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Distinct effects of 4-nonylphenol and estrogen-17β on expression of estrogen receptor α gene in smolting sockeye salmon

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Abstract

Xenoestrogens such as 4-nonylphenol (4-NP) have been shown to affect the parr–smolt transformation, but their mechanisms of action are not known. We therefore examined effects of 4-NP and estradiol-17β (E2) on expression of estrogen receptor (ER) α gene in the liver, gill, pituitary and brain of sockeye salmon to elucidate molecular mechanisms of 4-NP and E2 and developmental differences in response during smolting. Fish were treated twice within a week with 4-NP (15 and 150 mg/kg BW), E2 (2 mg/kg BW) or only vehicle at three stages of smolting, pre-smolting in March, early smolting in April and late smolting in May. The absolute amounts of ERα mRNA were determined by real-time PCR. The basal amounts of ERα mRNA peaked in April in the liver, gill and pituitary. In March, E2 extensively increased the amounts in the liver, while 4-NP had no effects at this stage. In contrast, 4-NP (but not E2) decreased liver ERα mRNA in April. 4-NP also decreased the amount of ERα mRNA in the gill in April. In the pituitary, 4-NP increased ERα mRNA in March but decreased it in May. There were no significant effects in the brain. Changes in basal ERα mRNA observed in this study indicate that estrogen responsiveness of tissues may change during salmon smolting. Furthermore, 4-NP and E2 have different effects on expression of ERα gene in the liver and gill during smolting, and the response is dependent on smolt stage.

Keywords: Estrogen receptor; Xenoestrogen; Nonylphenol; Salmon; Smolting; Liver; Gill; Seawater adaptation

1. Introduction

Smolting in anadromous salmonids is regarded as an adaptative event for transition from freshwater to seawater (Hoar, 1988; Boeuf, 1993). This process includes a series of developmental changes in many aspects of the physiology, biochemistry and behavior of the young salmon. These changes are affected by several endocrine factors including gonadal steroid hormones. Sex steroids have a fundamental, negative influence on smolting, antagonizing the morphological, metabolic and physiological changes that occur during smolting by as yet unknown mechanisms (Madsen and Korsgarrd, 1989). The levels of endogenous sex steroids are normally low during smolting stage, but they are elevated during sexual maturation of parr and may inhibit various physiological changes involved in smolting (Dellefors and Faremo, 1988; Lundqvist et al., 1990; Saunders et al., 1994). Ikuta et al. (1987) found that silvering of skin, occurrence of a black margin on the dorsal and caudal fins, slimness of the body and seawater tolerance

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that usually occur during smolting were not observed in the E2-administered masu salmon. These results support the notion that smolting and reproduction may be two physiological processes that antagonize each other.

Recently, the xenoestrogen 4-nonylphenol (4-NP) has been implicated in declines in wild salmon populations, possibly through its influence on the parr–smolt transformation and early seawater survival (Fairchild et al., 1999). 4-NP is an alkylphenol, predominantly occurring as a degradation product of nonylphenol theoxylate, which is widely used in plastic and rubber industry as non-ionic surfactants. The estrogenic effects of 4-NP have been documented in several species of fish by in vivo and in vitro studies (Jobling and Sumpter, 1993; Lech et al., 1996; Celius et al., 1999; Arukwe et al., 2002). 4-NP can bind to estrogen receptor (ER) and mimics estrogen to stimulate expression of ER-related genes such as vitellogenin (Vtg) and eggshell *zona radiata* protein (Zrp) genes (Arukwe et al., 2002). Therefore, it has been hypothesized that exposure of wild juvenile salmonids to 4-NP during the parr–smolt transformation could have an antagonistic effect on smolt physiology and compromise the ability of the fish to osmoregulate and survive in the marine environment. Indeed, injections of the smolts with E2 and 4-NP significantly reduced the gill Na^+, K^+-ATPase activity and impaired hypoosmoregulatory performance (Madsen et al., 1997). Furthermore, E2 and 4-NP treatment impaired not only smolt development but also survival after release, leading to a delay of downstream migration in Atlantic salmon (Madsen et al., 2004).

In vertebrates, the effects of estrogen are mainly mediated by ER. ER belongs to the nuclear receptor superfamily and is able to regulate transcription of target genes through binding to estrogen responsive elements (EREs) (Beato, 1991). Consequently, the expression of ER is essential for cellular estrogenic responses. In mammals, two subtypes of ER, ERα and ERβ have been characterized by molecular cloning. In teleosts, ERα was first characterized in rainbow trout (Pakdel et al., 1989) and then in other species such as tilapia (Tan et al., 1995), Japanese eel (Todo et al., 1996), red sea bream (Touhata et al., 1998), channel catfish (Xia et al., 1999), gilthead sea bream (Munoz-Cueto et al., 1999) and Atlantic salmon (Rogers et al., 2000). More recently, the presence of multiple ER subtypes, ERβ and ERγ, has been determined in various fish species (Tchoudakova et al., 1999; Hawkins et al., 2000; Ma et al., 2000; Patiño et al., 2000; Legler et al., 2002). In salmonids, however, ERα is the only subtype of ER expression that has been determined so far.

ERα gene is predominantly expressed in liver, ovary and pituitary, and is tightly related to vitellogenic and reproductive function (Nagler et al., 2000; Rogers et al., 2000; Menuet et al., 2001). Pakdel et al. (2000) described two ERα isoforms, long and short, that differ by an extra 45 amino acids in the N-terminal region in rainbow trout. These two ERα isoforms are expressed in different tissues: the long isoform is expressed in liver, ovary, pituitary and brain, whereas expression of the short isoform is restricted to the liver (Menuet et al., 2001). However, there is little information about the expression of ERα gene in the gill of any species, nor on the effects of E2 and 4-NP on ERα gene expression in salmon during smolting. In the liver, the treatment of rainbow trout with estrogen results in elevated expression of ERα gene preceding induction of vitellogenin gene (Pakdel et al., 1991; Salbert et al., 1993; MacKay et al., 1996). Similarly to E2, 4-NP has been shown to induce hepatic ER expression in vivo (Yadetie et al., 1999) and in vitro (Flouriot et al., 1995). However, there is no information concerning the effects of E2 and 4-NP on gill ERα gene expression, nor do we know how the response to E2 and 4-NP changes during development. Therefore, it is of considerable importance to examine expression of ERα gene in the gill, liver and other tissues and its changes after E2 and 4-NP treatment during smolting to clarify molecular mechanisms and developmental differences in response.

Here, we determined the levels of ERα gene expression in liver, gill, pituitary and brain at three different stages of smolting in sockeye salmon (*Oncorhynchus nerka*). Furthermore, the effects of E2 and 4-NP on expression of ERα gene were examined in these tissues. To determine the amounts of ERα mRNA, partial cDNA encoding ERα was isolated from sockeye salmon by reverse transcription-polymerase chain reaction (RT-PCR), and a highly sensitive, accurate quantitative assay for ERα mRNA was established by real-time PCR.

2. Materials and methods

2.1. Fish, treatment and sampling

One-year-old sockeye salmon (*O. nerka*) were reared at the Chitose Salmon Hatchery, National Salmon Resources Center (Hokkaido, Japan). Fish were maintained in outdoor tanks with flow of spring water (2 to 5 °C) under natural photoperiod. They were fed with dry pellets equivalent to 0.5% of body mass everyday in March, and to 3.0% in April and May. The following treatment and sampling procedures were conducted in March (pre-smolting), April (early smolting) and May (late smolting). At each experiment, 40 fish were randomly caught and measured for fork length and body weight (BW). Fork lengths of the fish were 11.4±0.9 cm in March, 11.4±1.1 cm in April and 14.0±1.7 cm in May. Body weights of the fish were 13.1±0.4 g in March, 13.3±0.4 g in April and 24.0±1.0 g in May. They were divided into four groups, and were transferred to plastic tanks (85 cm×33 cm×30 cm) with flow of spring water (10.5±0.5 °C) under natural photoperiod. They were injected intraperitoneally twice on day 0 and day 2 with 4-NP (branched form, Schenectady International Inc., Schenectady, New York, Japan) at 15 mg/kg BW and 150 mg/kg BW, E2 (Sigma-Aldrich, Tokyo, Japan) at 2 mg/kg BW and

Table 1
Primers and TaqMan probe used in this study

Name	Description	Sequence
ERα-F1	Forward primer for cloning	5′-CTCGCTTCCGCATGCTTAAACTGAAG-3′
ERα-R1	Reverse primer for cloning	5′-CATACTGGAGTACCCCGGAACCA-3′
ERα-F2	Forward primer for real-time PCR	5′-GCATGGAGCACCTTTACAGCA-3′
ERα-R2	Reverse primer for real-time PCR	5′-TGGAGGTGGTAGTGGTGGTAGA-3′
ERα-P	TaqMan probe for real-time PCR	$5'^{FAM}$-AATCCCCAGGCAAAGTGGCCCA-$^{TAMRA}3'$
ERα-RT	Primer for reverse transcription	5′-AGGCTGTCTTGCATCG-3′

vehicle (corn oil) only, and then sampled on day 7. They were anesthetized with 0.01% tricaine methanesulfonate (MS222) and an equal amount of sodium bicarbonate. The livers, gills, pituitaries and brains were taken out, frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

2.2. Cloning of sockeye salmon ERα cDNA by RT-PCR

Total RNA was extracted from an individual liver of sockeye salmon by the guanidium thiocyanate hot phenol-chloroform method (Chirgwin et al., 1979). RT reaction was performed on 3 μg of total RNA using an oligo(dT) primer (Amersham Biosciences Corp., NJ, USA) and SuperScript II RNase H^- RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. This cDNA was served as templates for PCR amplification with forward and reverse primers (ERα-F1 and ERα-R1, Table 1), which correspond to conserved sequences among other teleost ERαs. A PCR mixture contained 1× Expand High Fidelity buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, primers (1 mM each) and 0.53 units Expand High Fidelity Enzyme (Roche Applied Science, Mannheim, Germany). Amplification profile was: denaturation at 94 °C for 2 min, followed by 38 cycles of 94 °C for 15 s, 62 °C for 30 s, and 72 °C for 45 s, and finally additional 7 min at 72 °C. A PCR fragment of expected size (498 bp) was purified by a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and cloned into a pCR-Script Amp SK(+) cloning vector (STRATAGENE, CA, USA). Nucleotide sequence was determined by the dideoxy chain-termination method (Sanger et al., 1977) using an SQ-5500 DNA sequencer (Hitachi, Tokyo, Japan). Multiple cDNA clones containing the insert were subjected to sequence analysis.

2.3. Real-time PCR assay

Total RNA was extracted individually from the livers, gills, pituitaries and brains by the guanidium thiocyanate hot phenol-chloroform method. The concentrations of total RNA were determined by measurement of optical density at 260/280 nm and its quantity and also integrity were verified by gel electrophoresis. Total RNA of 150 or 200 ng was used for RT reaction in a mixture containing 500 mM dNTP, 0.2 mM ERα-RT (Table 1), 1× RT buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3), 5.5 mM $MgCl_2$, 4 U RNase inhibitor (TOYOBO, Tokyo), 12.5 U Multiscribe Reverse Transcriptase (PE Applied Biosystems, CA, USA). The reaction was performed at 25 °C for 10 min, at 48 °C for 30 min and at 95 °C for 5 min.

Real-time PCR was carried out with an ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Nucleotide sequences of primers and probes are shown in Table 1. The PCR reaction mixture contained 1× TaqMan Buffer A (PE Applied Biosystems), 25 mM $MgCl_2$, 2.3 mM dNTP, 100 nM each forward and reverse primers (Table 1), 130 nM of fluorogenic probe (Table 1) and 0.25 U of AmpliTaq Gold DNA polymerase. Amplification profile was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. To determine absolute amounts of ERα mRNA, standard sense RNA was in vitro synthesized by a MAXIscript (Ambion, Texas) using the sockeye salmon ERα cDNA. The synthesized RNA was quantified, serially diluted (3.5×10^3–3.5×10^8 copies/μl) and reverse transcribed in the same condition as total RNA to prepare standard cDNA. In the assay, several doses of standard cDNA were applied in triplicate and sample cDNA prepared from total RNA were applied in duplicate. Standard curve was linear in the range of 2×10^1 to 2×10^6 copies (r^2=0.997, Fig. 1). In each assay, a standard sample (sockeye salmon liver cDNA) was applied in triplicate to estimate coefficients of variation (CV) within and between runs. The intra-assay CV ranged 0.44–8.02% and inter-assay CV was 13.04%. The amounts of ERα mRNA were expressed as copies per microgram total RNA.

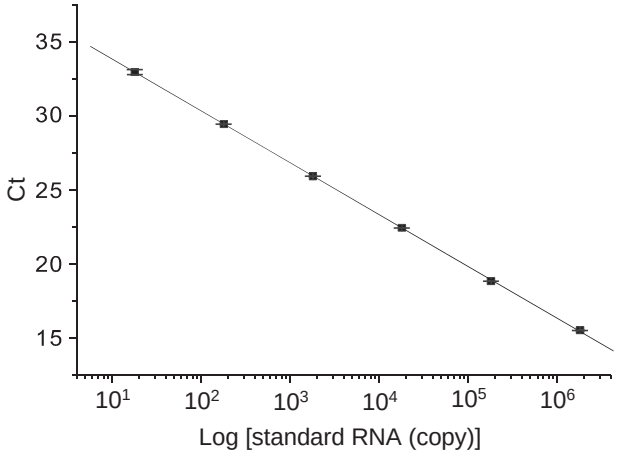

Fig. 1. Standard curve in real-time PCR assay for ERα mRNA.

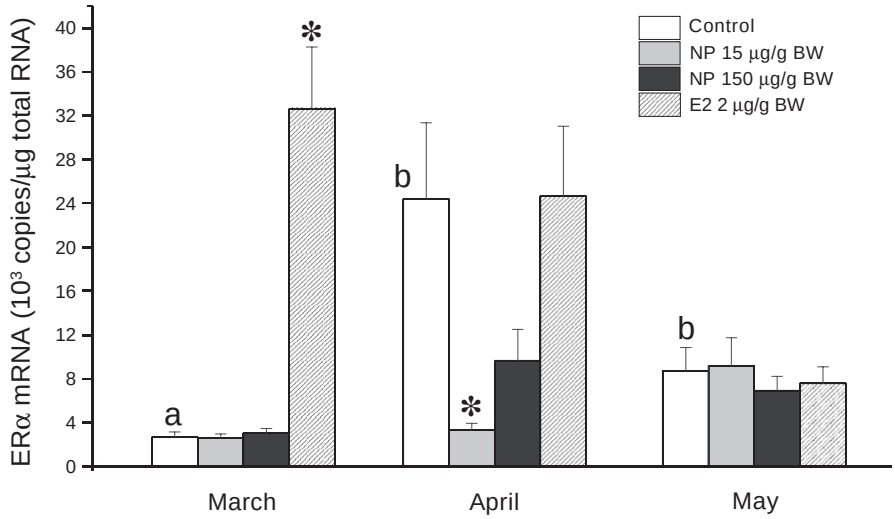

Fig. 2. Effects of 4-nonylphenol (NP) and 17β-estradiol (E2) on the amount of ERα mRNA in the liver of juvenile sockeye salmon during smolting. Different letters represent significant difference among control groups, $P<0.05$. *Significant difference between control and treatment group at each sampling time, $P<0.05$.

2.4. Statistical analysis

All data were expressed as means±S.E.M. One-way analysis of variance (ANOVA) and Tukey's post hoc tests were used for statistically significant differences among months in the control fish. Dunnett's pairwise multiple comparison *t*-test was applied to test the effects of 4-NP and E2 in each month.

3. Results

3.1. Isolation of partial ERα cDNA from sockeye salmon

A partial ERα cDNA was isolated from an individual liver by RT-PCR. This fragment consisted of 451 bp, sharing 98% of sequence identity with rainbow trout ERα cDNA. Recently, two isoforms of ERα had been determined and designated as short and long forms of ERα in rainbow trout (Pakdel et al., 2000). However, the real-time PCR assay in the present study detects both of these ERα isoforms because the primers and probe are designed based on a region that is complementary to both isoforms. The assay enabled us to determine the absolute amount of ERα mRNA in the wide range from 100 to 10^6 copies/reaction with high accuracy (Fig. 1).

3.2. ERα gene expression in the liver during smolting

The experiment fish experienced normal physiological changes during smolting (Ban and Yamauchi, 1991; Ban, 2004). Black margins of the caudal fin, a characteristic

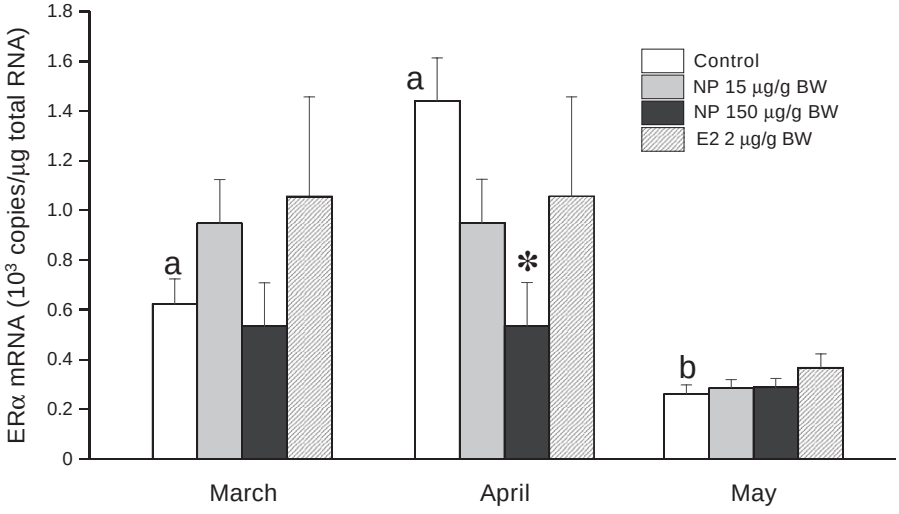

Fig. 3. Effects of 4-nonylphenol (NP) and 17β-estradiol (E2) on the amount of ERα mRNA in the gill of juvenile sockeye salmon during smolting. Different letters represent significant difference among control groups, $P<0.05$. *Significant difference between control and treatment group at each sampling time, $P<0.05$.

feature of smolts, were invisible in March, and became distinct in April but less clear in May. The activity of gill Na^+, K^+-ATPase increased from March to May, and plasma Na^+ levels after seawater transfer decreased from March to April (data not shown).

The basal amount of ERα mRNA in the liver changed considerably during smolting. It increased 9-fold from March to April, and then decreased slightly in May (Fig. 2). In March, treatment with E2 elevated the amount of ERα mRNA extensively, whereas 4-NP had no effect. In contrast, in April, 4-NP decreased the amount of ERα mRNA to levels similar to basal expression in March, whereas E2 had no effect at this stage. In May, both 4-NP and E2 had no effect on the amount of liver ERα mRNA.

3.3. ERα gene expression in the gill during smolting

The amount of ERα mRNA in the gill were much lower than that in the liver, approximately 1/5–1/30 of the liver (Fig. 3). Basal ERα mRNA more than doubled from March to April, then decreased to its lowest levels in May. In April, 4-NP decreased the amount of ERα mRNA to levels similar to basal expression in March. E2 showed similar effects, although the changes were not statistically significant. There were no significant effects of 4-NP and E2 treatments on gill ERα mRNA in March and May.

3.4. ERα gene expression in the pituitary and brain during smolting

The basal amounts of ERα mRNA in the pituitary and brain were similar to that of the liver. ERα mRNA increased in April in the pituitary, but showed no significant change in the brain. In the pituitary, 4-NP increased ERα mRNA in March, whereas 4-NP and E2 decreased the amounts in May. In the brain, both 4-NP and E2 had no effects on ERα mRNA at any stage (Fig. 4).

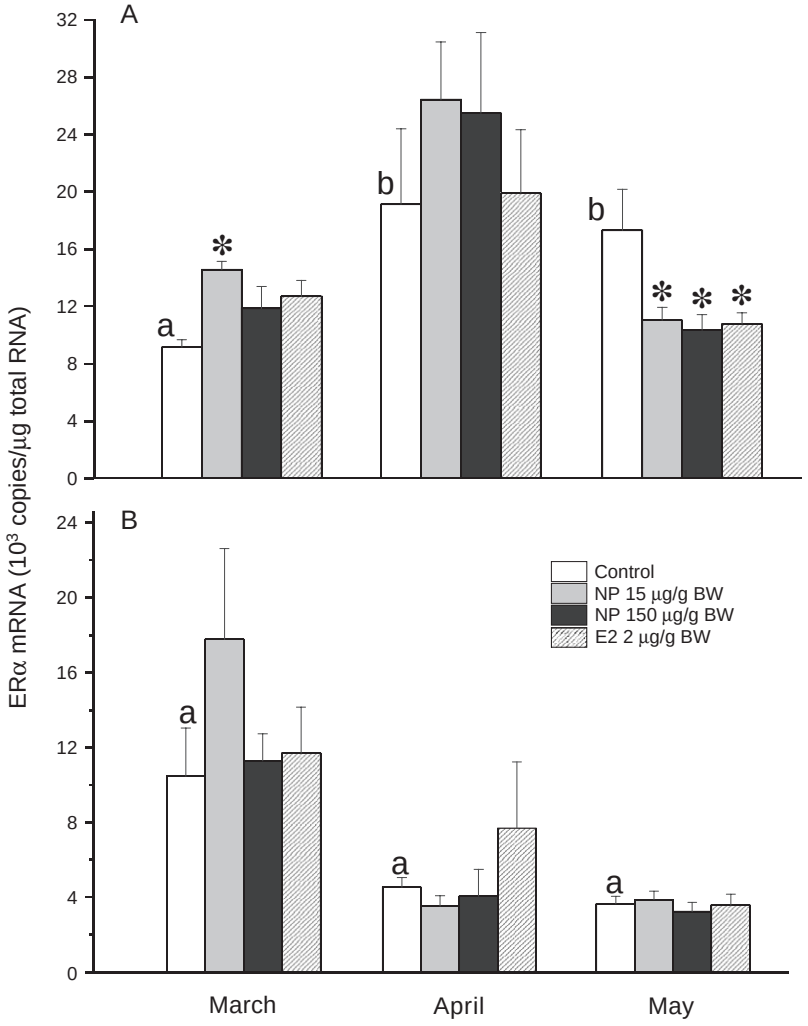

Fig. 4. Effects of 4-nonylphenol (NP) and 17β-estradiol (E2) on the amount of ERα mRNA in the pituitary (A) and brain (B) of juvenile sockeye salmon during smolting. Different letters represent significant difference among control groups, $P<0.05$. *Significant difference between control and treatment group at each sampling time, $P<0.05$.

4. Discussion

To determine the absolute amount of ERα mRNA in different tissues, we adopted a real-time PCR technique. The sensitivity of present assay is about 100 copies/well, which is sufficient to determine very low levels of ERα mRNA. Further, the ranges of intra- and inter-assay variations are comparable with those of radioimmunoassay. In this study, we determined the absolute amount of mRNA without using internal reference, such as β-actin and glyceraldehyde phosphate dehydrogenase. These housekeeping genes are not always expressed at constant levels and can be affected by experimental treatment and physiological conditions (Bustin, 2000; Tricarico et al., 2002). We verified integrity of total RNA extracted from various tissues by checking recovery of RNA in extraction and gel electrophoresis after extraction. Therefore, the real-time PCR method in the present assay is highly sensitive and reliable for quantification of low amounts of ERα mRNA in some tissues, such as the gill and brain.

The basal amount of ERα mRNA increased in April in the liver, gill and pituitary during smolting. The temporal increases of ERα gene expression during smolting may cause increased sensitivity to estrogens. The circulated levels of estrogen are normally low during smolting stage, but if levels are artificially increased, physiological processes involved in smolting may be antagonized and those in reproduction may be induced (Ikuta et al., 1987). Indeed, the treatment of juvenile salmon with E2 and 4-NP induced expression of various genes implicated in reproduction, such as Vtg, Zrp, and ER (Madsen et al., 1997, 2004; Yadetie et al., 1999; Arukwe et al., 2002; Moore et al., 2003). Even in natural conditions, Sower et al. (1984) reported that there was a slight increase in plasma estrogen of coho salmon smolts, and suggested there was a physiological action of these low but increasing levels of estrogen. The observed changes in basal ERα mRNA in the liver, gill and pituitary may relate to such a physiological action of estrogen during smolting.

In March, E2 elevated the amount of ERα mRNA extensively in the liver. This is in line with previous studies showing autoregulation of ER gene expression in the liver (Pakdel et al., 1991; Salbert et al., 1993; MacKay et al., 1996). However, in April and May, E2 had no such stimulatory effects on ERα gene. In April, the basal levels may reach their maximum and E2 could not further stimulate expression. The present results indicate that the regulation of ERα mRNA by E2 is strongly dependent on smolting stage. In addition, E2 had no effects on ERα mRNA in the gill, pituitary and brain. Salbert et al. (1993) reported in rainbow trout that E2 induced a strong increase in the amount of ERα mRNA in the liver, but a moderate increase in the brain and no changes in the pituitary. Therefore, these results clearly indicate that the effects of E2 on ERα gene expression are variable, depending on tissue and developmental stage.

In contrast to E2, 4-NP did not have any stimulatory effects on ERα mRNA in the liver, but decreased the amount in April to the basal level observed in March. The inhibitory effects of 4-NP were also apparent in the gill at this stage. It is acknowledged generally that 4-NP has estrogenic effects on endocrine system in various species (White et al., 1994). However, the present results indicate that the activity of 4-NP is not always as same as E2 at the molecular level. Using a microarray approach, Larkin et al. (2002) demonstrated that some genes of largemouth bass (*Micropterus salmoides*) were increased by E2 treatment, whereas with NP treatment the same genes were decreased. It is likely that 4-NP and E2 interact with the same part of a ligand-binding domain of ER, because of their competitive binding to the ER (White et al., 1994). However, the binding of 4-NP to the ER induces conformational changes in the ER that are different from those induced by the binding of E2 (Madigou et al., 2001). The different conformational changes induced by 4-NP may result in the subsequent different action on ERα gene expression. It is also possible that the different effects on ERα mRNA were influenced by different half-lives of the two compounds in vivo. In the present study, ERα mRNA was examined 7 days after the first injection, because in rainbow trout the E2-induced increase in ERα mRNA reached maximum 6 days after injection (Pakdel et al., 1991). Effects on ERα mRNA at different time points need to be determined to further clarify the differential susceptibility of ERα gene to 4-NP and E2.

In the present study, the 15 mg/kg 4-NP dose resulted in similar or stronger responses in the gill and pituitary than the 2 mg/kg E2. Furthermore, the effects of the 15 mg/kg 4-NP dose were more intense than that of the 150 mg/kg 4-NP in the liver and pituitary. Although 4-NP is usually a comparably weak xenoestrogen, showing about 1/1000th the potency of E2 in vivo studies, this study and the previous study (Yadetie et al., 1999) showed that 4-NP is more potent than reported for alkylphenols in fish, and the low dose of 4-NP may be potent enough to elicit maximum responses. A possible explanation for the apparent higher potency of 4-NP in the present study could be the direct administration method by i.p. injection used in the present study. A more rigorous dose–response study will be needed to calculate the precise potency of 4-NP relative to E2.

The inhibition of ERα mRNA by 4-NP is in direct conflict with the previous study by Yadetie et al. (1999), in which 4-NP stimulated ERα gene expression. This disagreement may be due to differences in the time course of the two studies. In the present study, ERα mRNA was examined 7 days after injection, whereas 4-NP-induced increase in ERα mRNA was observed 2 days after treatment in the previous study. It is also possible that the differences are due to differences in the developmental stages of fish used in the studies. We examined ERα mRNA in three different stages during smolting, and the inhibition by 4-NP was observed only in the early phase of smolting.

The negative action of 4-NP on ERα gene expression may cause desensitization of target cells to 4-NP, because 4-NP binds ER. Since 4-NP has various antagonistic effects on smolt physiology (Madsen et al., 1997, 2004; Moore et al., 2003), it is tempting to speculate that the negative response to 4-NP might be a protective function of target cells against manmade xenoestrogens but not against natural estrogens.

The gill is a major iono-regulatory organ in teleosts. During the parr–smolt transformation, gill chloride cells mature and their number increases, elevating the total amount of Na^+, K^+-ATPase and its activity (Ura et al., 1997). Expression of ERα has been found in gill and other osmoregulatory tissues such as kidney, and gut in Atlantic salmon (Rogers et al., 2000) and eelpout (Andreassen et al., 2003). However, the expression level in the gill was much weaker than in the liver. Nevertheless, basal amount of ERα mRNA increased in April, rendering the gill sensitive to estrogens. Indeed, 4-NP decreased the levels of ERα mRNA at this stage, while E2 showed a similar trend. Furthermore, in the present study both compounds tended to decrease Na^+, K^+-ATPase α subunit gene expression, whereas there were no apparent effects on Na^+, K^+-ATPase activity (unpublished data). In Atlantic salmon, E2 and 4-NP reduced the gill Na^+, K^+-ATPase activity as well as Na^+, K^+-ATPase α subunit gene expression (Madsen et al., 1997, 2004). These results indicate that the estrogenic compounds, particularly 4-NP, may influence osmoregulatory function via the gill in smolting salmon.

In the pituitary, the effects of 4-NP were stage-specific, stimulatory in March and inhibitory in May. 4-NP and E2 seemed to have similar effects on ERα mRNA. Estrogen plays a pivotal role in the regulation of development and function of the pituitary. In catfish, increased expression of ERα coincides with the period in which activation of gonadotropes takes place (Schulz et al., 1997; Teves et al., 2003). The increase of ERα mRNA in the present study may be related to the activation of gonadotropes in advance of onset of sexual maturation in later stages (Swanson, 1991).

4-NP and E2 had no significant effects on brain ERα mRNA during smolting, although the basal amount tended to decrease. Salbert et al. (1993) reported in rainbow trout that E2 increased the ERα mRNA in two nuclei of the hypothalamus, the nucleus lateralis tuberis and the nucleus preopticus periventricularis. In the present study, the amount of ERα mRNA was determined in whole brain and we cannot exclude the possibility that the expression of ERα gene is regulated by E2 or 4-NP in certain nuclei. Further study is needed to elucidate ERα gene expression and responses to 4-NP and E2 in the brain of smolting salmon.

In summary, we have found that basal amounts of ERα mRNA changed significantly in the liver and gill during smolting. This suggests that response to estrogen signaling will change during smolting. This is in fact what was found: the effects of 4-NP and E2 on ERα mRNA are strongly dependent on tissue and smolt stage. Furthermore, 4-NP and E2 have different effects on ERα mRNA in the liver and gill during smolting. Further work is necessary to determine what factors control these tissue and ontogenetic differences in response to estrogenic compounds.

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