, secreted chiefly from the pituitary, circulates throughout the body to stimulate the synthesis and secretion of IGF-1, primarily from the liver (Butler and LeRoith, 2001; Reindl and Sheridan, 2012; Wood et al., 2005). IGF-1 then acts on target cells to elicit effects such as cellular growth, proliferation, and differentiation (Butler and LeRoith, 2001; Reindl and Sheridan, 2012; Wood et al., 2005). Additionally, IGF-1 may have growth-modulating effects that are independent of GH (Duan and Xu, 2005). The GH-IGF-1 system has also been shown to play an important role in other physiological processes including energy metabolism, reproduction, and osmoregulation.

Despite extensive knowledge of the effects of EE on developmental and reproductive processes, very little is known about the effects of EE on organismal growth, particularly post-embryonic growth. This study used rainbow trout as a model to examine the effects of EE on growth because of their well-characterized GH-IGF-1 system (Reindl and Sheridan, 2012) and

because of their wide geographic distribution in aquatic habitats. Specifically, the aim of the present study was to elucidate the role of EEs, specifically 17β-estradiol (E2), β-sitosterol (βS), and 4-*n*-nonylphenol (NP), on post-embryonic organismal growth and to determine if the effects of EE are mediated at the level of the GH-IGF system.

Materials and Methods

Experimental 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. All procedures performed were in accordance with the *Guide for Care and Use of Laboratory Animals* (National Research Council, Washington, DC) and approved by the North Dakota State University Institutional Animal Care and Use Committee.

Experimental Conditions

To examine overall organismal growth, fish (ca. 50 g) were anesthetized with 0.05% (v/v) 2-phenoxyethanol, measured, weighed, and tagged with Visible Implant Elastomer (VIE) tags (Northwest Marine Technology, Inc., Turnwater, WA). Fish were transferred to 40-l glass aquaria (10 fish per tank) containing only fresh water. Feeding was recommenced 24 h after transfer to aquaria (2% body weight once per day) and continued throughout the 28-d exposure period; however, feeding was suspended 24 h prior to final sampling. Fish were allowed to recover for 48 h before EE were added to the tanks. Three estrogenic compounds were used:

17β-estradiol (Sigma, St. Louis, MO) at 10 µg/l, β-sitosterol (Calbiochem, San Diego, CA) at 100 µg/l, and 4-*n*-nonylphenol (AlfaAesar, Ward Hill, MA) at 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. Every other day one-half the volume of each tank was removed and replaced with fresh water containing the appropriate EE treatment (added so as to maintain the desired final concentration of each test agent) similarly to that described by Tremblay and Van der Kraak (1998). The water was well aerated and the tanks were kept at 14° C under a 12L:12D photoperiod. Over the course of the experiment, 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. Following the 28-d exposure, fish were again anesthetized with 0.05% (v/v) 2-phenoxyethanol, and weighed and measured according to tag location.

To investigate the molecular mechanisms affecting the GH-IGF axis, fish (ca. 50 g) were anesthetized with 0.05% (v/v) 2-phenoxyethanol, measured, weighed and transferred to 40-l glass aquaria (7 fish per tank) containing only fresh water. Feeding was recommenced 24 h after transfer to aquaria (2% body weight once per day) and continued throughout the 15-d exposure period; however, feeding was suspended 24 h prior to final sampling. Fish were allowed to recover for 48 h before EE were added to the tanks. Varying concentrations of the EE were used in different tanks: E2 (0.1 µg/l, 1.0 µg/l, 10 µg/l, 100 µg/l), βS (1.0 µg/l, 10 µg/l, 75 µg/l, 150 µg/l), and NP (1.0 µg/l, 10 µg/l, 75 µg/l, 150 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. Every other day one-half the volume of each tank was removed and replaced with fresh water containing the appropriate EE treatment (added so as to maintain the desired final concentration of each test agent) similar to the method described by Tremblay and Van der

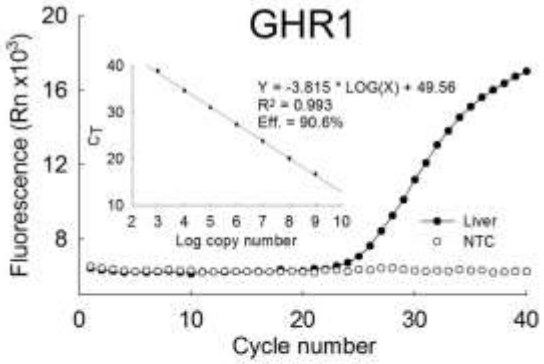
Kraak (1998). The water was well aerated and the tanks were kept at 14° C under a 12L:12D photoperiod. Over the course of the experiment, dissolved oxygen ranged from 8-10 mg/l and ammonia did not exceed 0.25 ppm. Discarded water was filtered through activated charcoal before disposal. At sampling, fish were again anesthetized with 0.05% (v/v) 2-phenoxyethanol. Blood was collected with heparinized glass capillary tubes from the severed caudal vessels and centrifuged (5000 x 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. The remainder of the liver was taken for a radioreceptor assay. All samples were immediately placed on dry ice and stored at -80° C.

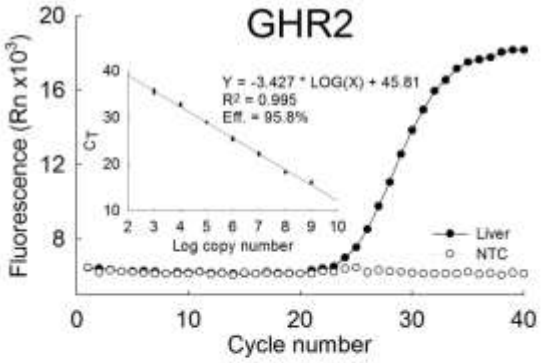
Quantitative Real-Time PCR

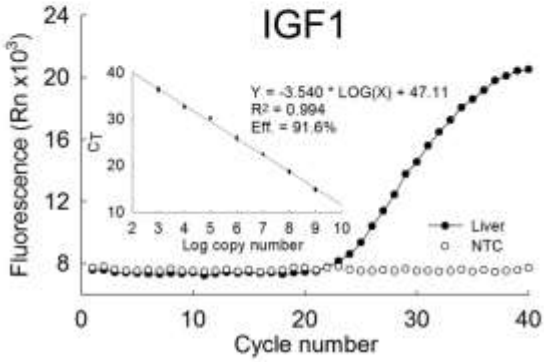
Frozen tissues were homogenized and total RNA was extracted using TRI reagent ® (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNA pellets were dissolved in 40-150 µL RNase-free deionized water. Total RNA was quantified by UV (A_{260}) spectrophotometry and then diluted with RNase-free deionized water to 100 ng/µl. RNA was reverse transcribed in a 10-µl reaction using 200 ng total RNA with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA) as described by the manufacturer's protocol.

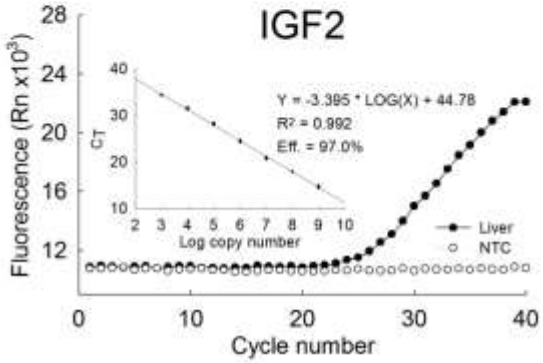
As described previously (Malkuch et al., 2008; Poppinga et al., 2007; Very et al., 2005), steady-state mRNA levels of GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B were determined by quantitative real-time PCR using a Stratagene Mx 3000p detection system (Stratagene, La Jolla, CA). Real-time PCR reactions were carried out for controls, standards, and samples in a 10 µl total volume (5 µl 2X Brilliant Master Mix; 0.5 µl of each gene-specific

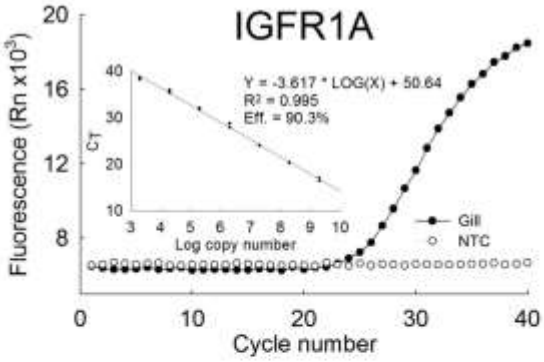
Figure 11. Quantitative real-time PCR of mRNAs encoding (A) growth hormone receptor 1 (GHR1) and GHR2, (B) insulin-like growth factor-1 (IGF-1) and IGF-2, (C) insulin-like growth factor receptor 1A (IGFR1A) and IGFR1B, and (D) vitellogenin (Vtg) obtained from rainbow trout. The amplification plot was used to determine threshold cycle number (C_T). A standard curve (inset) of serial dilutions of known amounts of each cDNA was used to determine sample copy number, and then normalized to β-actin levels. Representative amplification plots are shown for selected tissues; NTC, no-template control.

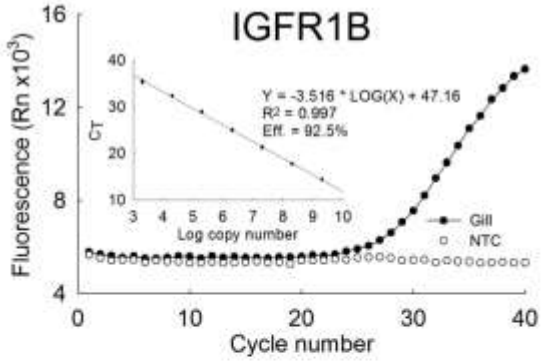

GHR1
Y = -3.815 * LOG(X) + 49.56
R² = 0.993
Eff. = 90.6%
Fluorescence (Rn x10³)
Cycle number
CT
Log copy number
Liver
NTC

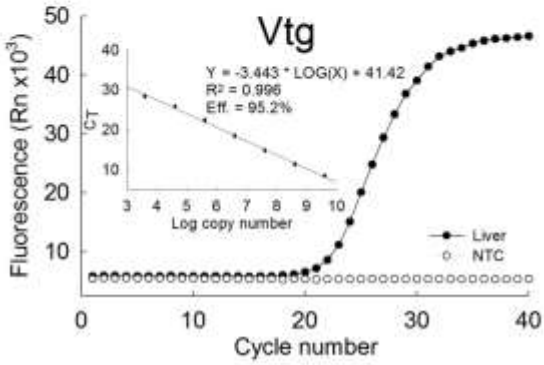

GHR2
Y = -3.427 * LOG(X) + 45.81
R² = 0.995
Eff. = 95.8%
Fluorescence (Rn x10³)
Cycle number
CT
Log copy number
Liver
NTC


IGF1
Y = -3.540 * LOG(X) + 47.11
R² = 0.994
Eff. = 91.6%
Fluorescence (Rn x10³)
Cycle number
CT
Log copy number
Liver
NTC


IGF2
Y = -3.395 * LOG(X) + 44.78
R² = 0.992
Eff. = 97.0%
Fluorescence (Rn x10³)
Cycle number
CT
Log copy number
Liver
NTC


IGFR1A
Y = -3.617 * LOG(X) + 50.64
R² = 0.995
Eff. = 90.3%
Fluorescence (Rn x10³)
Cycle number
CT
Log copy number
Gill
NTC


IGFR1B
Y = -3.516 * LOG(X) + 47.16
R² = 0.997
Eff. = 92.5%
Fluorescence (Rn x10³)
Cycle number
CT
Log copy number
Gill
NTC


Vtg
Y = -3.443 * LOG(X) + 41.42
R² = 0.996
Eff. = 95.2%
Fluorescence (Rn x10³)
Cycle number
CT
Log copy number
Liver
NTC

probe, forward primer, and reverse primer at concentrations optimized for each RNA species). Cycling parameters were set as follows: 95° C for 10 min, and 50 cycles of 92° C for 15 s plus 57° 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 following normalization to β-actin (Fig. 11). Cross hybridization was assessed by substituting alternate primer/probe sets in assays for each standard; no amplification was observed under these conditions.

cDNA standards for vitellogenin were synthesized by PCR. RT-PCR was used for the isolation of the cDNA sequence using an Advantage® 2 PCR Kit (BD Biosciences Clontech) following the manufacturer's protocol. PCR was carried out using cDNA from rainbow trout liver with appropriate primers (forward: 5'-CTCCCCTGTTCTCTGCCATTT-3'; reverse: 5'-ACTGCGTTGACTTCAGAAAACAA-3'). After an initial denaturation at 95° C for 1 min, a 30 cycle-PCR was performed consisting of denaturation (95° C for 30 s), annealing (65°C for 30 s) and extension (72° C for 1 min) cycles. PCR products were then identified by electrophoresis on an agarose gel (1%) in 1X Tris-borate-EDTA (TBE) buffer followed by ethidium bromide staining. Following confirmation of desired product, PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and inserted into JM109 cells. Following purification, plasmids (75 fmol) were sequenced by the CEQ 2000 Dye Terminator Cycle Sequencing with Quick Start Kit (Beackamn Coulter, Fullerton, CA, USA) according to the manufacturer's protocol. ABI Primer Express® Version 2 software was used to design gene-specific oligonucleotide primers for real-time PCR based on known vitellogenin sequence using GeneTool software (BioTools, Inc., Edmonton, AB, Canada) and subsequently synthesized by Sigma-Genosys (The Woodlands, TX, USA).

Steady-state mRNA levels of vitellogenin were determined by quantitative real-time PCR using PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Real-time PCR reactions were carried out for controls, standards, and sampled in a 10 µl total volume (5 µL 2X SYBR Green SuperMix, 0.5 µL mixed primers, 2.5 µL nuclease free water). Cycling parameters were set as follows: 95° C for 3 min, 40 cycles of 95° C for 15 s plus 61° C for 45 sec, and 1 cycle of 95° C for 1 min, 55° C for 30 sec, and 95° C for 30 sec. Sample copy number was calculated from the threshold cycle number (C_T), and the C_T was related to a gene-specific standard curve (Fig. 10). Real-time PCR of total RNA from liver showed a significant amplification of genes above no-template controls. Standard curves showed a linear relationship between log of the RNA copy number and C_T.

GH Binding

Hepatic microsomes were prepared from frozen liver and trout/salmon GH (sGH) was labeled with ^{125}I-labeled sGH as described by Grey et al. (1990). Protein determination was completed using the Bio-Rad, Inc. (Hercules, CA) dye-binding method for microplates. Bovine serum albumin was used as the standard.

Saturation binding assays were conducted on hepatocytes by incubating (14° C, 3 h) 400 µL of cell suspension and 50 µL of ^{125}-I-labeled sGH in either the presence or absence (for total binding) of cold sGH. Binding in the presence of sGH was taken as nonspecific binding. The incubation was terminated by centrifugation at 1,000 *g* (14° C, 15 min). Cells were washed three times with cold incubation medium to remove unbound hormone. The tubes were placed in 12 x 75-mm gamma tubes for determination of radioactivity (Beckman 5500 gamma counter).

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.

Results

Body Characteristics

Exposure to EE for 28 days had no effect on food intake (Table 3). However, EE exposure significantly reduced food conversion (Table 3), which resulted in lowered body condition and growth retardation. Estrogenic compounds inhibited growth in terms of both relative body weight and relative body length, and although E2 and βS had similar growth-inhibiting effects, the effects of NP were the most pronounced of the EEs tested (Fig. 12A, 12B). Interestingly, the hepatosomatic index (HSI) of EE-treated fish tended to be higher than that control fish, an effect that was significant E2-treated fish (Table 3). As a positive biological indicator of estrogen exposure, vitellogenin mRNA was measured and an increase in vitellogenin mRNA (Fig. 12C) for all EE-treated fish was observed.

Effects of EE on GHR mRNA and Functional Expression

The exposure of rainbow trout to EE for 15 days resulted in decreased levels of GHR mRNAs in liver. Two distinct GHR-encoding mRNAs are expressed in the liver, gill, red muscle, and white muscle of rainbow trout (Reindl and Sheridan, 2012). A significant decrease was observed in steady-state levels of mRNAs encoding GHR1 and GHR2 (Fig. 13A) in liver in a dose-dependent manner for E2-treated fish. βS- and NP-treated trout exhibited decreases of 75% and 72%, respectively, compared to control levels at the highest concentration for GHR1. Similarly,

Table 3. Body characteristics of rainbow trout exposed to environmental estrogens for 28 days.*

	After 28-day exposure			
Characteristic	Control	E2	βS	NP
Food consumption (g/day)	9.99 ± 0.01^{a}	$9.80 \pm .012^{a}$	9.91 ± 0.52^{a}	9.79 ± 0.08^{a}
Food conversion efficiency§	0.86 ± 0.13^{a}	0.59 ± 0.18^{b}	0.51 ± 0.11^{b}	0.15 ± 0.18^{c}
Condition factor†	1.30 ± 0.02^{a}	1.36 ± 0.03^{ab}	1.19 ± 0.03^{b}	1.13 ± 0.02^{c}
Hepatosomatic index‡	1.95 ± 0.07^{a}	2.36 ± 0.12^{b}	2.03 ± 0.35^{a}	1.98 ± 0.15^{a}

*Fish were immersed in fresh water (control) or fresh water containing 10 µg/l 17β-estradiol (E2), 100 µg/l β-sitosterol (βS), or 100 µg/l 4-nonylphenol (NP). Data are presented as means ± SEM (n=10); for a given characteristic, groups with different letters are significantly different ($p < 0.05$) from one another.

§Calculated as [total body weight gain/fish(g)/total food consumption/fish(g)]

†Calculated as [body weight/(body length)^3] x 100

‡Calculated as (liver weight/body length) x 100

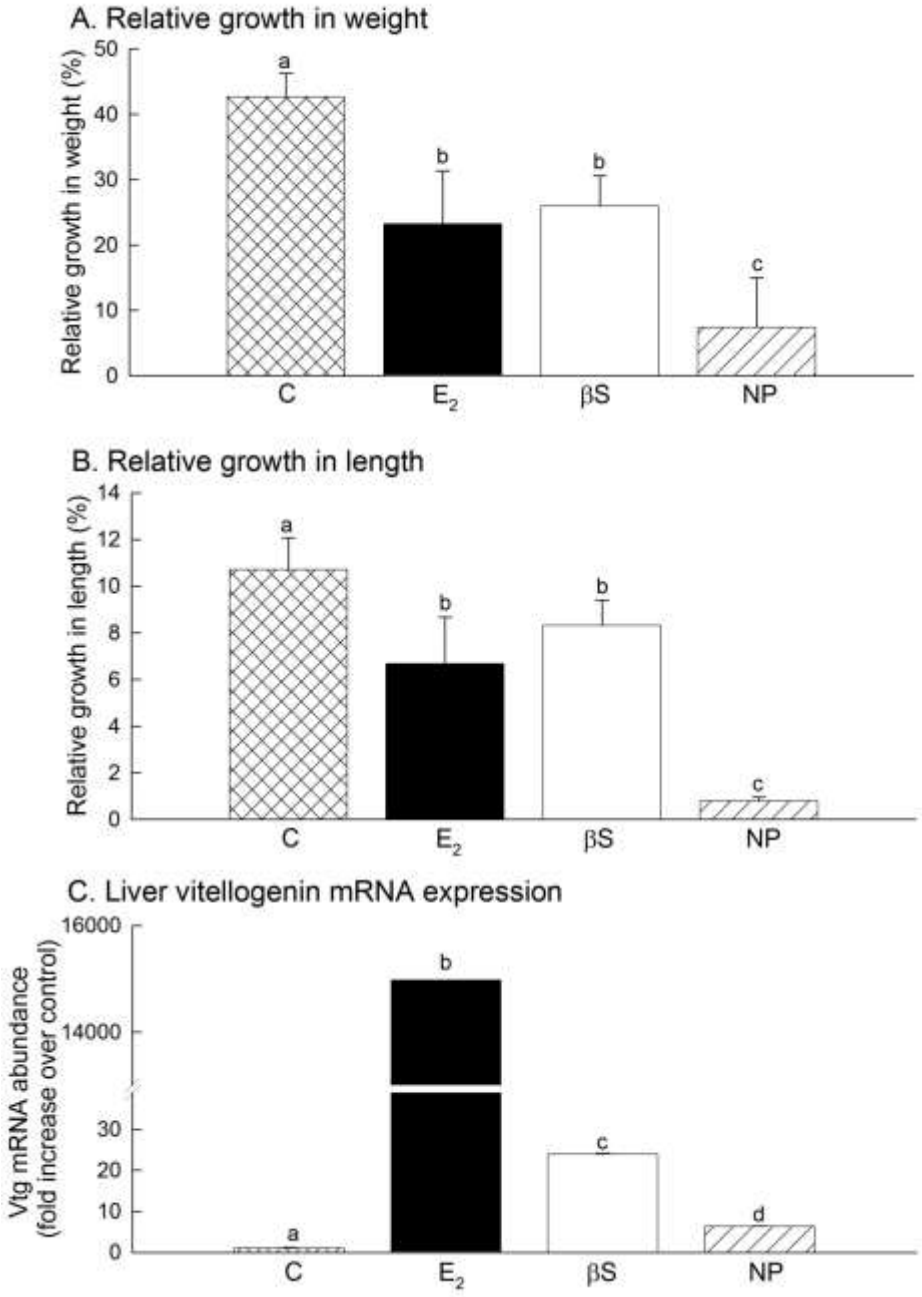
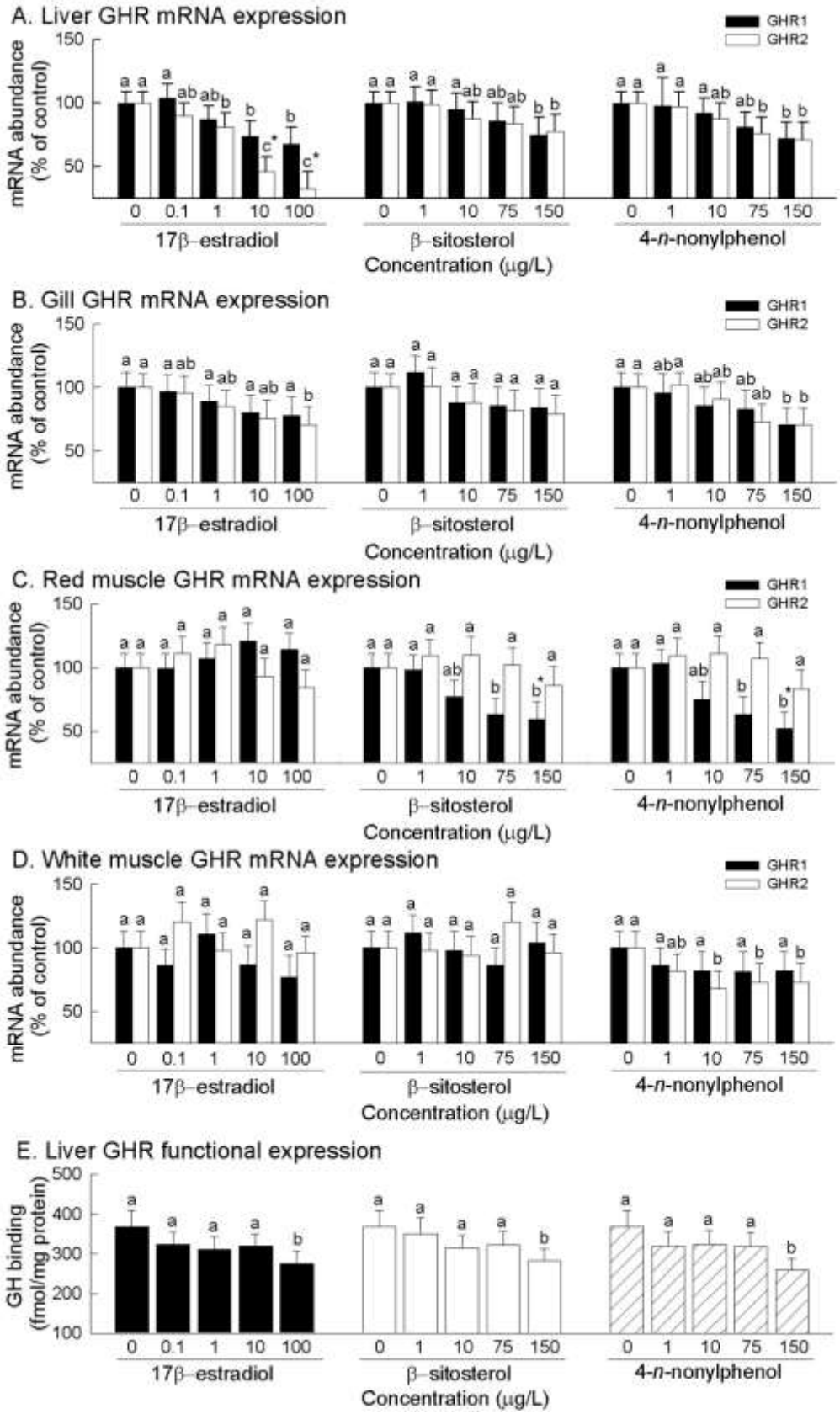


Figure 12. Effects of environmental estrogens (EE) on relative growth in (A) weight and (B) length, and (C) hepatic vitellogenin abundance following 28-day exposure to EE. Fish were exposed to varying concentrations of 17β-estradiol (E2), β-sitosterol (βS), or 4-nonylphenol (NP). Data are presented as mean ± SEM (n=7). Means with different letters are significantly different (p<0.05) from each other.

Figure 13. Effects of environmental estrogens (EE) on the abundance of (A) hepatic, (B) gill filament, (C) red muscle, and (D) white muscle growth hormone receptor 1 (GHR1) and GHR2 mRNAs and (E) liver binding capacity (Bmax) of GHRs in rainbow trout following 15-d exposure to EE. Fish were exposed to varying concentrations of 17β-estradiol (E2), β-sitosterol (βS), or 4-nonylphenol (NP). Data are presented as mean ± SEM (n=7). Means with different letters are significantly different ($p<0.05$) between the control and EE treatments for a given GHR subtype; *indicates a significant difference ($p<0.05$) between GHR subtypes within a given EE-treated group.


A. Liver GHR mRNA expression
B. Gill GHR mRNA expression
C. Red muscle GHR mRNA expression
D. White muscle GHR mRNA expression
E. Liver GHR functional expression
GHR1
GHR2
mRNA abundance (% of control)
GH binding (fmol/mg protein)
17β–estradiol
β–sitosterol
4-n-nonylphenol
Concentration (μg/L)

βS- and NP-treated groups showed decreased levels of GHR2, with βS decreasing to 78% and NP decreasing to 71% of control levels. Similar to results observed in liver, exposure of gill filaments to EE affected GHR1 and GHR2 (Fig. 13B) steady-state mRNA levels. GHR1 mRNA levels were suppressed, although not significantly, at high concentrations of E2 and βS when compared to the control fish. A dose-dependent decrease was observed in NP-treated fish for GHR1 and GHR2 mRNA levels in gill filaments. In red muscle, a dose-dependent decrease in βS-treated and NP-treated groups was observed for GHR1 mRNA levels (Fig. 13C). Notably, no significant decreases were observed in GHR2 mRNA levels in red muscle (Fig. 13C). White muscle, however, showed no significant decreased in GHR1 mRNA, whereas GHR2 mRNA (Fig. 13D) levels of NP-treated fish decreased to 73% of the control at the highest concentration of 150 μg/L.

Functional expression of GHR was determined by ^{125}-I-GH binding to microsomes. Binding capacity in hepatic microsomes was reduced at the highest concentrations in all EE-treated tissue (Fig. 13E), which was consistent with the mRNA expression data. Binding capacity in hepatic microsomes decreased to 75% (E2), 77% (βS), and 71% (NP) of control values (Fig. 13E). EE treatment had no effect on binding affinity in liver (data not shown).

Effects on IGF mRNA Expression

As was the case with GHRs, multiple rainbow trout tissues (e.g., liver, gill) possess two isoforms of IGF, IGF-1 and IGF-2 (Reindl and Sheridan, 2012). Exposure to EE suppressed hepatic and gill filament production of IGF mRNAs in a similar manner as that observed with GHR mRNAs. Hepatic IGF-1 and IGF-2 (Fig. 14A) mRNAs showed a dose-dependent decrease in all EE treatment groups, with the most pronounced effects being observed in E2- and NP-

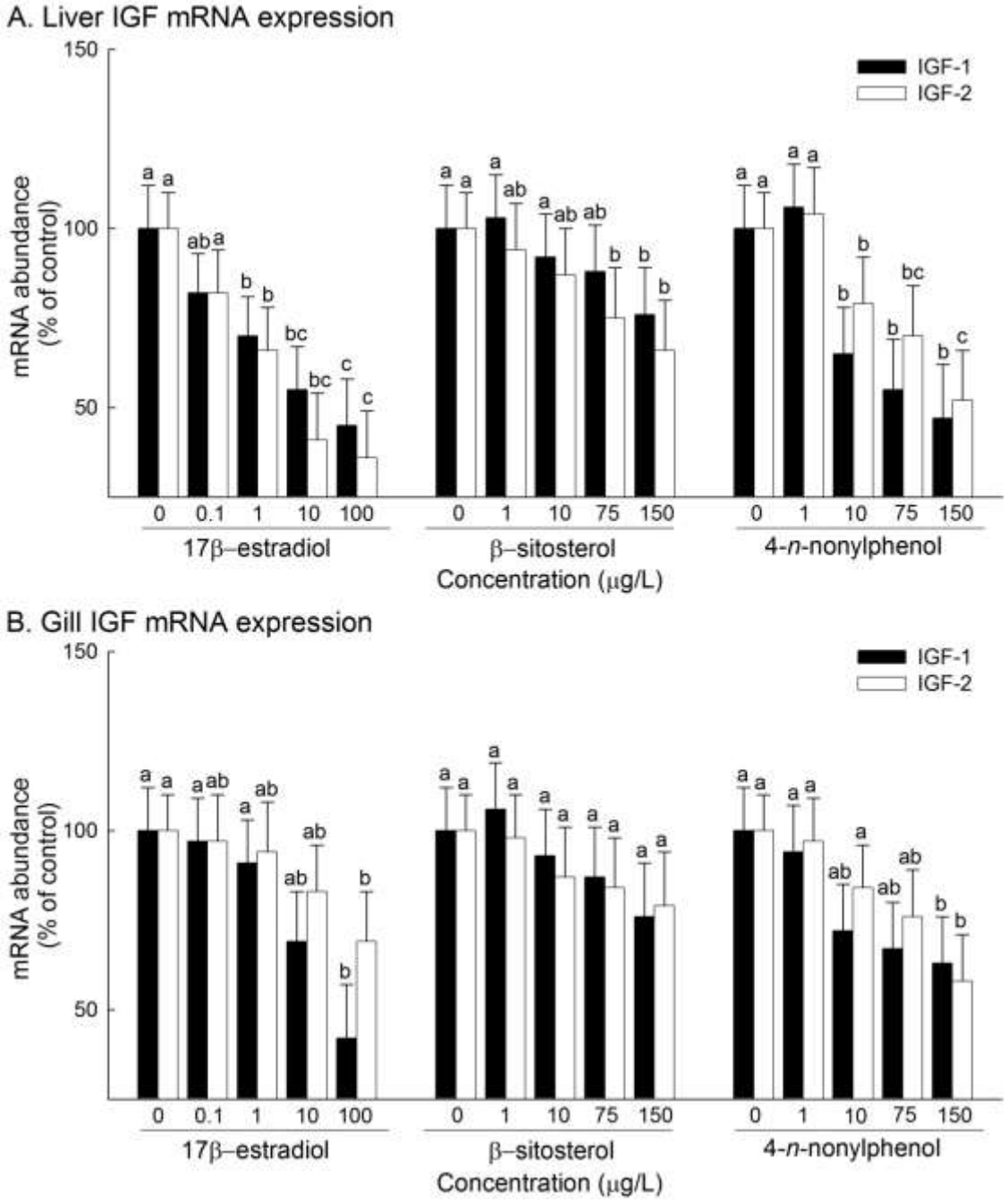


Figure 14. Effects of environmental estrogens (EE) on the abundance of (A) hepatic and (B) gill filament insulin-like growth factor-1 (IGF-1) and IGF-2 mRNAs in rainbow trout following 15-d exposure to EE. Fish were exposed to varying concentrations of 17β-estradiol, β-sitosterol, or 4-nonylphenol. Data are presented as mean ± SEM (n=7). Means with different letters are significantly different (p<0.05) for a given IGF type within a specific EE treatment group.

treated fish. The pattern of IGF mRNA expression observed in liver was similar to that observed in gill filaments, where IGF-1 and IGF-2 (Fig. 14B) mRNA expression decreased in a dose-dependent manner for E2- and NP-treated rainbow trout filaments. βS-treated fish, however, exhibited only a downward trend in IGF-1 and IGF-2 mRNA expression levels. At the highest concentrations IGF-1 and IGF-2 mRNA levels decreased to 76% and 70%, respectively, of the control values for βS.

Effects on IGFR mRNA Expression

Two types of IGFR1s, IGFR1A and IGFR1B, were expressed in gill, red muscle, and white muscle. Levels of both IGFR1s were greatest affected in gill filament, where IGFR1A decreased in a dose-dependent manner all treatment groups, except in βS treatment where mRNA levels decreased to only 83% of control values (Fig. 15A). No significant differences were observed in IGFR1B mRNA levels for any of the EE treatments, but all treatment groups exhibited a downward trend in IGFR1B mRNA (Fig. 15A). Effects on IGF sensitivity were less pronounced in muscle tissue. In red muscle a dose-dependent decrease in IGFR1A mRNA expression was observed in the NP-treated fish (Fig. 15B). Interestingly, IGFR1B mRNA expression decreased in a dose-dependent manner for both E2- and NP-treated fish (Fig. 15B). In white muscle, E2-treated fish exhibited decreased IGFR1A expression to levels that were 66% of the control (Fig.15C), whereas IGFR1B mRNA levels decreased to 77% of the control at the highest concentrations (Fig. 15C). For βS-treated fish, IGFR1A expression decreased to levels 77% of the control at 150 µg/l, whereas no significant decrease was present with IGFR1B mRNA. NP-treated fish had no significant differences among the concentrations.

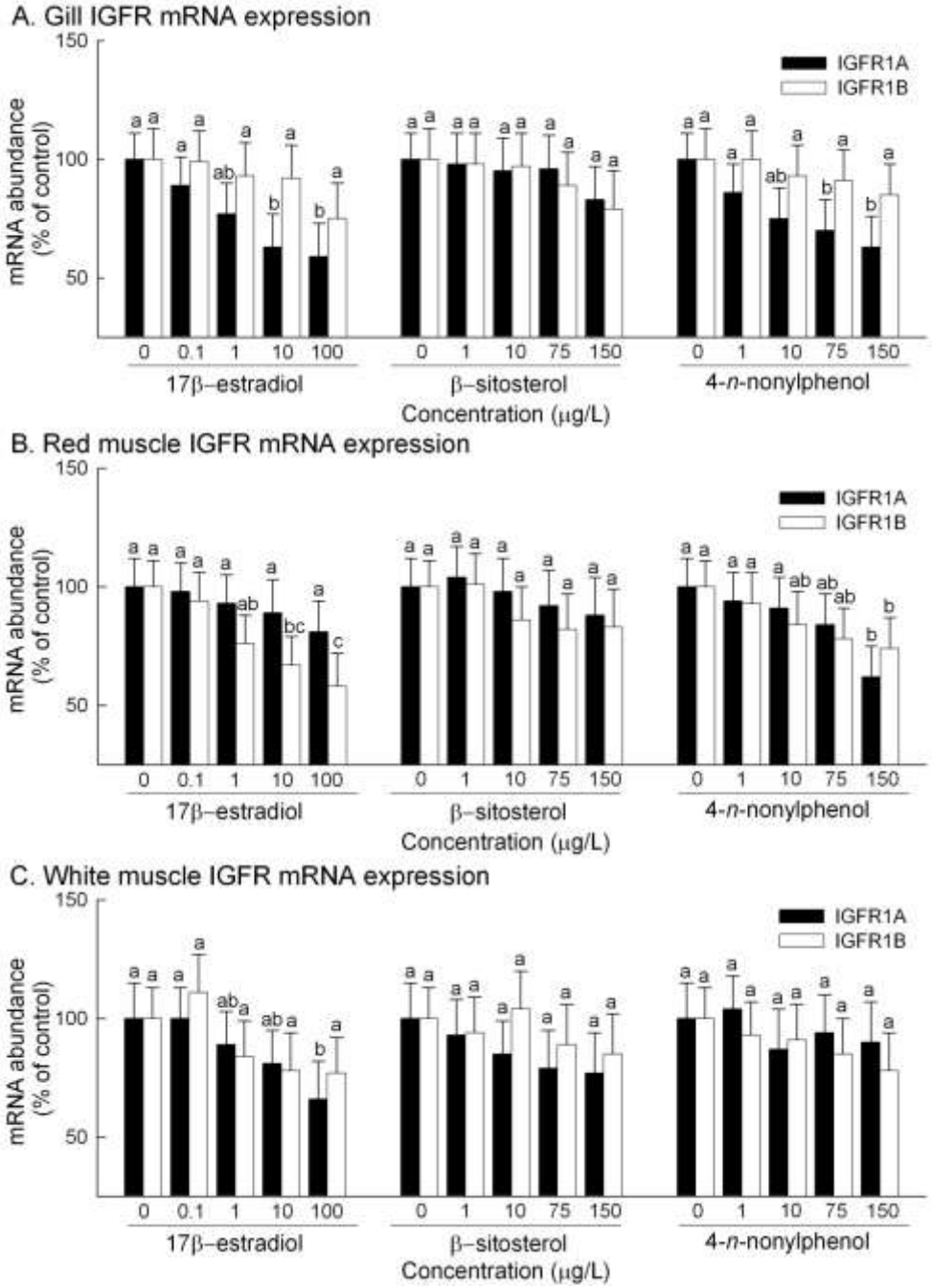


Figure 15. Effects of environmental estrogens (EE) on the abundance of (A) gill filament, (B) red muscle, and (C) white muscle IGF receptor 1A (IGFR1A) and IGFR1B mRNAs in rainbow trout following 15-d exposure to EE. Fish were exposed to varying concentrations of 17β-estradiol, β-sitosterol, or 4-nonylphenol. Data are presented as mean ± SEM (n=7). Means with different letters are significantly different (p<0.05) for a given IGFR subtype within a specific EE treatment group.

Discussion

Results from the current study indicate that EE inhibit post-embryonic growth, in terms of overall organismal growth (i.e. length, weight), and that the growth-inhibiting effects of EE result from alterations in peripheral aspect of the GH-IGF system, particularly GH sensitivity, IGF production, and IGF sensitivity. These effects were elicited at environmentally relevant concentrations of E2, βS, and NP (Blackburn and Wadlock, 1995; Cook et al., 1996; Hale et al., 2000).

In terms of overall organismal growth, E2, βS, and NP lead to decreased organismal growth in terms of relative growth (weight and length). This is in agreement with our previous study (Hanson et al., 2012) and other studies that found that exposure to EE led to significant decreases in growth in developing (e.g., first month post-hatch) rainbow trout (Ashfield et al., 1998) and in developing tilapia exposed 10-100 days post-fertilization (Shved et al., 2008). In addition, long-term exposure to E2 and NP has been shown to lead to reduced growth in Atlantic salmon smolts (Arsenault et al., 2004; Lerner et al., 2007). The effects of EE on organismal growth could be attributed to the exposure of rainbow trout during the juvenile life stage, a stage in which the fish are typically undergoing rapid growth (Mommsen, 2001). From a life history perspective, juveniles typically allocate energy resources towards growth prior to undergoing sexual maturation. Treatment at this critical life stage with a sex steroid such as estrogen, or a compound mimicking estrogen (e.g., β-sitosterol, nonlyphenol), may reprogram the fish to divert energy resources towards reproduction rather than growth.

Environmental estrogens reduced condition in fish, as in agreement with our previous study (Hanson et al., 2012) is which HSI was observed to increase in E2-treated fish. These data also are in agreement with previous findings by Carrera et al. (Carrera et al., 2007) in E2-treated

gilthead sea bream. Several estrogenic compounds act as agonists for the estrogen receptor and can induce the synthesis of vitellogenin, which could explain the increase observed in HSI particularly given our observation of an increase in vitellogenin mRNA for all EE-treated fish. While the current HSI data for βS- and NP-treated fish is not significantly different as previous studies have been (Hanson et al., 2012; Tremblay and Van der Kraak, 1998), the less robust response may simply be due to the lower affinity of βS and NP for the rainbow trout hepatic estrogen receptor when compared to endogenous estradiol (Tremblay and Van der Kraak, 1998; Cosnefroy et al., 2009).

The observed decrease in relative organismal growth was in agreement with our present results examining the GH-IGF system at the molecular level. The results of this study indicate that exposure of rainbow trout to EE disrupts the GH-IGF system at multiple tissue levels, as evidenced by several observations. First, EE attenuated hepatic GH sensitivity (e.g., GHR1, GHR2) in a concentration-dependent manner. Similar decreases in GH expression have been observed following chronic EE exposure in immediate post-hatch tilapia (Shved et al., 2008). As evidenced by a radioreceptor assay, EE also decreased functional expression of GHRs at the highest concentrations, which was in agreement with our mRNA data. GH also promotes hypo-osmoregulatory ability of fish in seawater (SW); E2 and NP resulted in osmoregulatory failure of SW Atlantic salmon in conjunction with reduced GH binding capacity (Lerner et al., 2012). It appears, then, that EE impact growth throughout an animal's life history, including during early development (embryonic; immediate post hatch) and post-embryonic juvenile stages. The age of an organism may play a critical role in the effects of EDCs, as liver detoxification mechanisms, DNA repair mechanisms, and the immune system are not fully developed at the earliest stages of life (Bern, 1992).

Exposure to EE suppressed hepatic and gill filament production of IGF mRNAs in a manner similar to that observed with GHR mRNAs. Chronic exposure to EE appears to elicit similar effects as acute exposure, as gilthead sea bream injected with E2 and *in vitro* exposure in rainbow trout also showed decreased hepatic IGF-1 and IGF-2 mRNA levels (Carnevali et al., 2005; Norbeck and Sheridan, 2011). This is in agreement with findings in Atlantic salmon and rainbow trout examining osmoregulatory dysfunction following exposure to E2 and NP that was attended by decreased plasma IGF-1 levels (Arsenault et al., 2004; Lerner et al., 2007; McCormick et al., 2005). Collectively, these data indicate that a negative feedback loop exists between E2 (and EE such as βS and NP) and peripheral elements of the GH-IGF system, which thereby decreases the growth-promoting actions of GH by affecting GH sensitivity and IGF synthesis.

Exposure to EE also suppressed gill filament and muscle production of IGFR mRNAs. The greatest effect of EE on IGFR1 expression was in gill filaments, where IGFR1A decreased in a dose-dependent manner in most treatment groups. While variable effects were observed in muscle tissue, generally there was a decrease in IGF sensitivity. This was in agreement with previous studies by our lab following *in vitro* treatment with E2 (Norbeck and Sheridan, 2012) and others in which ethinylestradiol exposure reduced IGFR mRNA in sexually maturing fathead minnows (Filby et al., 2007), suggesting that EE affect IGF sensitivity in adult organisms as well as juveniles.

These results indicate that EE inhibit post-embryonic growth by reducing GH sensitivity, IGF production, and IGF sensitivity. It should be noted that transient exposure to EE during early development can affect growth later in life. For example, transient NP exposure of trout hatchlings decreased growth up to one year after exposure (Ashfield et al., 1998). Such findings

are particularly alarming as recent evidence suggests that the epigenetic effects of EDCs are heritable across multiple generations, thus leading to a host of diseases states (Anway et al., 2005; Anway and Skinner, 2006).

The endocrine control of growth is complex and is closely related to other processes such as development, immunity, reproduction, behavior, and osmoregulation, all of which vary depending on the life stage of a teleost. Following exposure to EE, many of these processes may be disrupted as there is an endocrine interrelationship between and among these physiological processes. Osmoregulation, for example, which relies upon the GH-IGF system (Sakamota and McCormick, 2006) has been shown to be negatively affected by exposure to E2, βS, and NP in rainbow trout and Atlantic salmon (Hanson et al., 2012; Lerner et al., 2007) suggesting EE may affect the action of GH on multiple levels (i.e. growth-promoting, osmoregulatory). Due to the highly conserved nature of the GH-IGF system among vertebrates, this study has broad relevance to understanding how EE may affect other species, including humans.

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CHAPTER 3: ENVIRONMENTAL ESTROGENS INHIBIT THE EXPRESSION OF INSULIN-LIKE GROWTH FACTORS 1 AND 2 IN THE LIVER AND GILL OF RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) *IN VITRO*

Abstract

Rapid technological development over the past few decades has led to the increased introduction of an expanding array of chemicals into the environment via the air, water, and soil. Once in the environment, these compounds may pose a serious threat to terrestrial and aquatic vertebrates, as well as to humans. Fish in aquatic habitats are exposed to increasing concentrations and types of environmental contaminants, including environmental estrogens (EE). Previously, we exposed juvenile rainbow trout (ca. 50 g) *in vivo* to varying concentrations of 17β-estradiol (E2), β-sitosterol (βS), and 4-n-nonylphenol (NP) for 28 days (14 C; 12L:12D) and observed depressed food conversion, depressed growth, and altered expression of various elements of the growth hormone (GH)-insulin-like growth factor (IGF) system. In this study, we assessed the direct effects of EE on GH sensitivity as assessed by mRNA expression and functional expression of GH receptors (GHR), IGF production as assessed expression of IGF-1 and IGF-2 mRNAs, and IGF sensitivity as assessed by mRNA expression and functional expression of IGF receptors (IGFR) in selected tissues. Environmental estrogens tested affected the expression of GHRs in a tissue- and compound-related manner. By contrast, E2, βS and NP inhibited the expression of both IGF-1 and IGF-2 in a time- and concentration-related manner in liver. Although the response evoked by all of the EEs was similar for hepatic IGF-1 and IGF-2 mRNA expression, the potency and efficacy varied with EE; the rank order potency/efficacy was as follows: E2=NP> βS. E2, βS and NP also inhibited the expression of IGFR1A and IGFR1B mRNAs in a time- and concentration-related manner in gill and muscle; patterns for efficacy and

potency similar to those in liver also were observed in gill. These finding indicate that selected EEs can directly influence the growth of post-embryonic rainbow trout by inhibiting the synthesis of IGFs.

Introduction

Numerous physiological processes in vertebrates are mediated by the growth hormone (GH)-insulin-like growth factor-1 (IGF-1) system including growth, cellular differentiation, metabolism, reproduction, immune function, and behavior (Bjornsson et al., 2004; Duan, 1997; Hull and Harvey, 2000; Madsen and Bern, 1993; Mancera and McCormick, 1998; Norrelund, 2005). One of the primary roles of the GH-IGF system is modulating growth at the cellular level. GH is secreted from the pituitary and circulates throughout the organism bound to growth hormone binding protein (GHBP), where it binds to growth hormone receptors (GHR) to initiate the synthesis and secretion of IGF-1 primarily from the liver (Butler and LeRoith, 2001; Wood et al., 2005), but also from other sites (e.g., gill, muscle). IGF travels throughout the organism bound to insulin-like growth factor binding proteins (IGFBP) and binds to its receptors (IGFR) to stimulate cellular growth, proliferation, and differentiation.

Somatic growth has been shown to be affected by gonadal steroids (Leung et al., 2004). Specifically, estrogen has been implicated as playing a role in growth by modulating the GH-IGF system. For example, studies have shown that 17β-estradiol can increase plasma GH in rainbow trout and tilapia (Holloway and Leatherland, 1997; Melamed et al., 1995). Further evidence of the interaction between estrogen and growth has been observed as *in vivo* treatment with 17β-estradiol has been shown to decrease hepatic IGF-1 expression in tilapia, salmon, and gilthead sea bream (Carnevali et al., 2005; Davis et al., 2007; Lerner et al., 2007) as well as serum IGF-1 in tilapia and salmon (Davis et al., 2007; Lerner et al., 2007).

The increased production, use, and disposal of an expanding array of chemicals has led to increased introduction of these compounds into the environment through the air, soil, and water. These compounds originate from a multitude of sources (e.g., industry, agriculture, household cleaners, cosmetics, pharmaceuticals, personal care products, etc.), leading to continuous introduction and persistence of these chemicals in the environment (Daughton & Ternes, 1999). Increasing research shows the deleterious effects of environmental contamination on aquatic and terrestrial plants, fish, amphibians, birds, and mammals, including humans (Hester and Harrison, 1999), often as a result of disruption of the endocrine system.

Environmental estrogens (EE) are a specific class of environmental contaminants that include endogenous and synthetic animal estrogens (e.g., 17β-estradiol), phytoestrogens (β-sitosterol), mycotoxins (e.g., zearalenone), organochlorine pesticides (e.g., DDT), polychlorinated biphenyls (PCBs), and alkylphenol polyethoxylates (APEs; e.g., 4-nonylphenol) (Turner, 1999). EE are particularly harmful to aquatic wildlife, primarily fish, and cause a variety of detrimental developmental and reproductive effects (e.g., sex reversal, sterility, altered reproductive timing and behavior) that result in reduced fecundity (Kime, 1998).

There is a robust amount of knowledge on the effects of EE on developmental and reproductive processes. However, very little has been reported regarding the effects of EE on growth at the molecular level. This study used rainbow trout as a model to examine the effects of EE on growth because of their well-characterized GH-IGF-1 system. Specifically, the aim of the present study was to further elucidate the role of EE (specifically 17β-estradiol, β-sitosterol, and 4-n-nonylphenol) on growth at the molecular level and to determine if the effects of EE are mediated at the level of the GH-IGF axis *in vitro*.

Methods and Materials

Experimental Animals

Juvenile rainbow trout of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, USA and transported to North Dakota State University. Fish 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 before experimental manipulations. All procedures were performed in accordance approved by the North Dakota State University Institutional animal Care and Use Committee.

Experimental Conditions

To investigate the effects of EE on the GH-IGF system at the molecular level, fish (ca. 100 g) were anesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol, measured, weighed and bled via the severed caudal vessel. Tissues (gill arches, liver, and white muscle) were removed, perfused *ex vivo* with 0.75% (w/v) saline solution, and stored in ice-cold Hank's Buffered Salt Solution (in mM: 137 NaCl, 5.4 KCl, 4 $NaHCO_3$, 1.7 $CaCl_2$, 0.8 $MgSO_4$, 0.5 KH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, and 4 glucose; pH 7.6). Livers and muscle were cut into 1 mm^3 pieces while immersed in Hank's on ice. Individual gill filaments were dissected from the arches while immersed in Hank's on ice. All tissues were pooled and washed three times with Hank's Balanced Salt Solution with 0.24% BSA, 2 ml Gibco MEM amino acid mix (50x)/100 mL, and 1 mL Gibco nonessential amino acid mix (100x)/100 mL. Tissues were plated in 24-well culture plates (8-10 liver pieces, 12-15 gill filaments, 25-30 muscle pieces) containing 1-mL of Hank's medium with 0.24% BSA, 2 ml Gibco MEM amino acid mix (50x)/100 mL, and 1 mL Gibco nonessential amino acid mix (100x)/100 mL and preincubated at 14 °C under 100% O_2 while being shaken at 100 rpm in a gyratory shaker. Following the 3 h preincubation, media was

removed and tissues were subjected to either a time course or a dose course of three estrogenic compounds: 17β-estradiol (Sigma, St. Louis, MO), β-sitosterol (Calbiochem, San Diego, CA) and 4-*n*-nonylphenol (AlfaAesar, Ward Hill, MA). All compounds were dissolved in ethanol; the final concentration of ethanol did not exceed 0.007%. For the dose course the following treatments were administered for all tissues: E2 (0.1 µg/l, 1.0 µg/l, 10 µg/l, 100 µg/l), βS (1.0 µg/l, 10 µg/l, 75 µg/l, 150 µg/l), and NP (1.0 µg/l, 10 µg/l, 75 µg/l, 150 µg/l) for 6 h. A time course of 0 h, 3 h, 6 h, 12 h, and 24 h was used to examine long-term genomic effects at concentrations of 10 µg/l (E2) or 75 µg/l (βS, NP) on gill and liver. Due to tissue viability issues, a time course was not performed on muscle. Following incubation, tissues were immediately placed on dry ice and stored at -80° C for later mRNA and binding analyses.

Quantitative Real-Time PCR

Frozen tissues were homogenized and total RNA was extracted using TRI reagent ® (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNA pellets were dissolved in 40-150 µL RNase-free deionized water. Total RNA was quantified by UV (A_{260}) spectrophotometry and then diluted with RNase-free deionized water to 100 ng/µl. RNA was reverse transcribed in a 10-µl reaction using 200 ng total RNA with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA) as described by the manufacturer's protocol.

As previously described (Very et al., 2005; Poppinga et al., 2007; Malkuch et al., 2008), steady-state mRNA levels of GHR1, GHR2, 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). Real-time PCR reactions were carried out for controls, standards, and samples in a 10 µl total volume (5 µl 2X Brilliant Master Mix; 0.5 µl of each gene-specific

probe, forward primer, and reverse primer at concentrations optimized for each RNA species). Cycling parameters were set as follows: 95° C for 10 min, and 50 cycles of 92° C for 15 s plus 57° 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 following normalization to β-actin.

As previously described (Hanson et al., *in press*) steady-state mRNA levels of vitellogenin were determined by quantitative real-time PCR using PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Real-time PCR reactions were carried out for controls, standards, and sampled in a 10 µl total volume (5 µL 2X SYBR Green SuperMix, 0.5 µL mixed primers, 2.5 µL nuclease free water). Cycling parameters were set as follows: 95° C for 3 min, 40 cycles of 95° C for 15 s plus 61° C for 45 sec, and 1 cycle of 95° C for 1 min, 55° C for 30 sec, and 95° C for 30 sec. Sample copy number was calculated from the threshold cycle number (C_T), and the C_T was related to a gene-specific standard curve. Conditions were optimized for the dissociation curve indicating primer-dimer formation was absent.

Binding

Hepatic, gill filament, and muscle microsomes were prepared and trout/salmon GH (sGH) and IGF were labeled with ^{125}I-label (MP Biomedicals, Inc, Irvine, CA) as described previously (Gray et al., 1990, Parrizas et al., 1995). GH and IGF were purchased from GroPep, Ltd. (Adelaide, Australia). Protein determination was completed using the Bio-Rad, Inc. (Hercules, CA) dye-binding method for microplates. Bovine serum albumin was used as the standard.

Saturation binding assays were conducted on hepatocytes by incubating (14° C, 3 h) 400 µL of cell suspension and 50 µL of ^{125}-I-labeled sGH in either the presence or absence (for total binding) of cold sGH. Binding in the presence of sGH was taken as nonspecific binding. The incubation was terminated by centrifugation at 1,000 *g* (14° C, 15 min). Cells were washed with

cold incubation medium to remove unbound hormone. The tubes were placed in 12 x 75-mm gamma tubes for determination of radioactivity (Beckman 5500 gamma counter).

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.

Results

Effects of EE on Vitellogenin mRNA Expression

As a biological indicator of exposure to EE, vitellogenin mRNA levels were measured in hepatic tissue cultures. A significant increase was observed in all three EE-treated groups (Fig. 16) at the selected concentrations (10 µg/L E2, 75 µg/L βS, 75 µg/L NP). Specifically, E2-treated rainbow trout exhibited a 2186-fold increase (over control) in vitellogenin steady-state mRNA levels, whereas fish exposed to βS and NP displayed a 33- and 456-fold increase, respectively.

Effects of EE on mRNA and Functional Expression of GHRs

The exposure of rainbow trout tissue cultures to EE for various concentrations and times was tissue-and compound-dependent. Two distinct GHR-encoding mRNAs, GHR1 and GHR2, are expressed in the liver, gill, and white muscle of rainbow trout. E2 and NP decreased levels of GHR mRNAs in a time- (Fig. 17A) and concentration-(Fig. 17B) related manner in liver, while βS-treated liver pieces remained unchanged. A significant decrease was observed in steady-state levels of mRNAs encoding GHR 1 and GHR 2 (Fig. 17A) in liver after 6 h for E2- and NP-treated hepatocytes. By 24 h, E2- and NP-treated tissue decreased to 61% and 69% (GHR1) and 63% and 68% (GHR2), respectively, of control levels. At the highest concentrations, mRNA

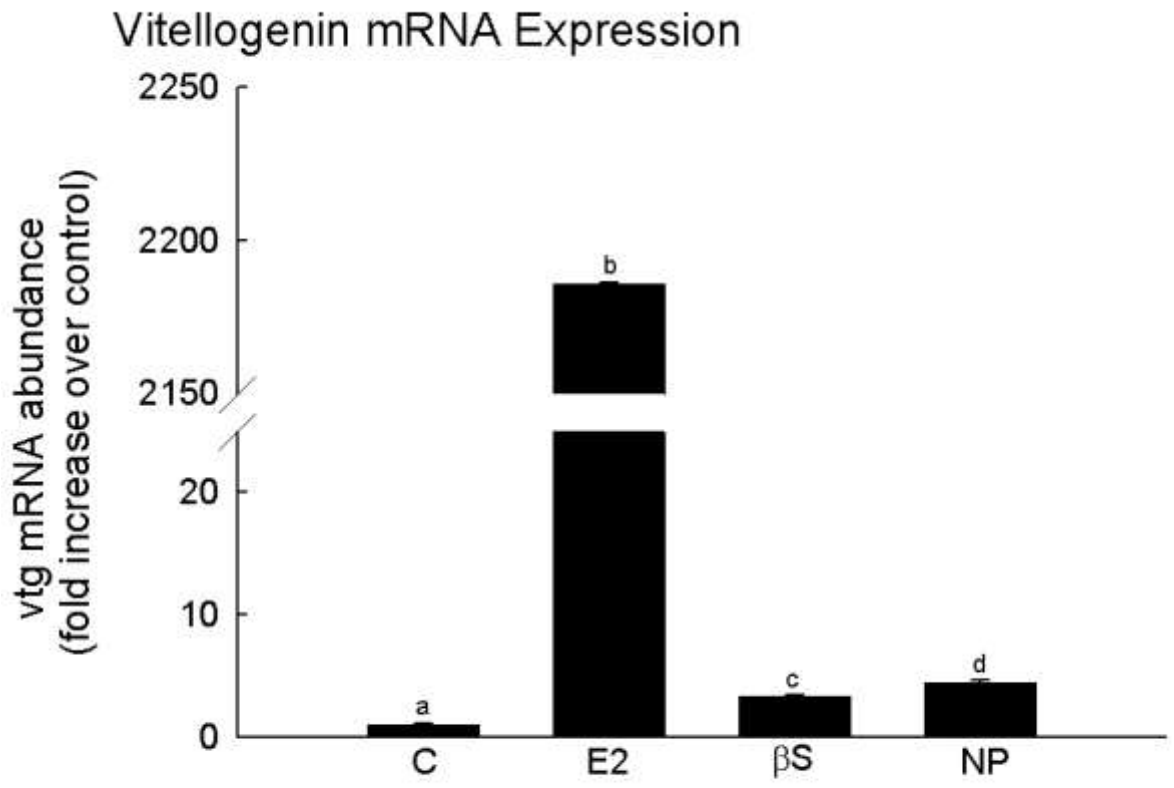

Figure 16. Effects of environmental estrogens (EE) on the abundance of hepatic vitellogenin mRNA for 10 µg/L 17β-estradiol (E2), 75 µg/L β-sitosterol (βS), or 75 µg/L 4-nonylphenol (NP) for 6 h. Data are presented as mean ± SEM (n=6). Means with different letters are significantly different ($p < 0.05$) different from each other.

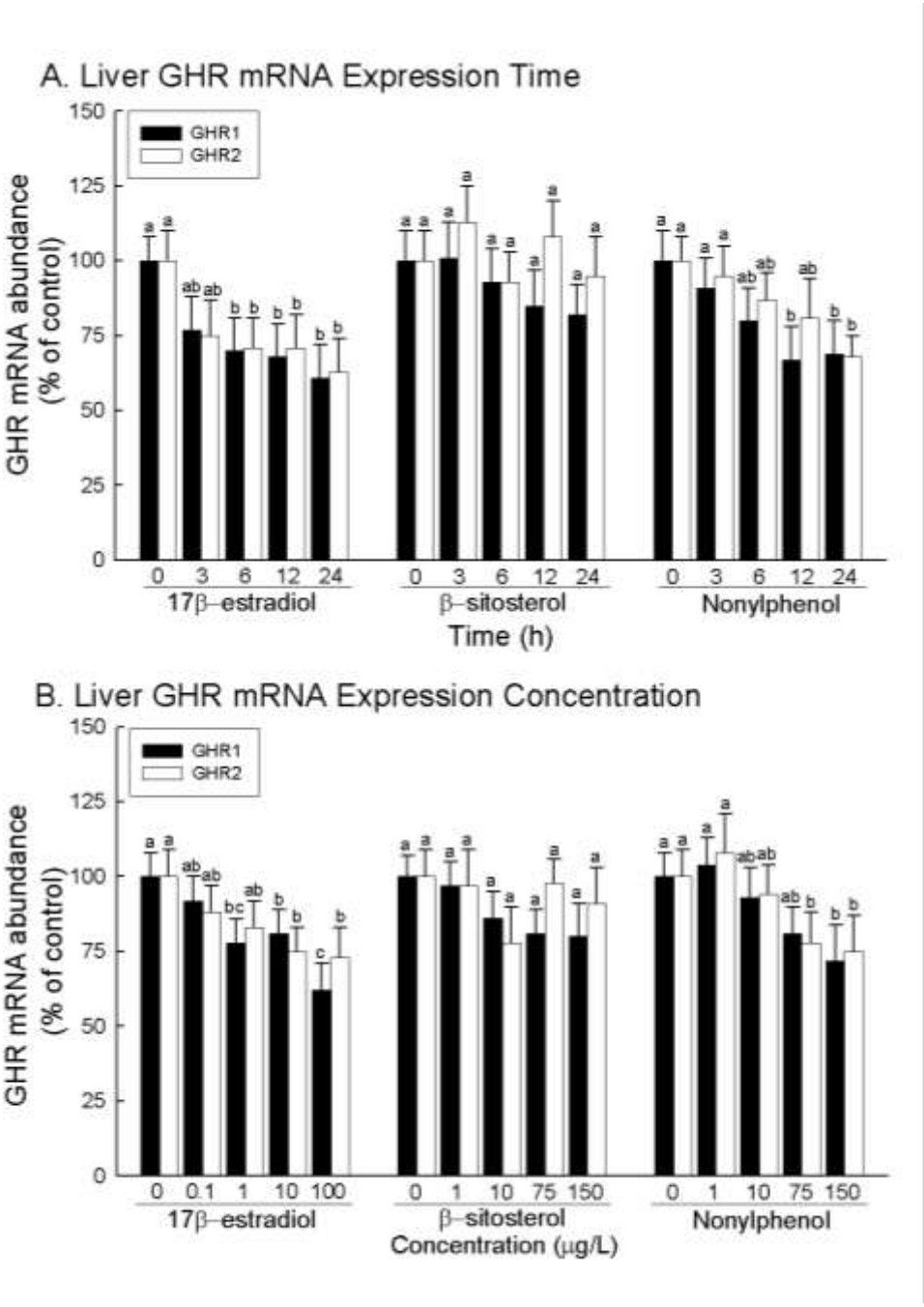

Figure 17. Effects of environmental estrogens (EE) on the abundance of hepatic growth hormone receptors (GHRs) in rainbow trout incubated *in vitro*. Liver pieces were incubated for (A) various times with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L 4-nonylphenol (NP) or (B) various concentrations for 6 h. Data are presented as mean ± SEM (n=6). For a given GHR subtype within a treatment, means with different letters are significantly different ($p < 0.05$) different from each other. * indicates a significant difference ($p < 0.05$) between two GHR subtypes within a treatment group.

abundance of GHR1 decreased to 62% and GHR2 mRNA abundance decreased to 73% of control values in E2-treated liver tissue, whereas GHR1 decreased to 72% of control values and GHR2 decreased to 75% of control values in NP-treated tissue (Fig. 17B). Exposure of gill filaments to EE did not appear to significantly suppress nor elevate GHR1 or GHR2 steady-state mRNA levels in a time- or concentration-dependent manner for any treatment group (Fig. 18A, 18B). A concentration-dependent decrease was observed for GHR1 mRNA abundance in muscle tissue following treatment with E2, βS, and NP. However, GHR2 mRNA suppression was only significant in E2- and NP-treated groups, where levels decreased to 74% and 62%, respectively, of control values (Fig. 19).

Functional expression of GHR was determined by ^{125}-I-GH binding to microsomes. Binding capacity in hepatic microsomes was reduced by over 40% in both E2- and NP-treated tissue (Fig. 20), which was consistent with the mRNA expression data. Binding capacity in gill, although suppressed, was not significantly different for any of the EE treatment groups (Fig. 20). Binding capacity in white muscle microsomes decreased to 73% (E2), 77% (βS), and 68% (NP) of control values (Fig. 20). Environmental estrogen treatment had no effect on binding affinity in liver, gill, or white muscle (data not shown).

Effects of EE on IGF mRNA Expression

The direct effects of EE on IGF-1 and IGF-2, the two forms of IGF found in rainbow trout, was also assessed *in vitro* for liver and gill tissue. Exposure to EE suppressed hepatic and gill filament production of IGF mRNAs in a concentration- and time-dependent manner for all EE tested. IGF-1 mRNA expression in hepatocytes declined rapidly with maximum suppression observed at 24 h, where mRNA abundance decreased to 34% (E2), 61% (βS), and 49% (NP) of control values (Fig. 21), while IGF-2 mRNA expression decreased to 38% (E2), 54% (βS), and

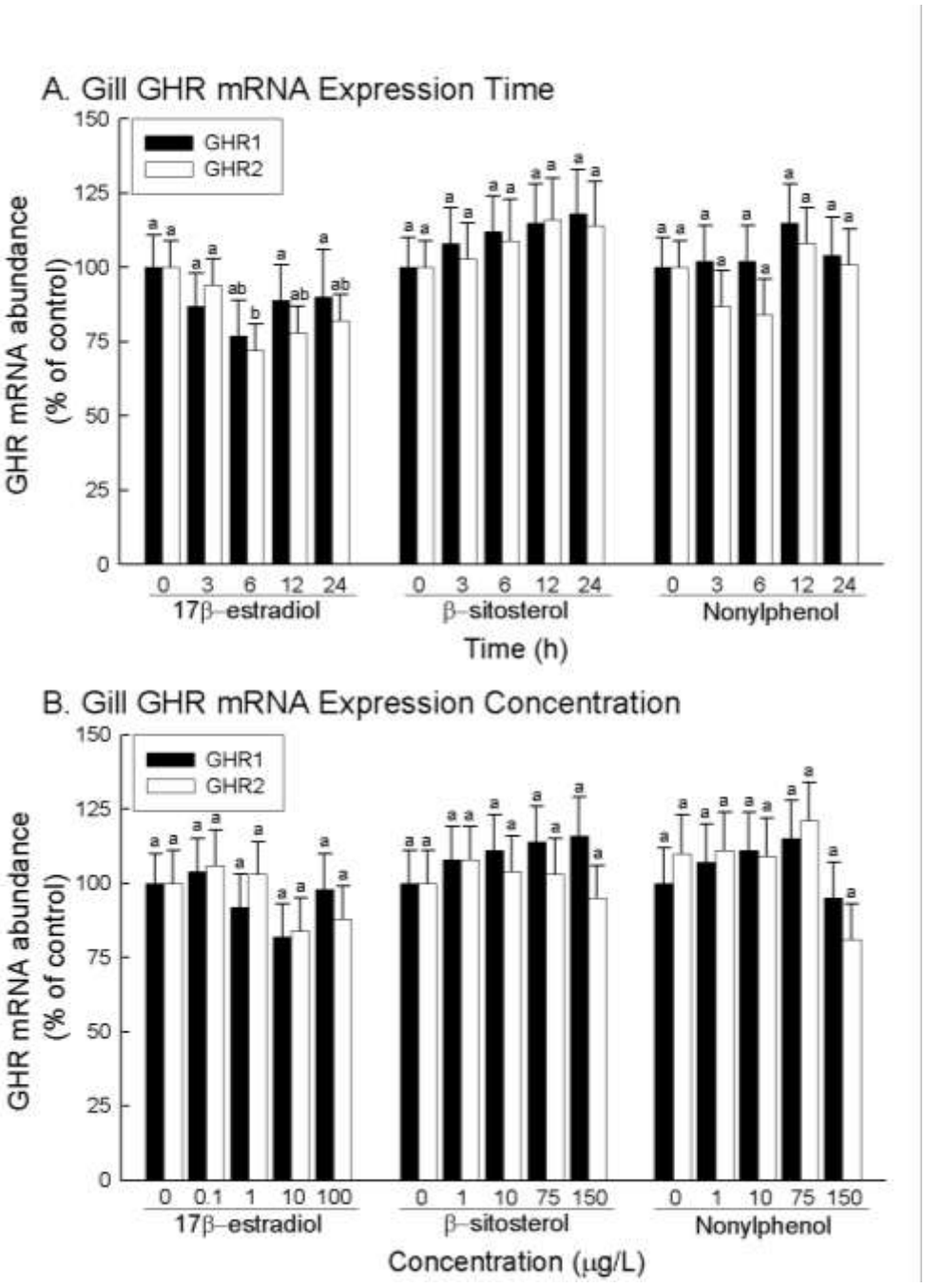

Figure 18. Effects of environmental estrogens (EE) on the abundance of gill filament growth hormone receptors (GHRs) in rainbow trout incubated *in vitro*. Gill filaments were incubated for (A) various times with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L 4-nonylphenol (NP) or (B) various concentrations for 6 h. Data are presented as mean ± SEM (n=6). For a given GHR subtype within a treatment, means with different letters are significantly different ($p < 0.05$) different from each other. * indicates a significant difference ($p < 0.05$) between two GHR subtypes within a treatment group.

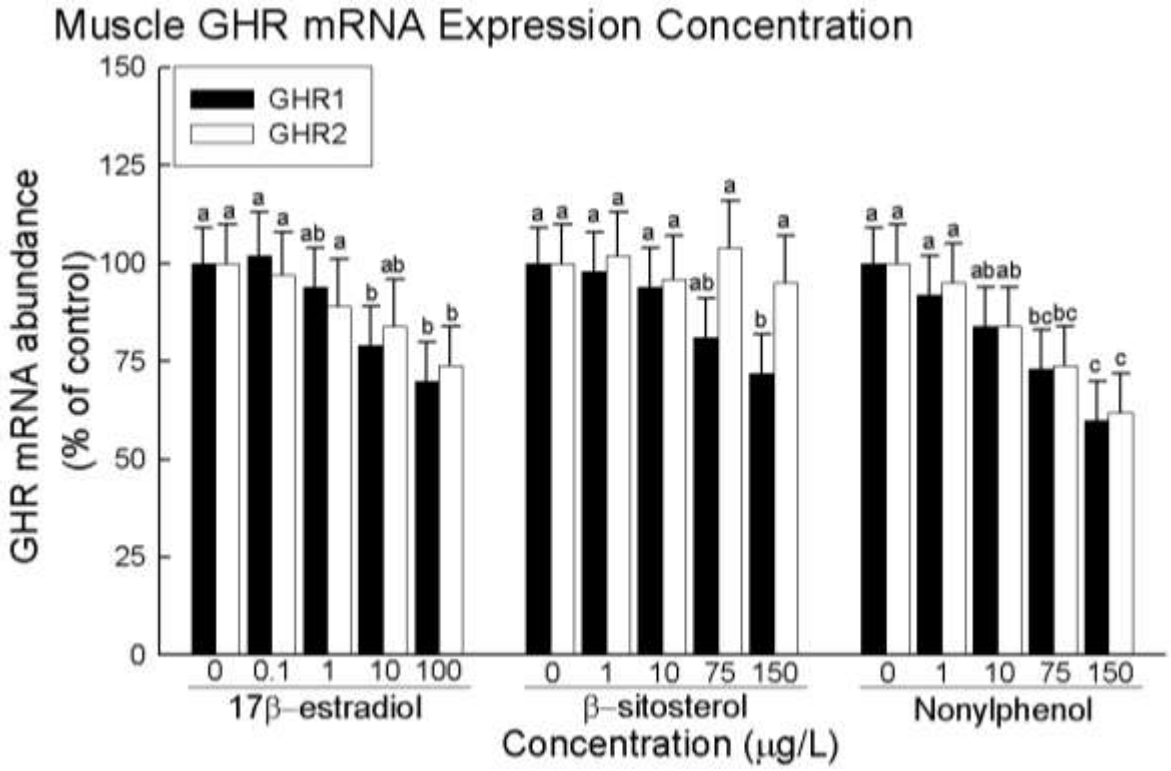

Figure 19. Effects of environmental estrogens (EE) on the abundance of the abundance of muscle growth hormone receptors (GHRs) in rainbow trout incubated *in vitro*. Muscle pieces were incubated for various concentrations for 6 h. Data are presented as mean ± SEM (n=6). For a given GHR subtype within a treatment, means with different letters are significantly different ($p < 0.05$) different from each other. * indicates a significant difference ($p < 0.05$) between two GHR subtypes within a treatment group.

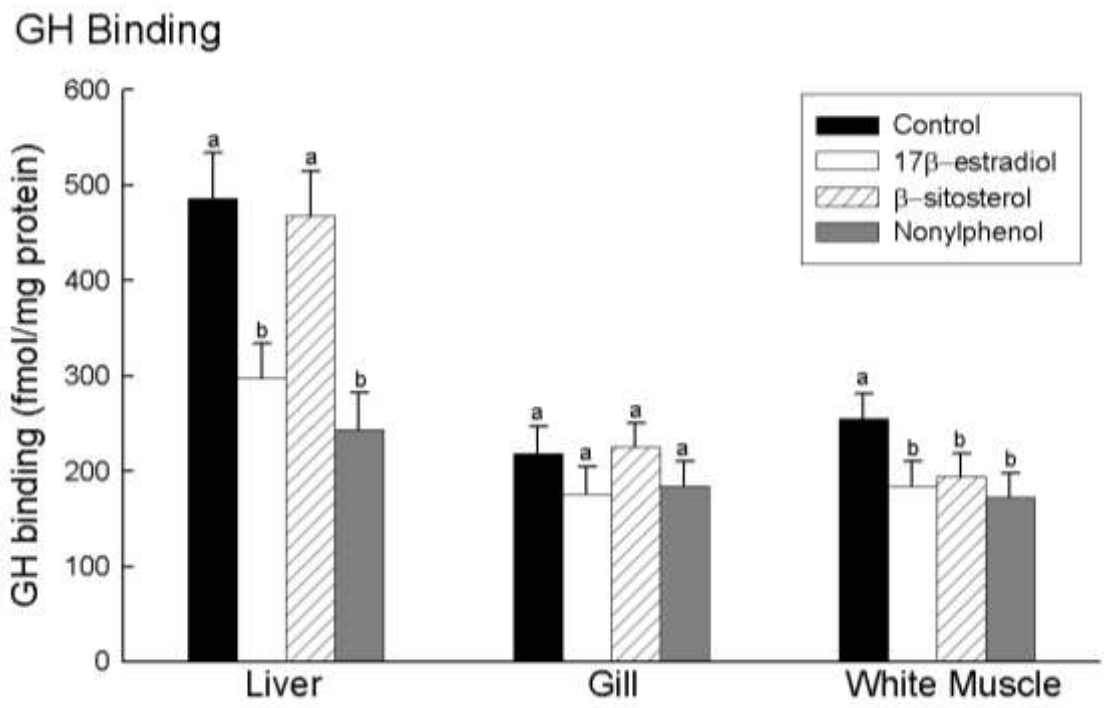

Figure 20. Effects of 17β-estradiol (E2), β-sitosterol (βS), or 4-nonylphenol (NP) on binding of ^{125}I-salmonid growth hormone (GH) to microsomes prepared from liver, gill filaments, and muscle of rainbow trout. Liver pieces, gill filaments, and muscle pieces were incubated *in vitro* with 10 µg/L 17β-estradiol (E2), 75 µg/L β-sitosterol (βS), or 75 µg/L 4-nonylphenol (NP) for 6 h. Illustrated is the binding capacity (Bmax) presented as mean ± SEM (n=6). For a given tissue, means with different letters are significantly ($p < 0.05$) different from each other.

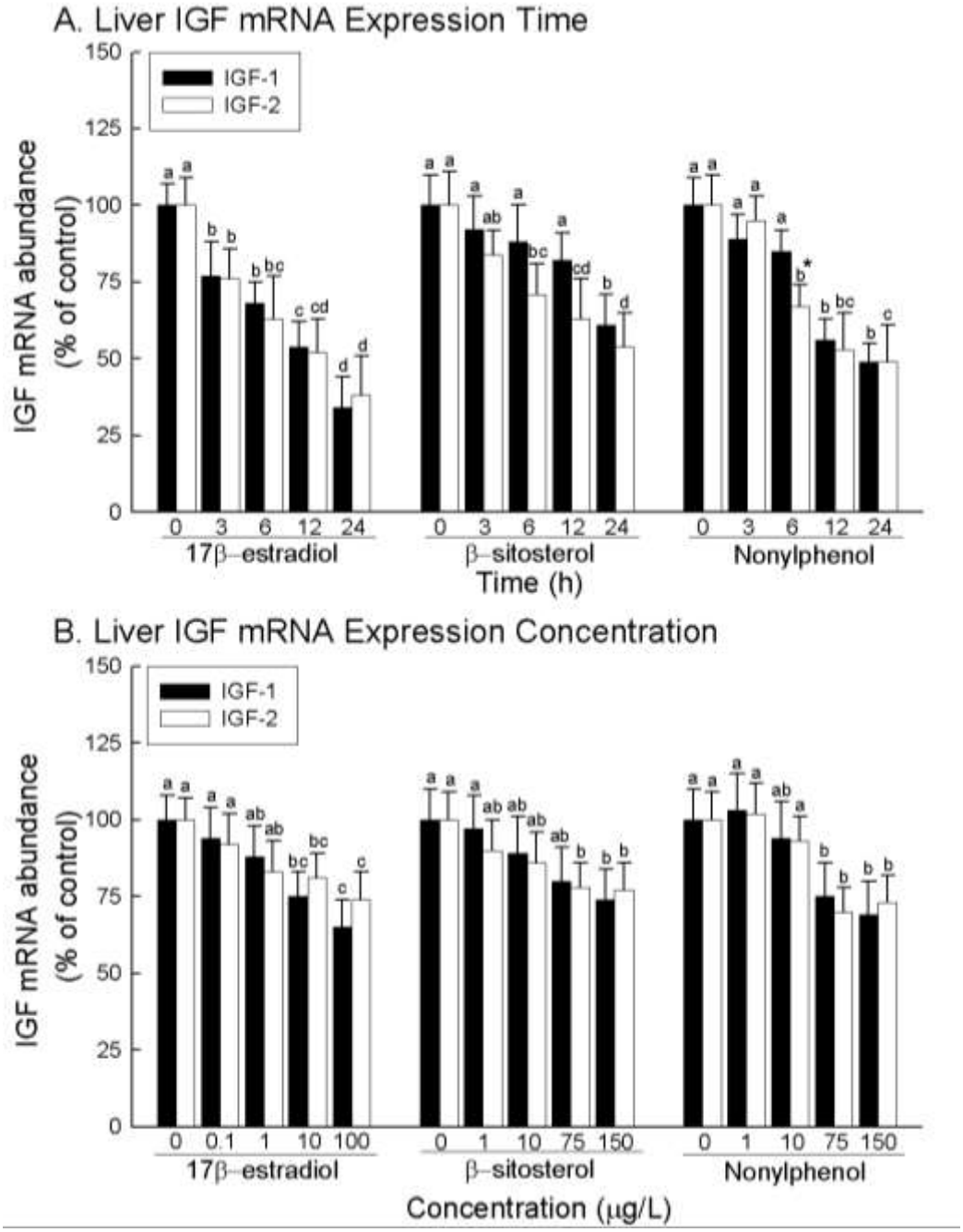

Figure 21. Effects of environmental estrogens (EE) on the abundance of the abundance of hepatic insulin-like growth factors (IGFs) in rainbow trout incubated *in vitro*. Liver pieces were incubated for (A) various times with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L 4-nonylphenol (NP) or (B) various concentrations for 6 h. Data are presented as mean ± SEM (n=6). For a given IGF type within a treatment, means with different letters are significantly different ($p < 0.05$) different from each other. * indicates a significant difference ($p < 0.05$) between two IGFs within a treatment group.

49% (NP) of control values (Fig. 21A). Similarly, expression of IGF-1 and IGF-2 mRNA was suppressed in a concentration-dependent manner for all three EE with maximum inhibition observed at 100 μg/L E2, 150 μg/L βS, and 150 μg/L NP (Fig. 21B). In gill, similar results were observed, with suppression of IGF-1 and IGF-2 generally apparent after 3 h and maximum suppression levels reached at 24 h (Fig. 22A) for E2- and NP-treated groups. In E2-treated fish, significant decreases in IGF-1 and IGF-2 were evident by 1 μg/L E2 and persisted through 100 μg/L E2 (Fig. 22B). In βS-treated fish IGF-1 was significantly down-regulated at concentrations of 75 μg/L and 150 μg/L βS (Fig. 22B). In NP-treated fish, IGF-1 and IGF-2 mRNA abundance was significantly lowered through the entire concentration course (Fig. 22B).

Effects of EE on mRNA and Functional Expression of IGFRs

Two types of IGFR1s, IGFR1A and IGFR1B, are expressed in gill and white muscle. Levels of IGFR1A and IGFR1B mRNA declined in a similar manner for E2- and NP-treated gill filaments, with maximum depression observed at 24 h (Fig. 23A). In E2-treated filaments, IGFR1A mRNA expression decreased to 61% of the control at 100 μg/L and NP-treated filaments showed a 65% decrease in IGFR1A mRNA expression when compared to control levels; βS, however, remain unchanged (Fig. 23A). IGFR1B mRNA expression was only depressed in the E2-treated gill filaments in the concentration course (Fig. 23B). IGFR1A expression significantly decreased in E2- and NP-treated muscle pieces at the highest concentrations, whereas IGFR1B mRNA expression was only significantly affected in NP-treated muscle tissue (Fig. 24).

Functional expression of IGFR was determined by ^{125}-I-IGF binding to microsomes. Binding capacity in gill microsomes was reduced significantly in NP-treated tissue (Fig. 25). Although

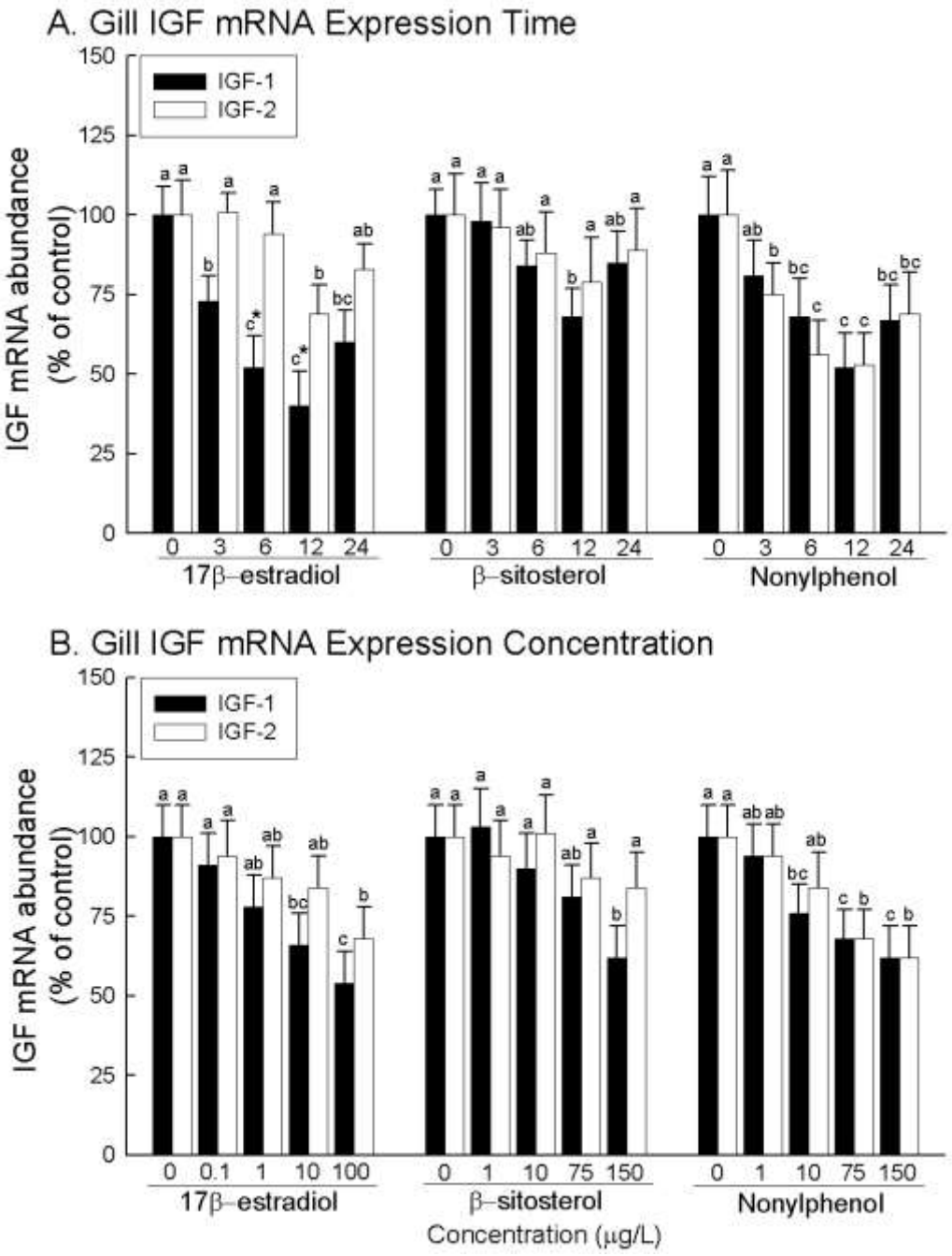

Figure 22. Effects of environmental estrogens (EE) on the abundance of the abundance of gill filament insulin-like growth factors (IGFs) in rainbow trout incubated *in vitro*. Gill filaments were incubated for (A) various times with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L 4-nonylphenol (NP) or (B) various concentrations for 6 h. Data are presented as mean ± SEM (n=6). For a given IGF type within a treatment, means with different letters are significantly different ($p < 0.05$) different from each other. * indicates a significant difference ($p < 0.05$) between two IGFs within a treatment group.

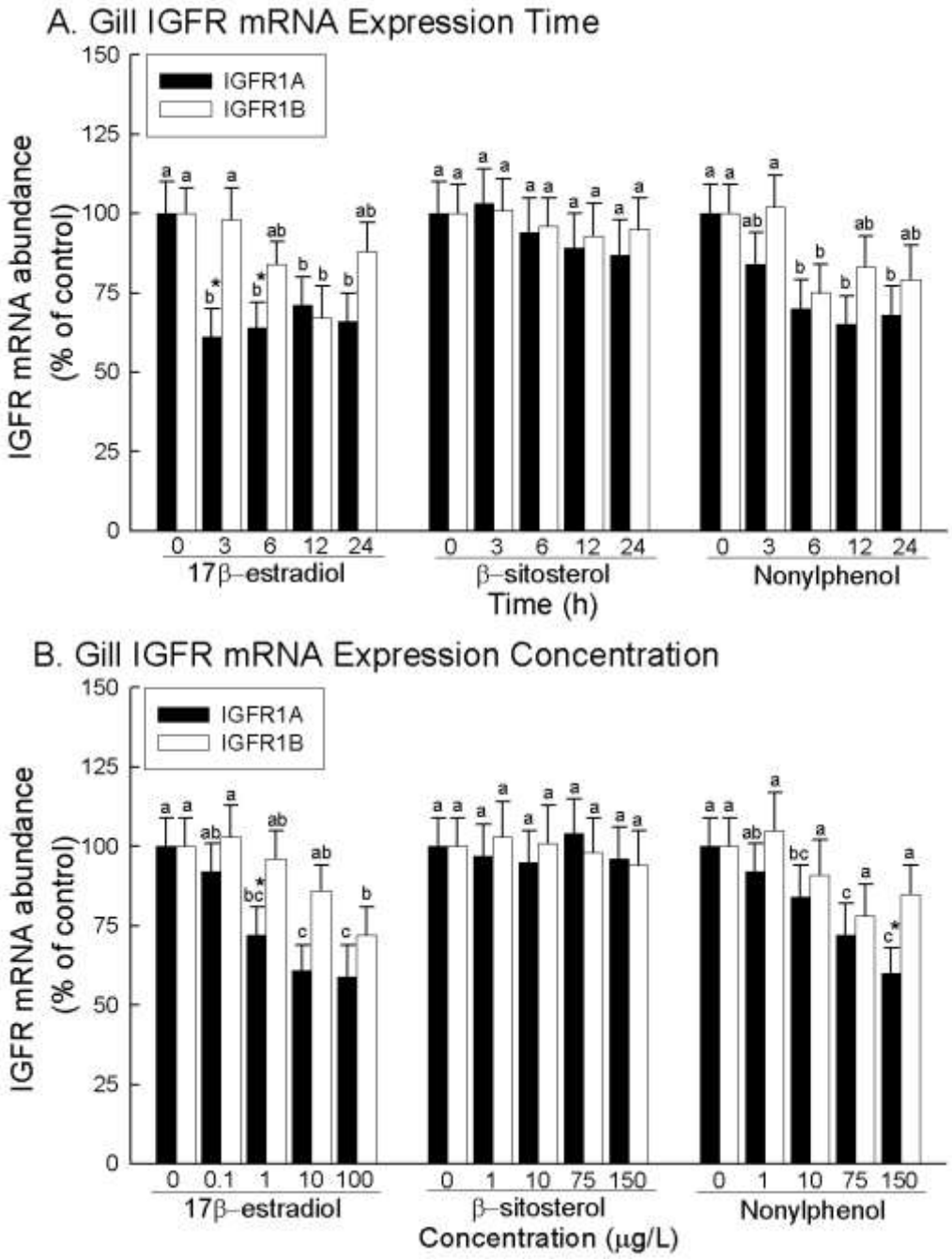

Figure 23. Effects of environmental estrogens (EE) on the abundance of the abundance of gill filament insulin-like growth factor receptors (IGFRs) in rainbow trout incubated *in vitro*. Gill filaments were incubated for (A) various times with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L 4-nonylphenol (NP) or (B) various concentrations for 6 h. Data are presented as mean ± SEM (n=6). For a given IGFR subtype within a treatment, means with different letters are significantly different ($p < 0.05$) different from each other. * indicates a significant difference ($p < 0.05$) between two IGFR subtypes within a treatment group.

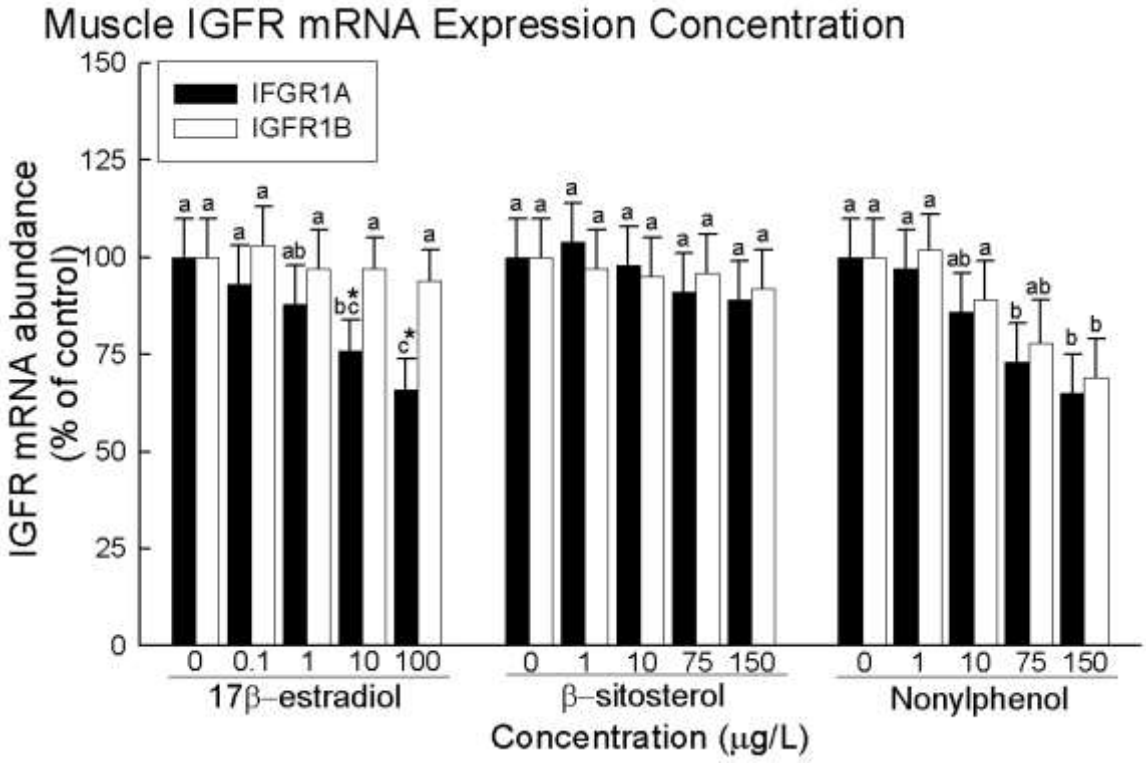

Figure 24. Effects of environmental estrogens (EE) on the abundance of the abundance of muscle insulin-like growth factor receptors (IGFRs) in rainbow trout incubated *in vitro*. Muscle pieces were incubated for various concentrations for 6 h. Data are presented as mean ± SEM (n=6). For a given IGFR subtype within a treatment, means with different letters are significantly different ($p < 0.05$) different from each other. * indicates a significant difference ($p < 0.05$) between two IGFR subtypes within a treatment group

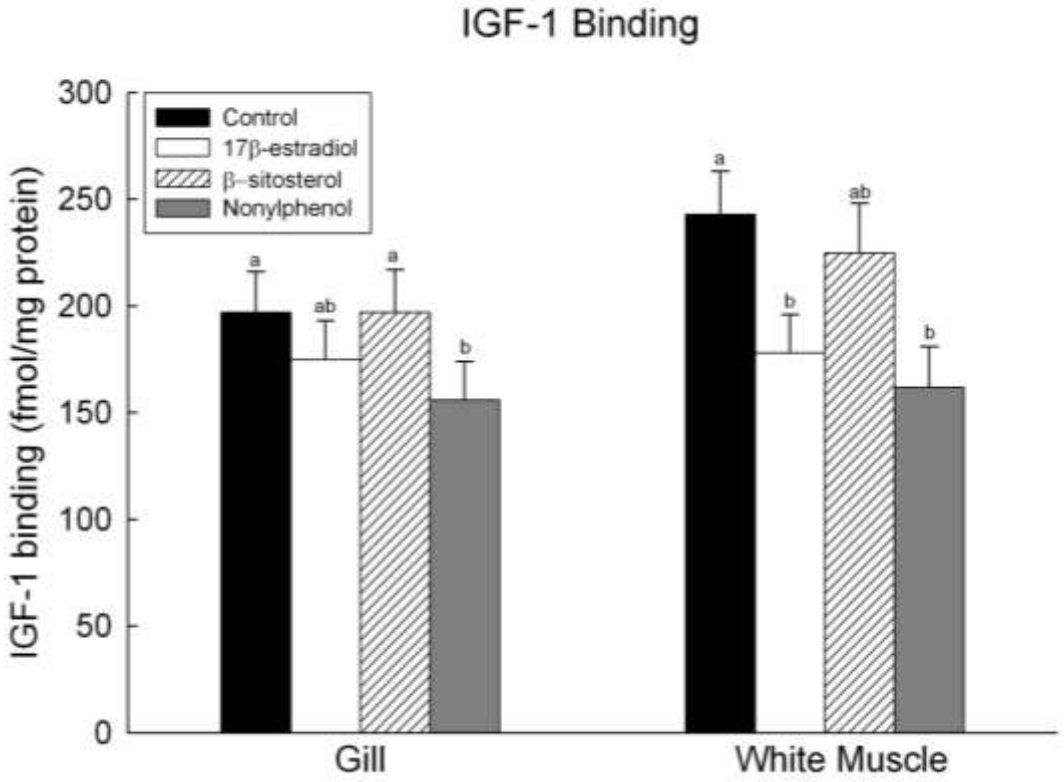

Figure 25. Effects of 17β-estradiol (E2), β-sitosterol (βS), or 4-nonylphenol (NP) on binding of ^{125}I-insulin-like growth factor (IGF) to microsomes prepared from gill filaments and muscle of rainbow trout. Gill filaments and muscle pieces were incubated *in vitro* with 10 µg/L 17β-estradiol (E2), 75 µg/L β-sitosterol (βS), or 75 µg/L 4-nonylphenol (NP) for 6 h. Illustrated is the binding capacity (Bmax) presented as mean ± SEM (n=6). For a given tissue, means with different letters are significantly ($p < 0.05$) different from each other.

suppressed, binding capacity in E2- and βS-treated tissues was not significantly decreased. Binding capacity in white muscle microsomes significantly decreased to 73% (E2) and 67% (NP) of control values (Fig. 25). EE treatment had no effect on binding affinity in gill or white muscle (data not shown).

Discussion

This study examined the influence of three EE, E2, βS, and NP, on the GH-IGF system in rainbow trout. Previously, we discovered that EE can decrease organismal growth in rainbow trout, which was in agreement with observations in platyfish and swordtail exposed to EE such as nonylphenol (Magliulo, et al., 2002). In order to further investigate this decrease in organismal growth, we examined the effects of EE on the GH-IGF system. The results of our study indicate that EE have a direct effect on growth at the molecular level in a tissue- and compound-dependent manner, lending support to our hypothesis that EE affect growth at the level of GH sensitivity, IGF synthesis, and IGF sensitivity.

As a biological indicator of exposure to EE, vitellogenin mRNA levels were measured (Kime et al., 1999). Our results indicate that the tissue cultures were exposed and responsive to EE. The ability of EE to modulate growth at the level of the GH-IGF axis was supported by several observations. First, E2 and NP were able to decrease steady-state levels of GHR-encoding mRNAs in liver; however, all three EE decreased steady-state levels of GHR1 and GHR2 in muscle. These effects were not uniform, as EE had no effect on expression of GHR1 or GHR2 mRNA in gill, which is in agreement with previous findings in our lab (Norbeck et al., 2011). Functional expression of GHRs, as assessed by ^{125}I-GH binding, was also significantly depressed in liver (following E2 and NP treatment) and muscle (following treatment with all three EE), while no effect was observed in gill, which is consistent with the mRNA data obtained. Second,

EE were able to suppress IGF-1 and IGF-2 mRNAs in liver and gill in both a time-and concentration-dependent manner, with the exception of IGF-2 mRNA abundance in βS-treated tissue. Lastly, EE were able to modulate IGFR1A and IGFR1B mRNA expression. These effects were most pronounced in IGFR1A for E2- and NP-treated gill and muscle tissue. E2 and NP were also able to significantly reduce binding capacity as assessed by ^{125}-I-IGF binding to IGFR. Collectively, these data indicate that EE may reduce sensitivity to GH, production of IGFs, and sensitivity to IGFs. These effects are not only compound specific, but also tissue- and isoform-specific. This may be the result of differential expression of receptors (e.g., GHRs, IFGRs) in teleosts with either similar or distinct function (Very et al., 2005; Saera-Vila et al., 2007).

Our results are in agreement with previous studies that have examined the effects of EE on different aspects of growth. For example, *in vitro* exposure of tilapia and rainbow trout to 17β-estradiol was shown to decrease expression of IGF-1 and IGF-2 mRNA in hepatocytes (Riley et al., 2004; Davis et al., 2008; Norbeck et al., 2011). Similarly, tilapia exposed to 17α-ethinylestradiol (EE2) had decreased levels of IGF-1 expression in liver (Shved et al., 2008). In the study by Shved and colleagues, GH mRNA was also inhibited by treatment with EE2. *In vivo* studies have also demonstrated a decrease in hepatic IGF-1 mRNA levels in fathead minnows exposed to E2 (Filby et al., 2006). Additionally, plasma levels of circulating IGF-1 were reduced in salmon and tilapia exposed to E2 or nonylphenol (Arsenault et al., 2004; Riley et al., 2004; McCormick et al., 2005; Davis et al., 2008), whereas GH plasma levels were either unaffected or increased in tilapia and rainbow trout exposed to E2 (Holloway & Leatherland, 1997; Davis et al., 2008). In mammals GHR expression appears to be estrogen-regulated in a tissue-dependent manner. For example, in rats treatment with estrogen has been shown to decrease brain expression of GHR mRNA (Bennet et al., 1996). However, a time- and concentration-dependent

increase in GH gene expression was observed in rat pituitary GH3 cells exposed to NP (Dang et al., 2009). The expression level differences between tissues and species may result from differential expression of the ER subtypes (Leung et al., 2004). Differences in GH and IGF mRNA expression may also be due to different stages of sexual maturation (and therefore different levels of endogenous estrogens), as both suppression and enhancement of GH-IGF elements have been observed in teleosts, particularly in gonadal tissues, depending on state of maturation (Gioacchini et al., 2005).

The interaction of EE with the GH-IGF system is complex, as multiple isoforms of ligands, receptors, and binding proteins exist. It is possible that EE can act not only directly on the GH-IGF axis (i.e., GH sensitivity, IGF sensitivity) to affect growth, but evidence also shows that binding proteins may be affected by EE. For example, Riley et al. (2004) found that two isoforms of IGF binding proteins in tilapia were differentially affected following E2 treatment, which was sex-dependent. Additionally, studies have shown that EE are capable of affecting thyroid hormone (Filby et al., 2006) in fish. As thyroid hormone is known to play a role in growth, this may be another avenue by which EE are eliciting effects on the GH-IGF axis.

Future studies should focus on elucidating the points of cross-talk between the estrogen (and other EE) and growth signaling pathways. Both βS and NP have been shown to be capable of binding to the estrogen receptor (Tremblay & Van Der Kraak, 1998) thereby forming an active transcription factor capable of binding to genes possessing an estrogen response elements. In addition, estrogen has been shown to interact with GHR signaling pathways (JAK-STAT, Erk, PI3K/Akt) (Leung et al., 2004). Interference with the JAK-STAT pathway, which plays a critical role in IGF-1 expression, and/or the Erk and PI3K/Akt pathways, which are necessary for IGFR action, could lend support to the observed effects of EE on organismal growth.

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CHAPTER 4: ENVIRONMENTAL ESTROGENS MODULATE THE GROWTH HORMONE-INSULIN-LIKE GROWTH FACTOR SYSTEM VIA SUPPRESSION OF GROWTH-RELATED SIGNALING CASCADES

Abstract

Endocrine disrupting compounds (EDCs) generally act by binding to and activating the estrogen receptor, thus eliciting estrogen-like effects in an organism. While there is growing evidence to support the observation that EDCs possess growth-inhibiting effects, the mechanisms by which these physiological effects occur are poorly understood. Further complicating the elucidation of these pathways is the apparent ability of estrogen and estrogen-like compounds to act in the classical genomic manner, as well as via the activation of nongenomic pathways. In this study, we used rainbow trout (*Oncorhynchus mykiss*) hepatocytes and gill filaments to assess the effects of EDCs, specifically environmental estrogens (EE), on growth-related signaling pathways *in vitro*. We examined the effects of 17β-estradiol (E2), β-sitosterol (βS), and 4-*n*-nonylphenol (NP) on the JAK-STAT, MAPK, and PI3K-Akt pathways at various concentrations (0-150 μg/L) and time points (0-180 min). Environmental estrogen treatment resulted in the deactivation of JAK-STAT, MAPK, and PI3K-Akt pathways in a time- and concentration-dependent manner. Maximum suppression for each pathway was rapid, typically occurring at 10 to 30 min. The effective concentrations for dephosphorylation of JAK-STAT, MAPK, and PI3K-Akt were compound- and tissue-dependent. The observed effects appeared to occur via an estrogen-dependent pathway, as indicated by treatment with an ER antagonist, ICI 182,780. Additionally, co-incubation with a pathway agonist (growth hormone) and EE linked the deactivation of these signaling pathways to suppressed growth hormone receptor 1 (GHR1), GHR2, insulin-like growth factor-1 (IGF-1), IGF-2, insulin-like growth factor receptor 1A

(IGFR1A), and IGFR1B mRNA expression levels. In agreement with the observed results, suppressor of cytokine signaling 2 (SOCS-2) mRNA expression was elevated. Collectively, these findings suggest biological pathways by which EE may regulate growth-related gene expression.

Introduction

Endocrine disrupting compounds (EDCs) are exogenous compounds found in an assortment of common materials such as pesticides, personal care products, plastics, and detergents that alter the normal physiology of an organism or its progeny. The increased production, use, and disposal of an expanding array of EDCs has led to increased introduction of these compounds into the environment, which is of concern because of their well-known detrimental effects (e.g., tumorigenesis, reproductive abnormalities, etc.). Environmental estrogens (EE) are a specific class of EDCs that include endogenous and synthetic animal estrogens (e.g., 17β-estradiol), phytoestrogens (β-sitosterol), mycotoxins (e.g., zearalenone), organochlorine pesticides (e.g., DDT), polychlorinated biphenyls (PCBs), and alkylphenol polyethoxylates (APEs; e.g., 4-nonylphenol) (Turner, 1999).

The growth hormone (GH)-insulin-like growth factor-1 (IGF-1) system modulates a number of physiological processes such as growth, cellular differentiation, metabolism, reproduction, immune function, and behavior (Bjornsson et al., 2004; Duan, 1997; Hull & Harvey, 2000; Madsen & Bern, 1993; Mancera & McCormick, 1998; Norrelund, 2005). The GH-IGF system primarily regulates growth at the cellular level. Briefly, GH is secreted from the pituitary and circulates throughout the organism bound to binding proteins, where it can then bind to its receptors (GHRs) primarily on the liver (Butler & LeRoith, 2001; Wood et al., 2005) to activate signaling pathways including JAK-STAT, MAPK, and PI3K and subsequently induce the synthesis and secretion of IGF-1 (Reindl et al., 2011). Additionally, IGF-1 can be secreted to a

lesser extent in tissues such as gill and muscle. IGF, once bound to its receptors (IGFRs) can stimulate cellular growth, proliferation, and differentiation in target tissues via activation of pathways including MAPK and PI3K.

Gonadal steroids, such as estrogen, have been shown to affect somatic growth in vertebrates (Leung et al., 2004) by modulating components of the GH-IGF system. For example, studies have shown that *in vivo* treatment with 17β-estradiol can cause an increase in plasma GH in rainbow trout and tilapia (Holloway and Leatherland, 1997; Melamed et al., 1995). The interplay between estrogen and the GH-IGF system has also been observed at the level of IGF-1 expression, as 17β-estradiol has been shown to decrease hepatic IGF-1 expression in tilapia, salmon, and gilthead sea bream (Davis et al., 2007; Riley et al., 2004; Lerner et al., 2007; Carnevali et al., 2005) and IGF-1 serum levels in tilapia and salmon (Davis et al., 2007; Lerner et al., 2007). In addition, we previously observed that both *in vivo* and *in vitro* treatment with 17β-estradiol causes a decrease in both GHR and IGFR synthesis and sensitivity, as well as IGF synthesis (Hanson et al., *in press*).

While EE are now known to modulate growth at the level of the GH-IGF axis, very little has been reported regarding the cellular mechanisms behind these actions. Both β-sitosterol and 4-*n*-nonylphenol have been shown to be capable of binding to the estrogen receptor (Tremblay & Van der Kraak, 1998) thereby forming an active transcription factor capable of binding to genes possessing an estrogen response element. In addition, estrogen has been shown to interact with GHR signaling pathways (JAK-STAT, Erk, PI3K/Akt) and proteins such as suppressor of cytokine signaling-2 (SOCS-2) (Leung et al., 2004). While steroidal compounds are known to work via classic genomic mechanisms, evidence has also emerged indicating that EE can activate rapid nongenomic responses in the cell at low levels (Bulayeva and Watson, 2004). This study

chose rainbow trout as a model system to examine the mechanistic effects of EE on growth at the level of the GH-IGF system because of their well-characterized GH-IGF signaling system and their multiple isoforms of hormones as well as receptor subtypes (Reinecke et al., 2005; Klein and Sheridan, 2008). Specifically, the aim of the present study was to further elucidate the molecular mechanisms of EE (specifically 17β-estradiol, β-sitosterol, and 4-n-nonylphenol) that are responsible for the suppression of the GH-IGF system (i.e., GH sensitivity, IGF sensitivity) and subsequently, organismal growth.

Materials and Methods

Materials

17β-estradiol, DMSO, and an ER antagonist (ICI 182, 780) were purchased from Sigma (St. Louis, MO, USA), β-sitosterol was purchased from Calbiochem (San Diego, CA), and 4-*n*-nonylphenol was purchased from AlfaAesar (Ward Hill, MA). GH was purchased from GroPep, Ltd. (Adelaide, Australia). Phospho-specific and total antibodies for MAPK, Akt, Jak2, STAT5, horseradish peroxidase-linked anti-rabbit IgG antibody, and cell lysis buffer were obtained from Cell Signaling Technology (Beverly, MA, USA). Molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA, USA).

Experimental Animals

Juvenile rainbow trout of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, USA and transported to North Dakota State University. Fish 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 and were fed to satiation twice daily with AquaMax Grower (PMI Nutrition International, Inc., Brentwood, MO), except 24 before

experimental manipulations. All procedures were performed in accordance approved by the North Dakota State University Institutional Animal Care and Use Committee.

Experimental Conditions

To investigate the signaling pathways disrupted by EE, fish (ca. 30 g) were anesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol, measured, weighed and bled via the severed caudal vessel. Tissues (gill arches, liver) were removed, perfused *ex vivo* with 0.75% (w/v) saline solution, and stored in ice-cold Hank's Buffered Salt Solution (in mM: 137 NaCl, 5.4 KCl, 4 $NaHCO_3$, 1.7 $CaCl_2$, 0.8 $MgSO_4$, 0.5 KH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, and 4 glucose; pH 7.6). Livers were cut into 1 mm^3 pieces while immersed in Hank's on ice. Additionally, individual gill filaments were dissected from the arches while immersed in Hank's on ice. All tissues were pooled and washed three times with Hank's Balanced Salt Solution with 0.24% BSA, 2 ml Gibco MEM amino acid mix (50x)/100 mL, and 1 mL Gibco nonessential amino acid mix (100x)/100 mL. Tissues were plated in 24-well culture plates (8-10 liver pieces, 12-15 gill filaments) containing 1-mL of Hank's medium with 0.24% BSA, 2 ml Gibco MEM amino acid mix (50x)/100 mL, and 1 mL Gibco nonessential amino acid mix (100x)/100 mL and preincubated at 14 °C under 100% O_2 while being and shaken at 100 rpm in a gyratory shaker. For the first experiment, following the 3 h preincubation, media was removed and tissues were subjected to either a time-course or a concentration-course of three estrogenic compounds: 17β-estradiol (Sigma, St. Louis, MO), β-sitosterol (Calbiochem, San Diego, CA) and 4-*n*-nonylphenol (AlfaAesar, Ward Hill, MA). All compounds were dissolved in ethanol; the final concentration of ethanol did not exceed 0.007%. For the dose course the following treatments were administered for all tissues: E2 (0.1 µg/l, 1.0 µg/l, 10 µg/l, 100 µg/l), βS (1.0 µg/l, 10 µg/l, 75 µg/l, 150 µg/l), and NP (1.0 µg/l, 10 µg/l, 75 µg/l, 150 µg/l) for 30 min. A time course of 0 min,

5 min, 10 min, 30 min, 60 min, and 180 min was used to examine rapid signaling effects at concentrations of 10 µg/l (E2) or 75 µg/l (βS, NP) on gill and liver. Following incubation, tissues were immediately placed on dry ice and stored at -80° C for later mRNA and western analyses.

For the second experiment, following the 3 h preincubation, media was removed and each of the tissues was subjected to treatment with or without an ER antagonist, ICI 182,780 (10^{-6} M), for 2 h. Following the 2 h incubation period, EE (E2, βS, NP) treatment was administered at concentrations of 10 µg/L (E2), 75 µg/L (βS), and 75 µg/L (NP). The control group received DMSO (Sigma, St. Louis, MO). For mRNA analysis, tissues were collected 6 h after EE treatment.

For the third experiment, following the 3 h preincubation, media was removed and the hepatocytes and gill filaments were co-incubated with a pathway agonist, growth hormone (GH) (Reindl et al., 2011), at 100 ng/mL, and EE (E2, βS, NP) at concentrations of 10 µg/L (E2), 75 µg/L (βS), and 75 µg/L (NP) for 15 min for signaling analysis. Following incubation, tissues were immediately placed on dry ice and stored at -80° C for later western analyses.

Quantitative Real-Time PCR

Frozen tissues were homogenized and total RNA was extracted using RNAzol reagent ® (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNA pellets were dissolved in 75 µL RNase-free deionized water. Total RNA was quantified by UV (A_{260}) spectrophotometry and then diluted with RNase-free deionized water to 200 ng/µl. RNA was reverse transcribed in a 10-µl reaction using 200 ng total RNA with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA) as described by the manufacturer's protocol.

As previously described (Very et al., 2005; Poppinga et al., 2007; Malkuch et al., 2008), steady-state mRNA levels of GHR1, GHR2, 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). Real-time PCR reactions were carried out for controls, standards, and samples in a 10 µl total volume (5 µl 2X Brilliant Master Mix; 0.5 µl of each gene-specific probe, forward primer, and reverse primer at concentrations optimized for each RNA species). Cycling parameters were set as follows: 95° C for 10 min, and 50 cycles of 92° C for 15 s plus 57° 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 following normalization to β-actin.

ABI Primer Express® Version 2 software was used to design SOCS-2-specific oligonucleotide primers (forward: 5'-CACACAACGGGACGGTTCA-3'; reverse: 5'-CCTGGTGGCGTTGTTGATG-3') for real-time PCR based on known SOCS-2 sequence using and subsequently synthesized by Sigma-Genosys (The Woodlands, TX, USA). Steady-state mRNA levels of SOCS-2 were determined by quantitative real-time PCR using PerfeCTa SYBR® Green SuperMix (Quanta Biosciences, Gaithersburg, MD, USA). Real-time PCR reactions were carried out for controls, standards, and sampled in a 10 µl total volume (5 µL 2X SYBR Green SuperMix, 0.5 µL mixed primers, 2.5 µL nuclease free water). Cycling parameters were set as follows: 95° C for 3 min, 40 cycles of 95° C for 15 s plus 61° C for 45 sec, and 1 cycle of 95° C for 1 min, 55° C for 30 sec, and 95° C for 30 sec. Sample copy number was calculated from the threshold cycle number (C_T). Conditions were optimized for the dissociation curve indicating primer-dimer formation was absent.

Western Blotting

Activation of signaling pathways in hepatocytes and gill filaments was measured from experiment three by homogenizing the tissues with 100 µL 1 x cell lysis buffer (Cell Signaling Technology). Lysates were subsequently incubated on ice for 10 min and centrifuged at 16,000 g

for 10 min at 4° C. The protein concentration of the supernatant was determined using the Bradford protein assay for microplates (Bio-Rad Laboratories). Samples containing 50 µg total protein were then separated on a 7.5% SDS-PAGE gel and transferred to a 0.45 µm nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% nonfat milk in TBS-Tween20 (TBS-T) at room temperature for 1 h. Membranes were washed three times with TBS-T and incubated with the indicated rabbit antiserum (1:000) in 5% BSA TBS-T buffer overnight (4° C). Membranes were then washed with TBS-T and incubated at room temperature with goat-anti-rabbit IgG-HRP antiserum (1:2000) for 1 h, washed, and visualized using ECL detection system (GE Healthcare, Buckinghamshire, UK) and bands were quantified with a FluorChem FC2 imager (Alpha Innotech). Membranes were stripped under reducing conditions (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 for 30 min at 50° C with agitation), washed twice with a large volume of TBS-T, and the immunodetection protocol was repeated with the control antiserums. Antibodies were validated as previously described (Reindl et al., 2011).

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.

Results

EE Selectively Deactivate/Activate Signaling Pathways

The deactivation of biological signaling pathways by EE was studied in hepatocytes and gill filament lysates from fish tissues treated *in vitro* probed with phospho-specific and control antibodies. Phospho-JAK2, phosopho-STAT5, phospho-Akt, and phospho-Erk were all detected

in the treated hepatocytes and gill filaments. Environmental estrogens deactivated JAK2, STAT5, Akt, and MAPK in a concentration- and time-related manner (Fig. 26, Fig. 27, Fig. 28, Fig. 29). Maximum suppression of JAK2 and STAT5 was rapid and occurred within 10 min (Fig. 26, Fig. 28). Deactivation of Akt, a downstream target of PI3K, and Erk was less rapid, with the lowest degree of phosphorylation observed at 30 min and with suppressive effects persisting through 180 min (Fig. 27, Fig. 29). Progressively lower degrees of phosphorylation were observed following treatment with the concentration course (Fig. 26, Fig. 27, Fig. 28, Fig. 29). The observed effects were both compound- and tissue-dependent. Phosphorylation of JAK2 and STAT5 was significantly suppressed at concentrations as low as 10 μg/L (E2) and 1 μg/L (NP) in hepatocytes (Fig. 26). β-sitosterol, however, did not have significant concentration effects. Marginally more robust effects were observed in JAK2 and STAT5 phosphorylation levels in gill filaments, where phosphorylation was significantly inhibited at levels as low as 0.1 μg/L (E2) and 1 μg/L (NP) (Fig. 28). Akt and Erk similarly had reduced phosphorylation states in liver tissue, where values decreased to 61% (E2), 82% (βS), and 53% (NP) of control values for Akt, and 67% (E2), 78% (βS), and 63% (NP) of control values for Erk (Fig. 27). In gill filaments, treatment with E2, βS, and NP produced Akt phosphorylation states that were 64%, 87%, and 61%, of control values, respectively (Fig. 29). Similar results were observed for Erk phosphorylation where values were depressed to 65% (E2), 87% (βS), and 63% (NP) of control values at the highest treatment concentrations.

ER-Dependence and Linkage of Cell Signaling Elements to EE-inhibited Growth Components

The involvement of the JAK-STAT, PI3K-Akt, and MAPK pathways appeared to be ER dependent, as indicated by the co-incubation of EE and ICI 182,780 in liver and gill filaments. In

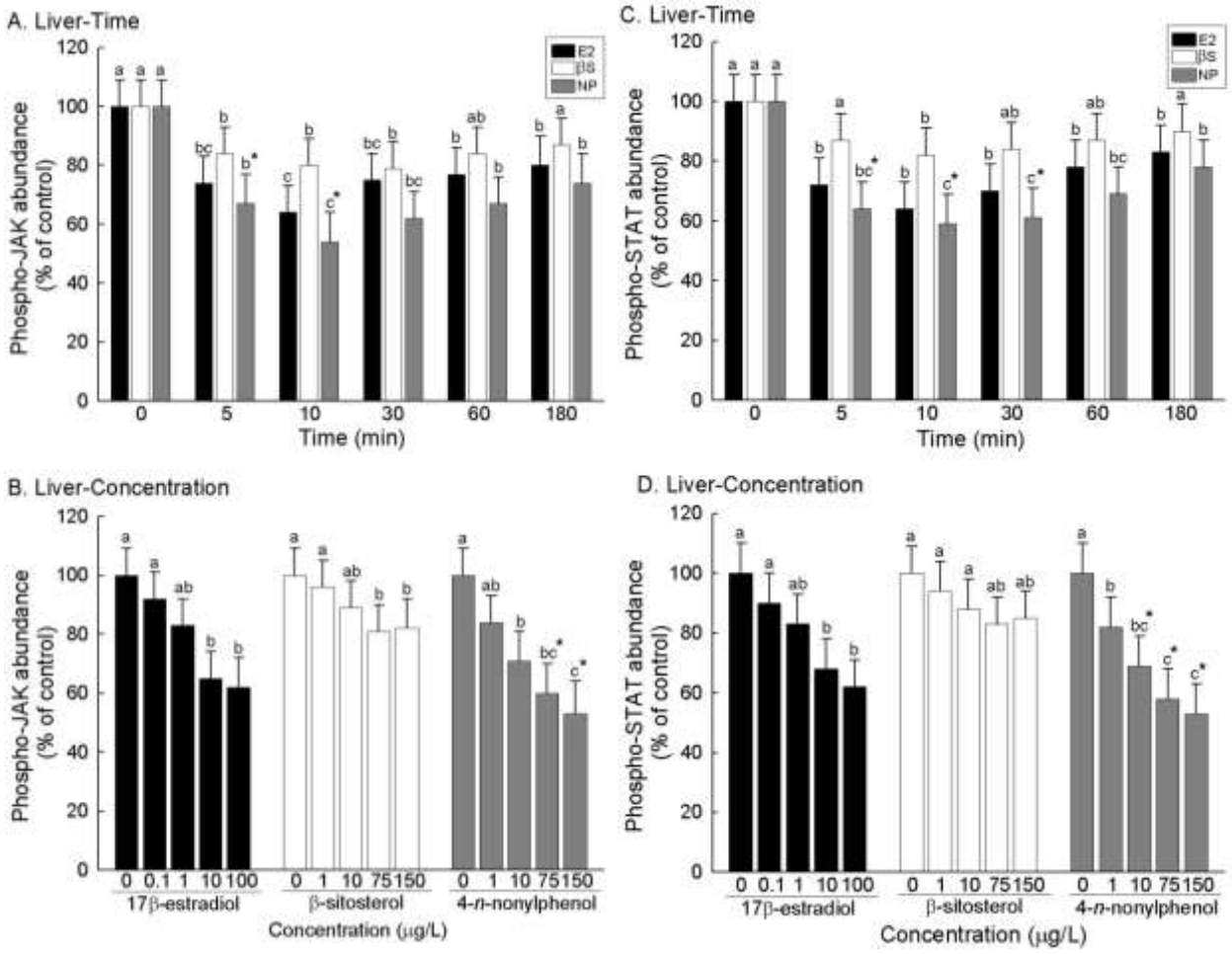


Figure 26. Effects of environmental estrogens (EE) on the abundance of phosphorylated JAK2 and STAT5 in hepatocytes. (A and C) Time-dependent phosphorylation of JAK2 and STAT5 respectively in cells incubated with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L nonylphenol (NP). (B and D) Concentration-dependent phosphorylation of JAK2 and STAT5 respectively in cells incubated in the presence of E2, βS, or NP for 30 min (control is 0 μg/L EE). Cell lysates were separated by SDS-PAGE followed by western immunoblotting and quantified with a FluorChem imager. The abundance of phosphorylated JAK2 and STAT5 was normalized to total JAK2 and STAT5. Data are presented as means ± SEM (n=4). Means with different letters are significantly different ($p < 0.05$).

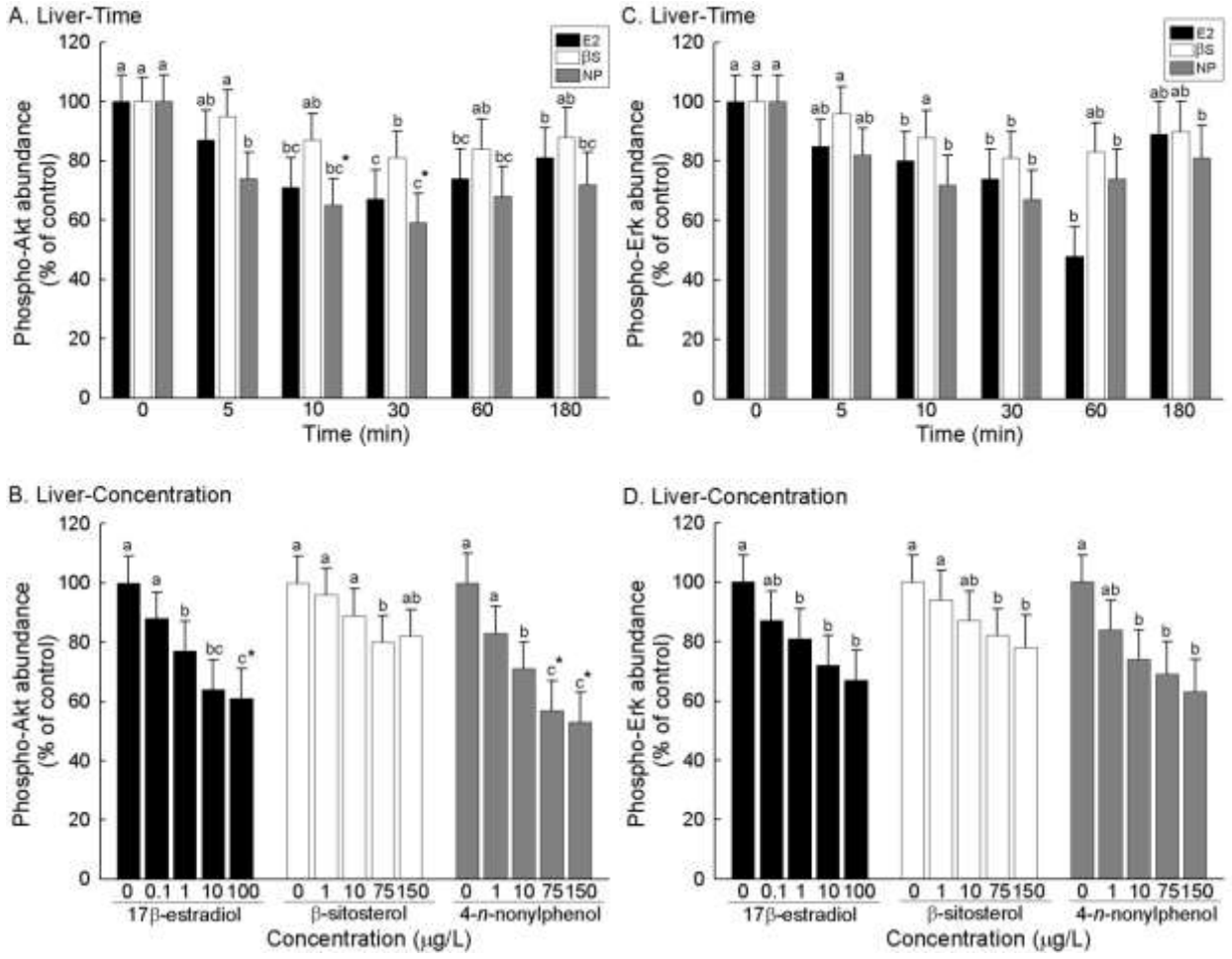


Figure 27. Effects of environmental estrogens (EE) on the abundance of phosphorylated Akt and Erk in hepatocytes. (A and C) Time-dependent phosphorylation of Akt and Erk respectively in cells incubated with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L nonylphenol (NP). (B and D) Concentration-dependent phosphorylation of Akt and Erk respectively in cells incubated in the presence of E2, βS, or NP for 30 min (control is 0 μg/L EE). Cell lysates were separated by SDS-PAGE followed by western immunoblotting and quantified with a FluorChem imager. The abundance of phosphorylated Akt and Erk was normalized to total Akt and Erk. Data are presented as means ± SEM (n=4). Means with different letters are significantly different ($p < 0.05$).

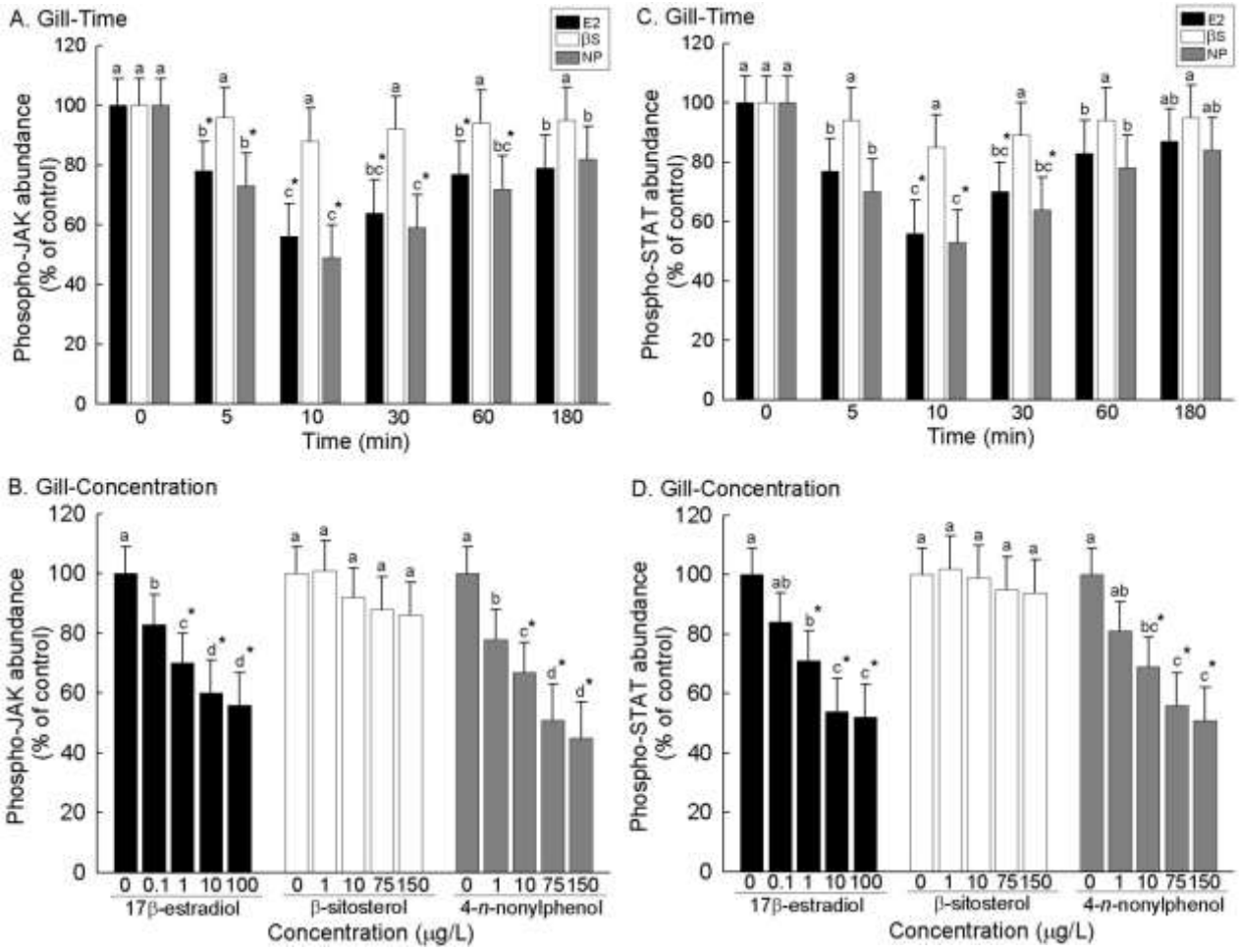


Figure 28. Effects of environmental estrogens (EE) on the abundance of phosphorylated JAK2 and STAT5 in gill filaments. (A and C) Time-dependent phosphorylation of JAK2 and STAT5 respectively in cells incubated with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L nonylphenol (NP). (B and D) Concentration-dependent phosphorylation of JAK2 and STAT5 respectively in cells incubated in the presence of E2, βS, or NP for 30 min (control is 0 μg/L EE). Cell lysates were separated by SDS-PAGE followed by western immunoblotting and quantified with a FluorChem imager. The abundance of phosphorylated JAK2 and STAT5 was normalized to total JAK2 and STAT5. Mean are presented as means ± SEM (n=4). Means with different letters are significantly different ($p < 0.05$).

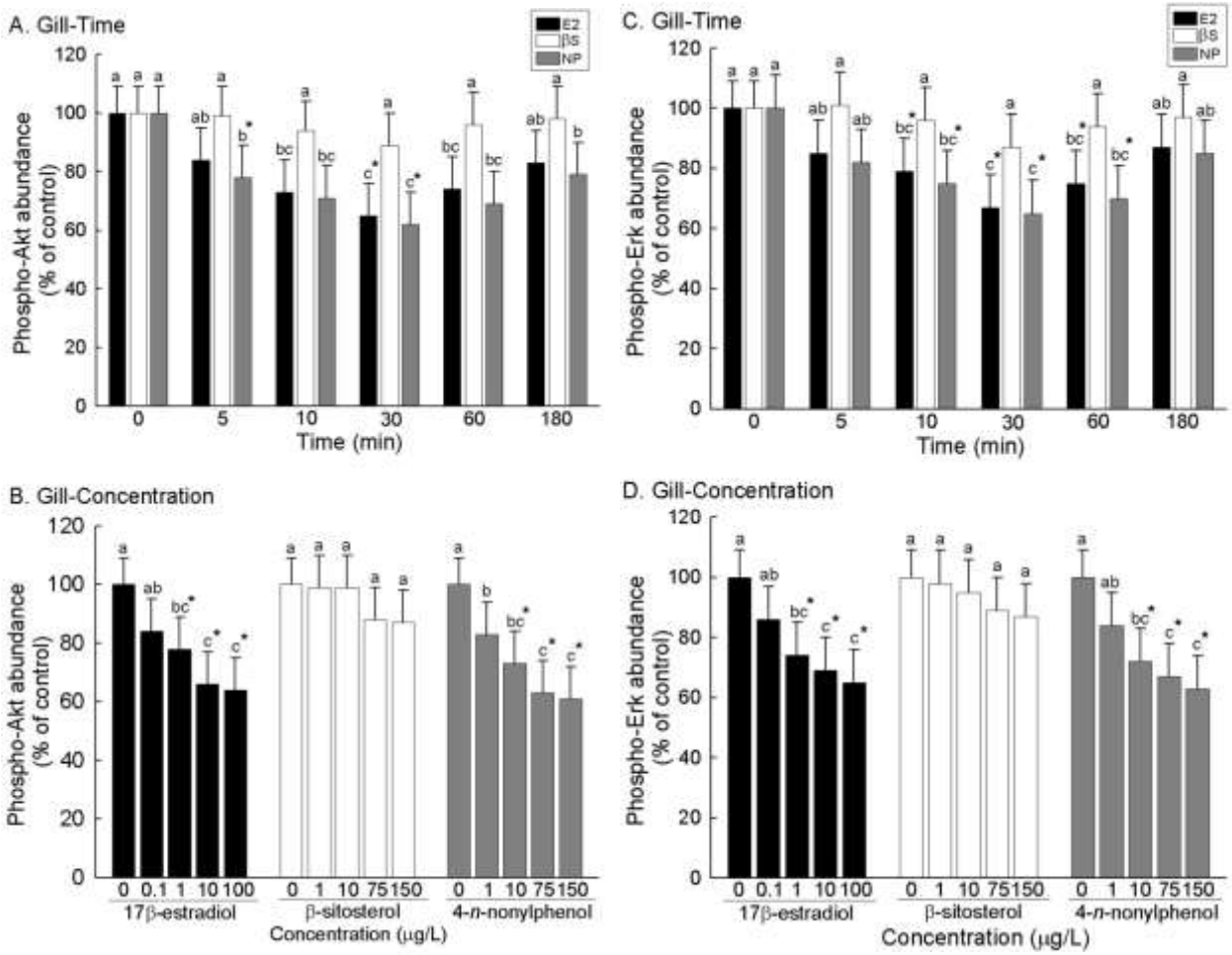


Figure 29. Effects of environmental estrogens (EE) on the abundance of phosphorylated Akt and Erk in gill filaments. (A and C) Time-dependent phosphorylation of Akt and Erk respectively in cells incubated with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L nonylphenol (NP). (B and D) Concentration-dependent phosphorylation of Akt and Erk respectively in cells incubated in the presence of E2, βS, or NP for 30 min (control is 0 μg/L EE). Cell lysates were separated by SDS-PAGE followed by western immunoblotting and quantified with a FluorChem imager. The abundance of phosphorylated JAK2 and STAT5 was normalized to total Akt and Erk. Data are presented as means ± SEM (n=4). Means with different letters are significantly different ($p < 0.05$).

hepatocytes, steady-state mRNA expression of GHR1 and GHR2 was suppressed in all of the EE-treated groups. The effects were most pronounced following E2 and NP treatment. Co-incubation with ICI 182,780, led to an increase in expression of both GHR1 and GHR2 to near control levels (Fig. 30). Similarly, IGFs were significantly suppressed to levels that were 69% (E2), 76 % (βS), and 71% (NP) of control values for IGF-1 and 71% (E2), 78 % (βS), and 75% (NP) of control values for IGF-2 when treated with EE alone. Treatment with ICI 182,780 restored IGF-1 mRNA expression to 98%, 102%, and 101% of control values following E2, βS, and NP treatment, respectively. Similarly, in the ICI 182,780-treated cells, IGF-2 steady-state mRNA expression levels increased to values that were 94% (E2), 104% (βS), and 97% (NP) of control values (Fig.30). SOCS-2 mRNA expression was additionally examined in liver where values increased to 124%, 108%, and 116% of control values following treatment of hepatocytes with E2, βS, and NP, respectively (Fig. 32).

In gill, the most pronounced effects were observed on IGF and IGFR mRNA expression (Fig. 31), where treatment with E2, βS, and NP significantly suppressed IGF and IGFR mRNA levels, an effect that could be reversed via treatment with ICI 182,780 co-incubation. Significant effects on GHR expression were observed only for E2-treated gill filaments, where levels decreased to 82% of the control value, and could be rescued to 98% of control values by ICI 182,780 treatment (Fig. 31). In gill, SOCS-2 mRNA expression increased following EE treatment to levels that were up to 168% (E2) of control values. Co-incubation with ICI 182,780 lead to a suppression of SOCS-2 mRNA expression to levels near control values (109% in E2-treated groups) (Fig. 32).

We examined the linkage of specific cell signaling pathways to EE-inhibited GH, IGF, and IGFR expression in hepatocytes and gill filaments. Following stimulation with a known pathway

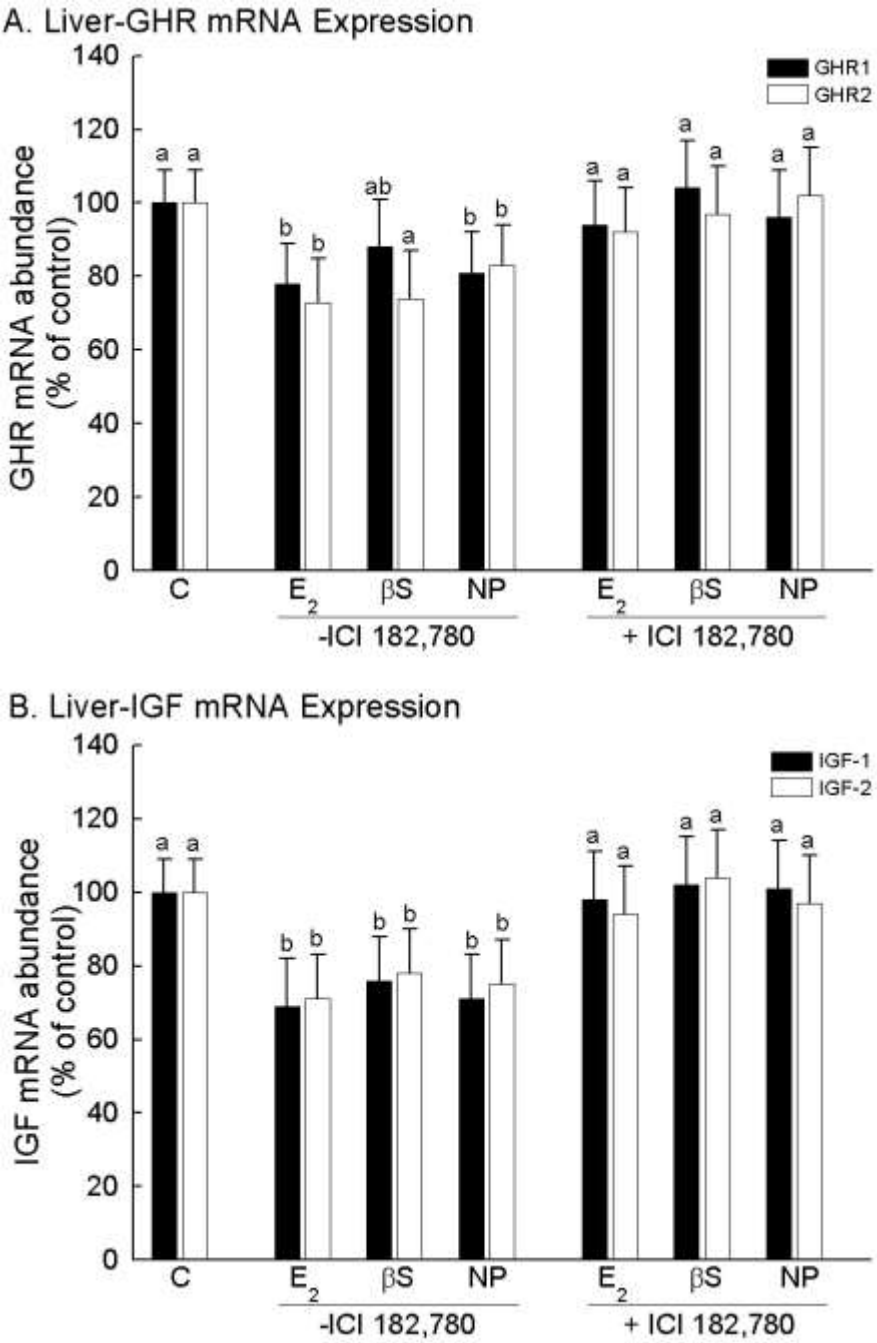
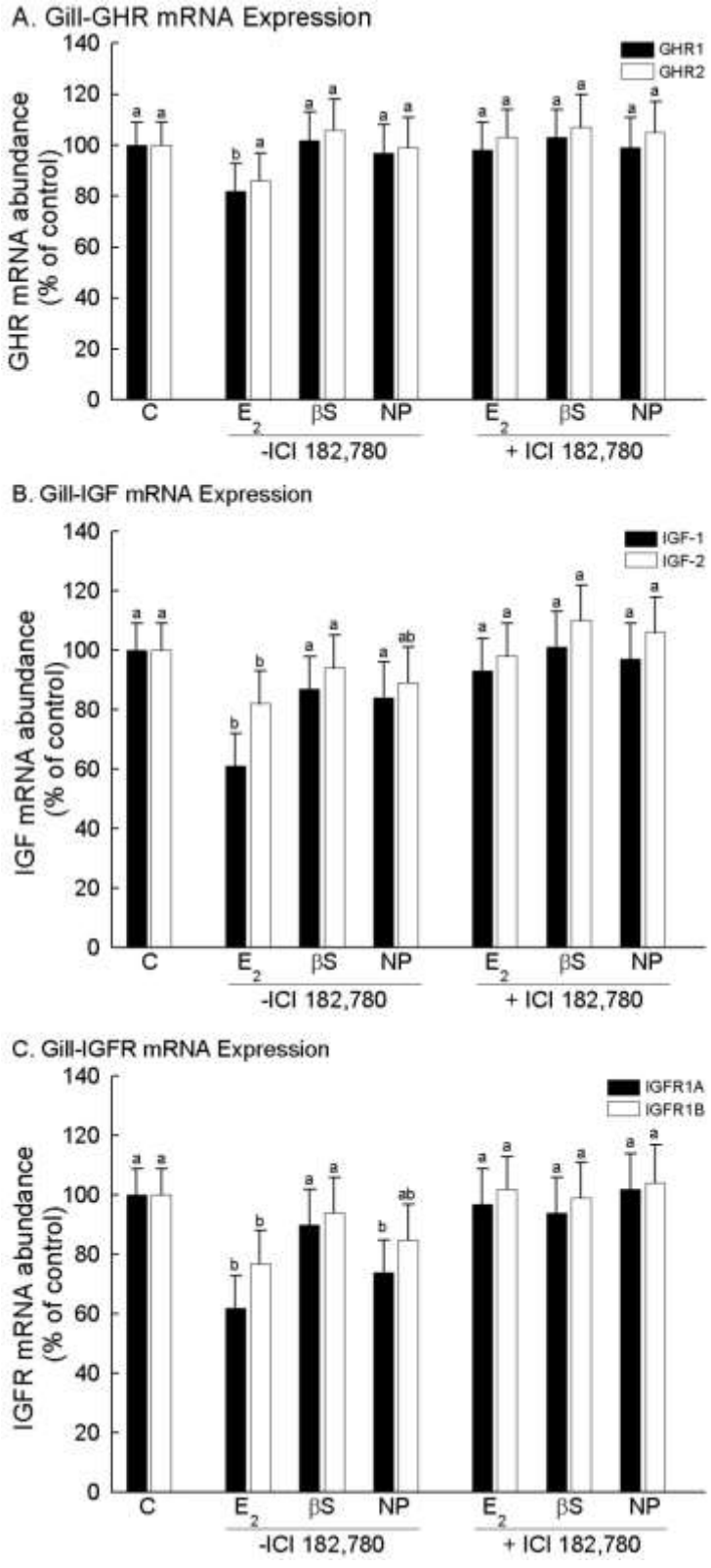


Figure 30. Effects of environmental estrogens (EE) and an estrogen receptor antagonist, ICI 182,780 on the abundance of (A) growth hormone receptors (GHRs) and (B) insulin-like growth factors (IGFs) in hepatocytes. Tissues were incubated for 2 h in the presence or absence of ICI 182,780, followed by 6 h with 10 µg/L 17β-estradiol (E2), 75 µg/L β-sitosterol (βS), or 75 µg/L nonylphenol (NP). Data are presented as mean ± SEM (n=6). For a given gene subtype, means with different letters are significantly different ($p < 0.05$) different from each other.

Figure 31. Effects of environmental estrogens (EE) and an estrogen receptor antagonist, ICI 182,780, on the abundance of (A) growth hormone receptors (GHRs), (B) insulin-like growth factors (IGFs), and (C) insulin-like growth factor receptors (IGFRs) in gill filaments. Tissues were incubated for 2 h in the presence or absence of ICI 182,780, followed by 6 h with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L nonylphenol (NP). Data are presented as mean ± SEM (n=6). For a given gene subtype, means with different letters are significantly different ($p < 0.05$) different from each other.


A. Gill-GHR mRNA Expression
GHR1
GHR2
GHR mRNA abundance (% of control)
140
120
100
80
60
40
20
0
C
E2
βS
NP
E2
βS
NP
-ICI 182,780
+ ICI 182,780
B. Gill-IGF mRNA Expression
IGF-1
IGF-2
IGF mRNA abundance (% of control)
C. Gill-IGFR mRNA Expression
IGFR1A
IGFR1B
IGFR mRNA abundance (% of control)

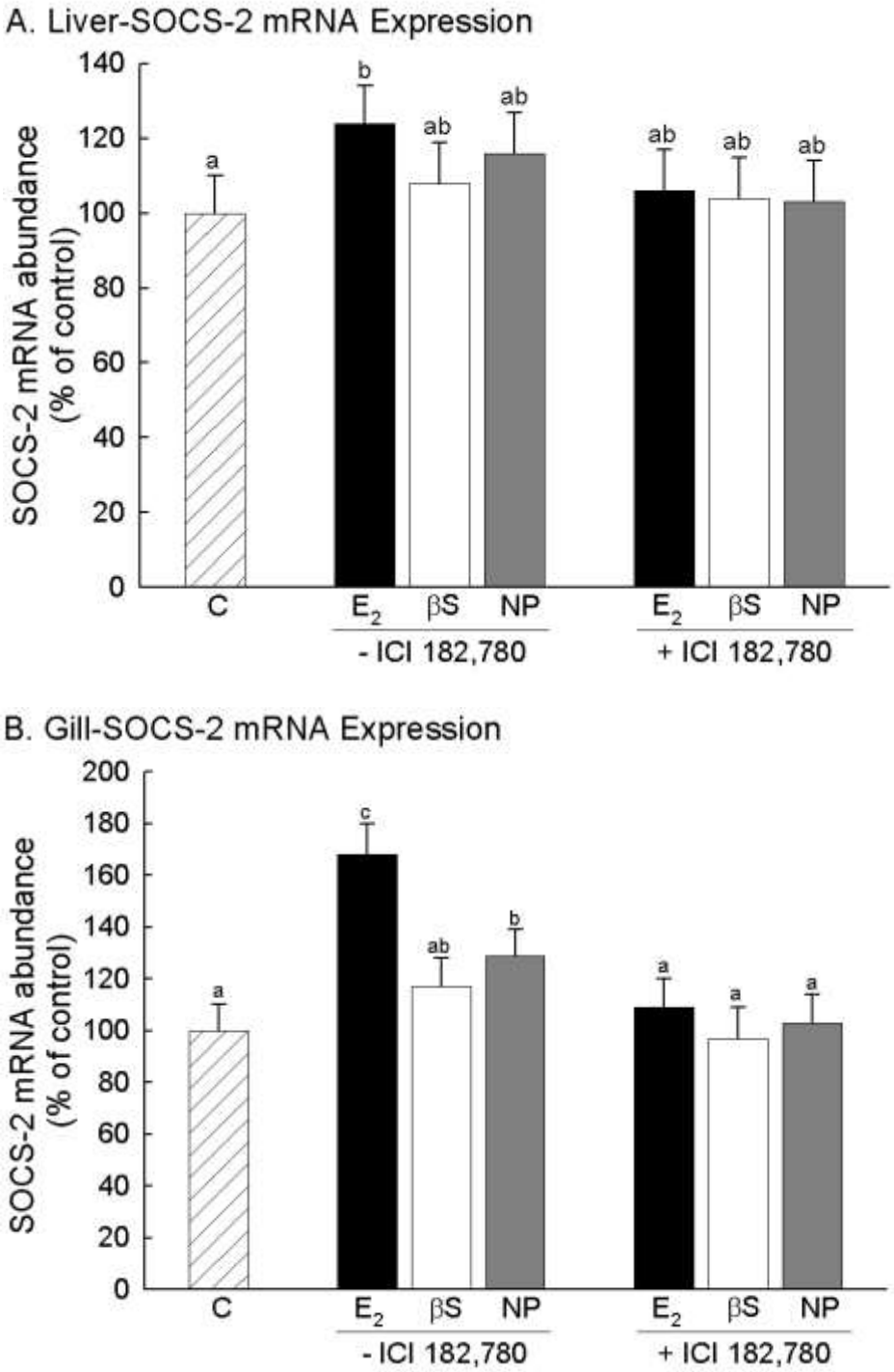


Figure 32. Effects of environmental estrogens (EE) and an estrogen receptor antagonist, ICI 182,780 on the abundance of suppressor of cytokine signaling 2 (SOCS-2) expression in (A) hepatocytes and (B) gill filaments. Tissues were incubated for 2 h in the presence or absence of ICI 182,780, followed by 6 h with 10 µg/L 17β-estradiol (E2), 75 µg/L β-sitosterol (βS), or 75 µg/L nonylphenol (NP). Data are presented as mean ± SEM (n=6). For a given gene subtype, means with different letters are significantly different ($p < 0.05$) different from each other.

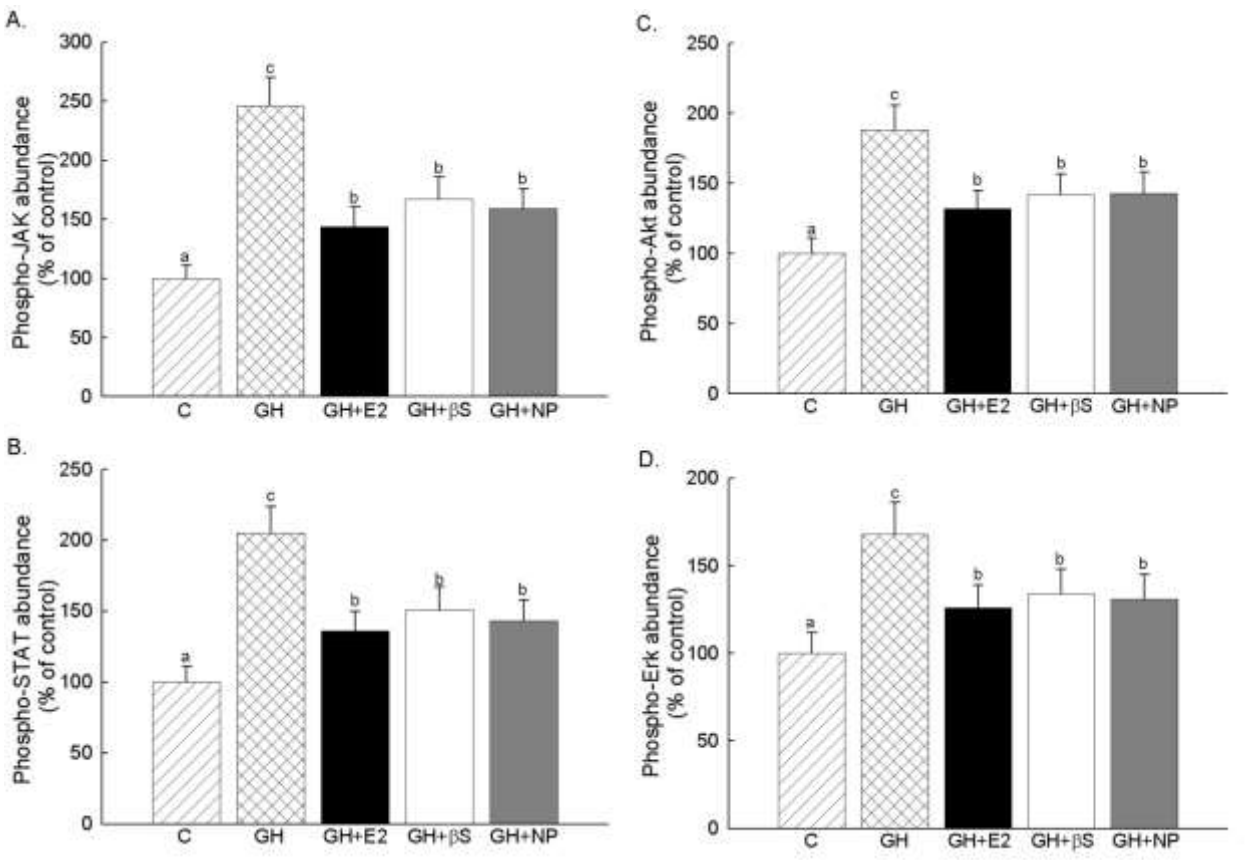


Figure 33. Blockade of GH-stimulated activation by environmental estrogens (EE). Effects on the phosphorylation of (A) JAK2, (B) STAT5, (C) Akt, and (D) Erk in hepatocytes incubated with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L nonylphenol (NP) for 15 min following GH stimulation. Cell lysates were separated by SDS-PAGE followed by western immunoblotting and quantified with a FluorChem imager. The abundance of phosphorylated JAK2, STAT5, Akt, and Erk was normalized to total JAK2, STAT5, Akt, and Erk, respectively. Data are presented as mean ± SEM (n=4). Means with different letters are significantly different ($p < 0.05$).

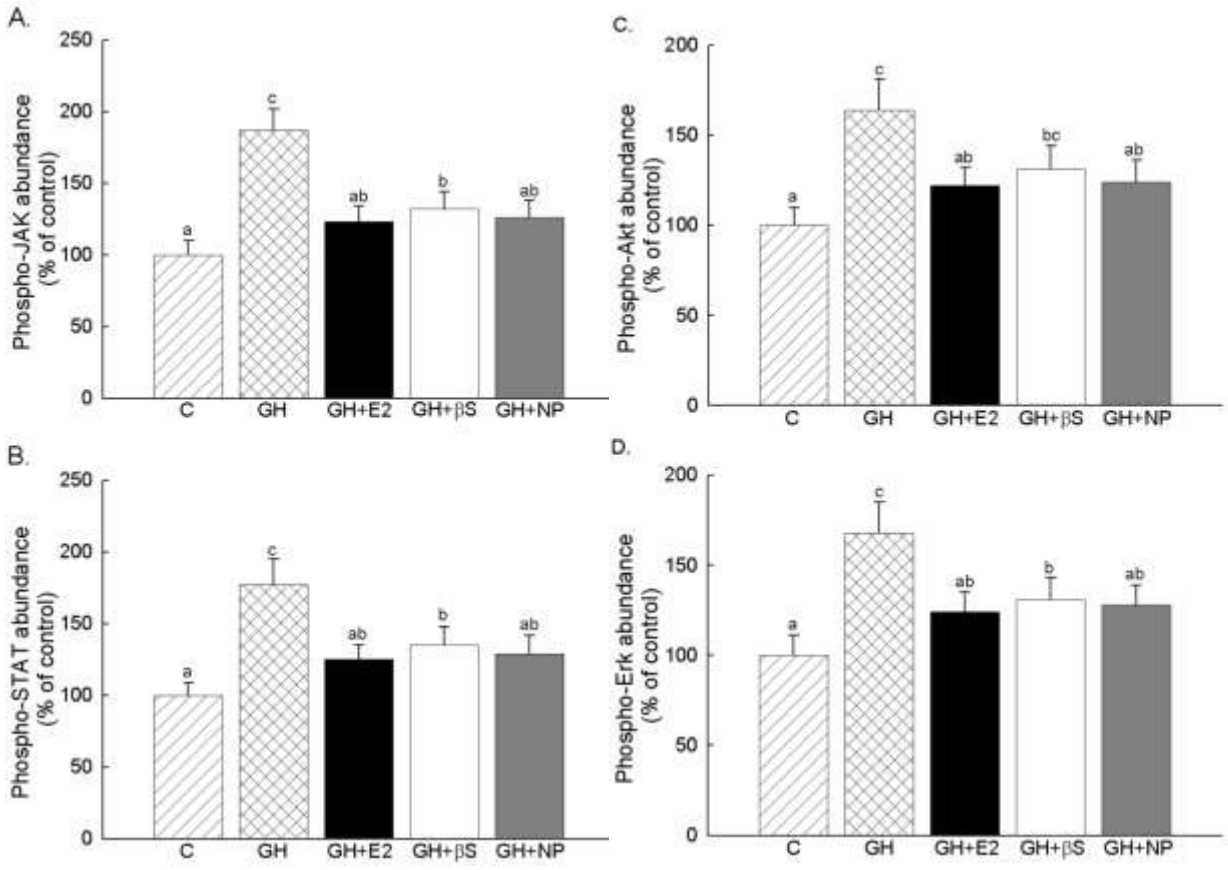


Figure 34. Blockade of GH-stimulated activation by environmental estrogens (EE). Effects on the phosphorylation of (A) JAK2, (B) STAT5, (C) Akt, and (D) Erk in gill filaments incubated with 10 μg/L 17β-estradiol (E2), 75 μg/L β-sitosterol (βS), or 75 μg/L nonylphenol (NP) for 15 min following GH stimulation. Cell lysates were separated by SDS-PAGE followed by western immunoblotting and quantified with a FluorChem imager. The abundance of phosphorylated JAK2, STAT5, Akt, and Erk was normalized to total JAK2, STAT5, Akt, and Erk, respectively. Data are presented as mean ± SEM (n=4). Means with different letters are significantly different ($p < 0.05$).

agonist (GH) phosphorylation states of JAK-STAT, PI3K-Akt, and Erk were increased as expected. Following treatment with EE, there was a significant suppression in GH-stimulated pathway activation for JAK2, STAT5, Akt, and Erk in hepatocytes (Fig. 33). Similarly, GH-stimulated phosphorylation of the JAK2, STAT5, Akt, and Erk pathways in gill filaments was significantly blocked following treatment with E2, βS, and NP.

Discussion

Environmental estrogens can bind to the estrogen receptor in mammals and teleosts, albeit with a much lower affinity than estrogen (Bonefeld-Jorgensen et al., 2001; Tremblay and Van der Kraak, 1998; Knudsen and Pottinger, 1999; Sumpter and Jobling, 1993). While EDCs can bind to the ER, the elucidation of signaling mechanisms has been complicated by a host of factors including multiple ER subtypes (ERα, ERβ in mammals; ERα1, ERα2, ERβ1, ERβ2 in rainbow trout), tissue-specific distribution of the receptors, and the ability of estrogen to produce both genomic and nongenomic responses in the cell.

The results of the present study confirm the inhibitory effects of EE on GH sensitivity, IGF synthesis, and IGF sensitivity and support our initial hypothesis that the JAK-STAT, MAPK, and PI3K-Akt pathways in part regulate the inhibition of GHR mRNAs, IGF mRNAs, and IGFR mRNAs in EE-treated hepatocytes and gill filaments of rainbow trout (Fig. 35). These findings help to establish mechanisms through which EE exert growth suppressing actions.

GH and IGF signaling involve the activation of signaling cascades, including JAK-STAT, ERK, and PI3K/Akt. Environmental estrogens inhibited the phosphorylation of ERK 1/2, Akt, JAK, and STAT in hepatocytes and gill filaments in a time-and concentration-dependent manner. These effects were ER-dependent, as treatment with each of the compounds and the ER antagonist, ICI 182,780, reestablished mRNA expression levels that were similar to those

observed in control tissues. Additionally, treatment with EE blocked GH-induced pathway activation of JAK-STAT, Erk, and PI3K/Akt in hepatocytes and gill filaments, thus linking these cell signaling elements to the previously observed suppression of GHR, IGF, and IGFR mRNA expression.

Environmental estrogens have been shown to have a variety of effects on signaling mechanisms in cells and the interplay of EEs and growth signaling pathways is complex. Our study demonstrated that JAK2, STAT5, Akt, and Erk represent targets for rapid, nongenomic responses to EEs in rainbow trout hepatocytes and gill filaments. This is in agreement with what others have found in ovariectomized hypophysectomized rats, where JAK-STAT activation was inhibited by E2 in a dose-dependent manner (Murphy and Friesen, 1988). Our results were also in agreement with a study in mussel hemocytes that found that NP and bispenol A (BPA) significantly decreased the phosphorylation state of STAT5 (Canesi et al., 2004). However, several studies have found contradictory results to those observed in our study. For example, NP, coumestrol, endosulfan, dichlorodiphenyldichloroethylene (DDE), and dieldrin were shown to stimulate a rapid membrane-initiated cascade in a prolactinoma cell line via a membrane bound ER (Bulayeva and Watson, 2004). Similarly, BPA, octylphenol, NP, and E2 rapidly (within 30 minutes) activated ERK1/2 and PI3K signaling cascades in rat pituitary cells (Dang et al., 2009). Dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) were also shown to be capable of activating the receptor tyrosine kinase pathways such as JAK-STAT (Enan et al., 1998; Shen and Novak, 1997).

The majority of studies in vertebrates have been in mammalian cell lines, not teleosts, thus representing one possible avenue for the observed differences in activation/deactivation of signaling pathways. There are four ER isoforms in rainbow trout (Nagler et al., 2007), whereas

in mammals only two isoforms exist. Teleost and tetrapod ER amino acid homology is as low as 32% for the A/B domain and 36% for the E domain (Socorro et al., 2000), and thus may have differential physiological functions. These ERs are differentially expressed and may exhibit different binding affinities for EE, thus activating or repressing different signaling pathways. Moreover, it is plausible that there are specific ER subtype-effector pathway linkages responsible for the contrasting effects. While results (time and concentration, activation and inhibition) may be variable, it is evident that EDCs can disrupt signaling pathways to affect physiological endpoints such as cellular growth and proliferation.

One possible mechanism by which EE reduce pathway activation may be through regulation of SOCS proteins. In our study, EE were shown to increase SOCS-2 mRNA expression in an ER-dependent fashion, an effect that was in agreement with what has previously been observed following exposure to endogenous estradiol (Tollet-Egnell et al., 1999; Paul et al., 2000; Leong et al., 2004; Rico-Bautista et al., 2004). As E2 is able to up-regulate SOCS-2 mRNA expression, it is reasonable to suggest that EEs are capable of eliciting the same results on SOCS-2.

In the context of growth-related signaling cascades, E2 can stimulate SOCS-2 expression and thus inhibit GH signaling via the JAK-STAT pathway, specifically JAK2 (Jelinsky et al., 2003; Leung et al., 2003; Leung et al., 2004). SOCS-2 may also play a role in IGF-1 signaling, as SOCS-2 has been shown to bind to IGFRs (Dey et al., 1998). Through the inhibition of JAK2 or IGF-1 signaling elements, SOCS-2 regulation may influence multiple effector pathways downstream of the GHR and IGFR. In mammals, there are eight SOCS family members, SOCS1-7 and cytokine-inducible SH2-containing protein (CISH). In rainbow trout, there are 7 known SOCS genes (Wang et al., 2010), however less is known about how SOCS functions in teleosts. It is possible that isoforms other than SOCS-2 may be contributing to the observed

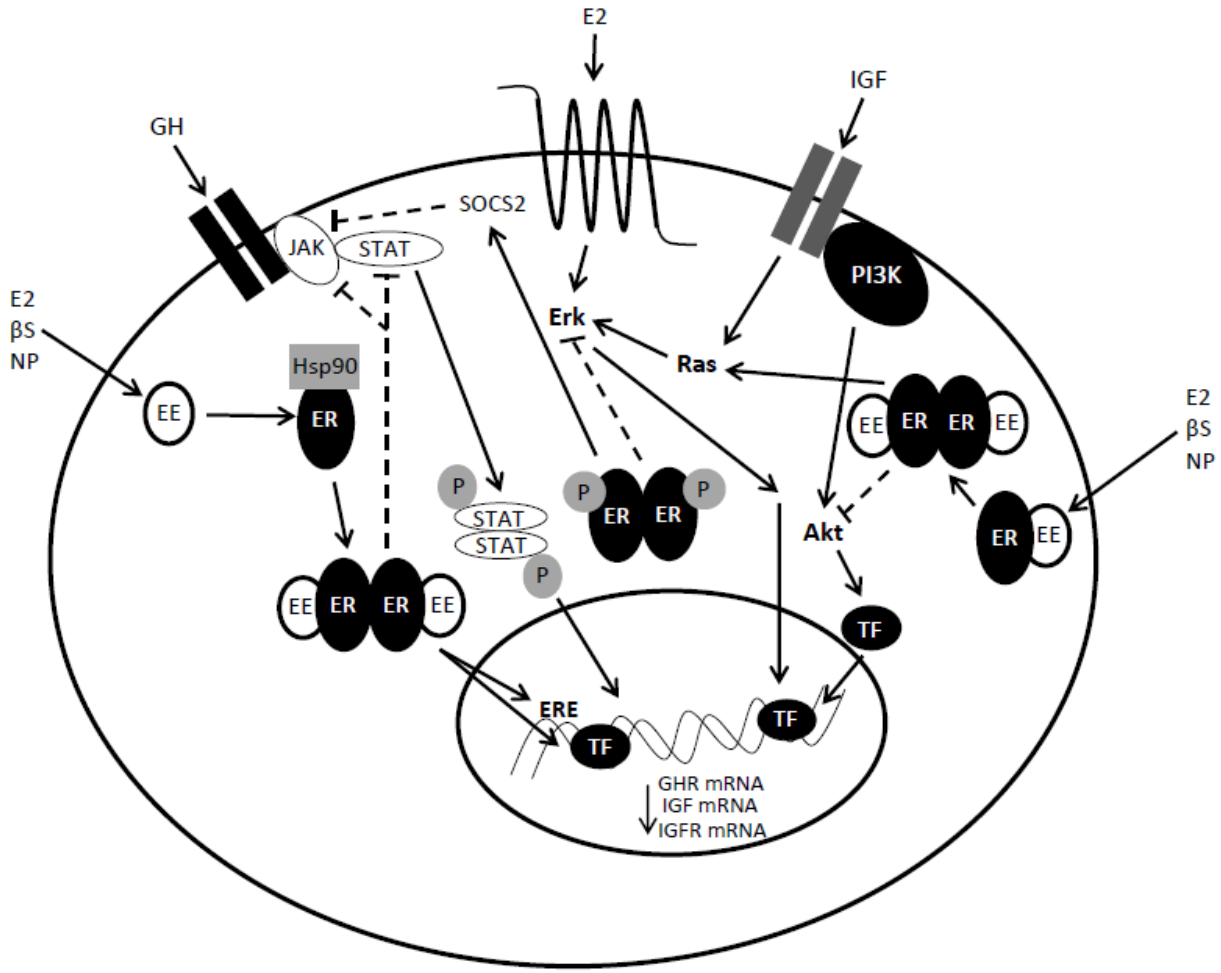


Figure 35. Schematic diagram of environmental estrogens (17β-estradiol, β-sitosterol, and 4-*n*-nonyphenol) signaling mechanisms in juvenile rainbow trout hepatocytes and gill filaments. Estradiol, E2; heat shock protein 90, Hsp90; estrogen receptor, ER; phosphorylated, P; transcription factor, TF; estrogen response element, ERE; growth hormone, GH; insulin-like growth factor, IGF.

effects on the regulation of growth-related signaling. For example, in a murine hepatoma cell line, SOCS-1 and SOCS-6 were shown to inhibit insulin receptor tyrosine kinase activity and the insulin-dependent activation of ERK1/2 and Akt kinase (Mooney et al., 2001).

The dependence of growth-related gene expression (i.e., depressed GHR, IGF, and IGFR mRNA expression) on the ER in hepatocytes and gill filaments was in agreement with what others have observed. Dang et al. (2009) found that pituitary cells treated with NP, BPA, or E2 exhibited increased GH mRNA and protein expression, an effect that could be blocked by co-incubation with an ER antagonist (ICI 182,780). Thus, whether EE are stimulatory or inhibitory on GH-IGF system components, the ER appears to be an obligatory component in signal transmission.

We have linked the EE-related suppression in GHR, IGF, and IGFR mRNA expression to pathways known to be involved in growth processes. Cross-talk with the GH-IGF system may be explained by multiple observations. First, from a genomic perspective, transcription factors such as AhR, Sp1, Sp3, MSY-1, C/EBP, and various STATs may be involved in the interplay of EE and growth signaling (Schwartzbauer and Menon 1998; Wang & Jiang 2005; Wood et al., 2005; Schwartzbauer et al., 1998; Yu et al., 1999; Elango et al., 2006). For example, IGFR expression has been shown to be capable of activation via AP-1 and Sp1 (Fujimoto et al., 2004; Sarfstein et al., 2006), two transcription factors known to interact with ER.

Second, direct interaction of the ER with signaling pathways has been evidenced by the co-immunoprecipitation of ERα with different components of the MAPK pathway in MCF-7 cells, thus indicating direct action on Ras/Erk kinases (Migliaccio, et al., 1996). Similarly, E2 bound to ERα has been observed to induce the phosphorylation of the IGFR in human embryonic kidney cells and thus alter Erk1/2 signaling (Kahlert et al., 2000). In endothelial cells, E2 treatment ERα

has been shown to bind the p85α regulatory subunit of PI3K, thus activating the PI3K/Akt signaling cascades (Simoncini et al., 2000; Hisamoto et al., 2001). Furthermore, IGF-1 can phosphorylate ERα, thus activating the receptor and allowing the ER complex to elicit its physiological effects (Lanzino et al., 2008). In mammalian cell lines, cross-talk has been observed between estrogen receptors, IGF, and IGFR (Kahlert et al., 2000; Klotz et al., 2002; Mendez et al., 2006), thus eliciting downstream signaling effects and regulating the expression of proteins involved in IGF-1 signal transduction (Bernard et al., 2006). Further evidence supporting the cross-talk between EDCs and growth pathways was provided by Yu et al. (2012), as E2 was able to rapidly stimulate MAPK signaling and induced the phosphorylation of ERα in cells with a functional IGFR versus in those without. Collectively, there are several points of interaction that exist between E2 and GH-IGF signaling and it appears as though bidirectional cross-talk may be responsible for our observed results. Furthermore, cross-talk between pathways such as MAPK, PI3K/Akt, and JAK-STAT has been observed. Previously, our lab found that JAK2 is critical for GHR signal propagation through ERK or PI3K/Akt in trout hepatocytes. This was evidenced via the complete inhibition of ERK and Akt activation when hepatocytes were treated with a JAK2 inhibitor (1,2,3,4,5,6-hexabromocyclohexane) (Reindl et al., 2011). In the context of growth hormone signaling, it is reasonable to suggest that the effects of EE on ERK and PI3K phosphorylation may simply be due to the downstream nature of these kinases in relation to JAK.

Complicating the elucidation of the cross-talk between these pathways is that many of these studies have investigated effects of EDCs using immortal cancer cell lines, which undergo growth and proliferation at an accelerated rate and thus may not accurately portray EE action on natural somatic growth. Additional problems arise in generalizing the response(s) of cells to

EDCs, as many of these response(s) appear to follow a non-traditional dose-response curve (Watson et al., 2007) and seem to be both tissue- and compound-dependent.

We have demonstrated for the first time the inhibition of signaling pathways by EE. There is a dearth of information regarding the negative regulation of JAK-STAT, PI3K-Akt, and MAPK by EDCs, thus future studies should focus on further elucidating the mechanisms (i.e., alternative pathways, transcription factors, epigenetic alterations) by which these compounds are inhibiting growth.

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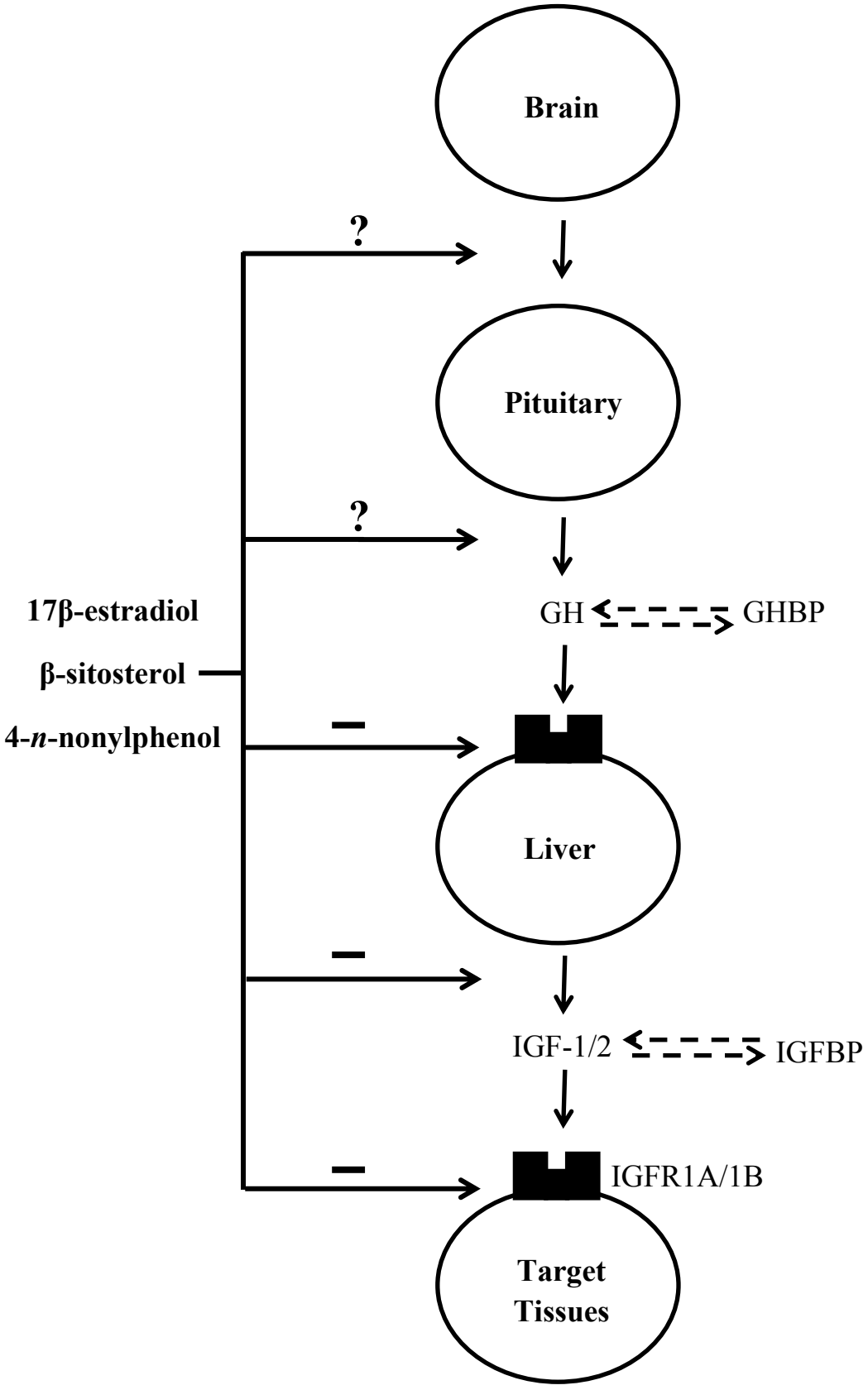
GENERAL CONCLUSIONS

Endocrine disrupting compounds are a diverse group of chemical compounds that have been shown to have deleterious effects on a host of physiological processes, including reproduction, osmoregulation, and growth (Hester and Harrison, 1999; Kime et al., 1998; Madsen et al., 2004, McCormick et al., 2005; Lerner et al., 2007). Our work focused primarily on the effects of EDCs, specifically EE, on growth. We have shown that 17β-estradiol, β-sitosterol, and 4-*n*-nonylphenol directly modulate overall organismal growth and growth at the molecular level through inhibitory actions on GH sensitivity, IGF synthesis, and IGF sensitivity (Fig. 35). In addition to EE affecting the GH-IGF system in terms of growth at the organismal and molecular level, the components of this system were affected in terms of osmoregulatory ability as well. The effects in our studies appeared to be tissue- and compound-dependent.

In terms of osmoregulatory ability, aqueous EE exposure was associated with elevated plasma chloride levels and suppressed GHR1, GHR2, IGF-1, IGF-2, IGFR1A, and IGFR1B mRNA expression following aqueous exposure to E2, βS, and NP in tissues including liver, gill, white muscle, and red muscle over the course of the salt water challenge. E2, βS, and NP abolished or attenuated normal salinity-induced changes in the expression of GHR, IGF, and IGFR1 mRNAs, indicating that EE reduce salinity adaptation by inhibiting components of the GH-IGF system.

Environmental estrogen treatment *in vivo* significantly reduced food conversion, body condition (i.e., condition factor), HSI, and body growth. Environmental estrogen-inhibited growth resulted from alterations in peripheral elements of the GH-IGF system. *In vivo* and *in vitro*, E2, βS, and NP reduced the expression of GHR, IGF, and IGFR mRNA and protein; however, the effects varied in an EE-, compound- and tissue-specific manner. The findings of our *in vivo* study were the most pronounced in terms of effects on the GH-IGF system and

Figure 36. Schematic representation of the growth hormone-insulin-like growth factor system in vertebrates and known negatively regulated targets (growth hormone sensitivity, insulin-like growth factor synthesis, insulin-like growth factor sensitivity) of endocrine disrupting compounds. growth hormone, GH; growth hormone binding protein, GHBP; growth hormone receptor, GHR; insulin-like growth factor, IGF; insulin-like growth factor binding protein; insulin-like growth factor receptor, IGFBP; endocrine disrupting compounds, EDCs.


Brain
?
Pituitary
?
17β-estradiol
β-sitosterol
4-n-nonylphenol
GH
GHBP
Liver
IGF-1/2
IGFBP
IGFR1A/1B
Target Tissues

indicate that EE disrupt post-embryonic growth by reducing GH sensitivity, IGF production, and IGF sensitivity.

E2, βS, and NP treatment resulted in the deactivation of JAK-STAT, MAPK, and PI3K-Akt pathways in a time- and concentration-dependent manner. Maximum suppression for each pathway was rapid, typically occurring at 10 to 30 min. The effective concentrations for dephosphorylation of JAK-STAT, MAPK, and PI3K-Akt were compound- and tissue-dependent, with the most pronounced effects being observed in hepatocytes. The observed effects appeared to occur via an estrogen-dependent pathway and the deactivation of these pathways was linked to suppressed GHR, IGF, and IGFR mRNA expression levels. The findings suggest biological mechanisms by which EE may regulate growth-related gene expression.

Based upon the collective results of this dissertation, EE appear to directly modulate the mRNA expression and functional expression of GHRs, and the expression of IGF mRNA in the liver. Although transcriptional effects were observed in gill and muscle, the effects of all three EE appeared to be most pronounced on IGF mRNA expression in the liver. This may indicate the primary mechanism by which EE are inhibiting growth. The evidence suggests that EE affect the GH-IGF system at the level of the liver and at the level of target tissues; however, it is possible that the effects on the target tissues are downstream effects occurring from the actions of EE on the liver. Additionally, the possibility remains that the changes in mRNA expression of IGFs are not transduced into changes in plasma protein levels of IGF *in vitro* or *in vivo*. The same possibility should be considered when examining IGF and IGFR functional expression *in vivo*.

Endocrine disrupting chemicals are thought to elicit their physiological effects primarily via nuclear receptors. Elucidating the multifaceted effects of EDCs has proved to be difficult for a host of reasons, including the ability of estrogen to signal via genomic and nongenomic

mechanisms, the numerous ER subtypes and the tissue-specific distribution of these subtypes, and the varying milieu of proteins in tissues (Matthews and Gustafsson, 2003; Nagler et al., 2007; Routledge et al., 2000). Effective concentrations of EDCs are largely variable and chronic versus acute exposure may bring about diverse physiological actions. In addition to the differentially expressed receptors, the receptors may be linked to different effector pathways, and ultimately result in different physiological endpoints. For example, in metabolic processes (e.g., glucose and lipid metabolism), ERα has been linked to anti-lipogenesis and glucose tolerance, while ERβ has been linked to impaired glucose and lipid homeostasis (Faulds et al., 2012).

Furthermore, variable effects between this study and studies by others may arise from differences in length of exposure, age and health of the organism, and type of exposure (i.e., aqueous versus dietary). The effects of EE on organismal growth could be attributed to the exposure of rainbow trout during the juvenile life stage, a stage in which the fish are typically undergoing rapid growth (Mommsen, 2001). From a life history perspective, juveniles typically allocate energy resources towards growth prior to undergoing sexual maturation. Treatment at this critical life stage with a sex steroid such as estrogen, or a compound mimicking estrogen (e.g., β-sitosterol, nonlyphenol), may reprogram the fish to divert energy resources towards reproduction rather than growth. By decreasing the growth rate of these organisms, and thus perhaps maturation rates, the lifetime fitness of an organism may be altered.

Future Studies

The data here focused only on the primary source of IGFs, the liver, and a few target tissues (i.e., gill and muscle). As the control of growth is initiated in the brain, effects of EE on other endocrine organs such as the hypothalamus and pituitary should be examined to determine the primary site of action for EE. It is possible that the observed results were merely downstream

effects of negative regulation at the level of the hypothalamus and pituitary by EE. Another aspect of growth regulation that should be examined is the effects of EE on GHBP and IGFBP, as these two proteins are responsible for the bioavailability of GH and IGF (Baumann et al, 1988; Edens and Talamantes, 1998). Previous studies in mammals have indicated that co-incubation with E2 and GH increased GHBP mRNA and GHBP protein levels (Carmignac et al., 1993; Contreras and Talamantes, 1999). Similarly, E2 stimualted the release of IGFBPs (25 kDa and 30 kDa) from hepatocytes in tilapia (Riley et al, 2004) and IGFBP-1 in humans (Paassilta et al., 2000), thus it is reasonable to suggest that EE may in part exert their physiological effects on growth through binding proteins. In addition to looking at binding proteins, it is possible that estrogen and EE may exert effects on IGF-3 expression. Ethinylestradiol negatively affected IGF-3 mRNA expression in a sex-dependent manner in the gonads of tilapia (Berishvili et al., 2010). While IGF-3 appears to be gonad-specific, the effects of EE on this form of IGF may assist in understanding the overall picture of the effects of EE on growth at all levels. Effects on transcription factors such as Sp1, Sp3, and MSY-1 should also be considered in future studies examining EE action on growth processes, as these transcription factors have been shown to regulate GHR promoter activity and GHR synthesis (Schwartzbauer et al., 1998; Yu et al., 1999).

The influence of EE on other hormonal systems involved in growth including reproduction, thyroid hormones, and metabolism, should additionally be further examined in order to determine the interplay between and among these systems. As exposure to combinations of EDCs may dramatically affect cellular function (mixtures may be 1000 times more potent than singularly acting EDCs (Arnold et al., 1996; Benachour et al., 2007; Kortenkamp, 2008), expanded research should focus on the possible synergistic actions of these compounds. Lastly, future studies should examine other signaling mechanisms and transcription factors that may be

altered by EE, as well as any epigenetic modifications to the genes responsible for somatic growth.

Summary

Our understanding of the complex mechanisms by which EE modulate growth at the organismal and molecular level is still rudimentary. The multitude of physiological processes that are negatively affected by EE are indicative of the consequences associated with a world that is undergoing rapid technological development. While it is unfortunate that the adverse effects of compounds such as DES and DDT did not come to light sooner, with adequate research and understanding, a ban was implemented on these harmful compounds. Interestingly, the ban on DDT is thought to be one of the primary reasons that the bald eagle population has recovered after being on the brink of extinction (Stokstad, 2007). Avoiding hazard in the design of new chemicals ("green chemistry") may be one beneficial practice to the reduction of exposure to EDCs. Programs such as the Tiered Protocol for Endocine Disruption (TiPED) promote green chemistry and help identify a chemical's endocrine disrupting effects early in the design process via in silico, cell-specific, and whole organismal-based assays. Programs such as TiPED may be extremely beneficial to organismal health; unfortunately, it is not possible to detect every EDC as the understanding of these compounds and their effects on physiology is still advancing (Schug et al., 2013). Ultimately, this work will inform policies and practices aimed at reducing the impact of EE on our environment and on wildlife and human health.

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