ZOOLOGICAL SCIENCE **24**: 824–828 (2007)

Effects of 17β-Estradiol on Estrogen Receptor α and β mRNA Expression in Tissues of the Olive Flounder (*Paralichthys olivaceus*)

Cheol Young Choi*

Division of Marine Environment and BioScience, Korea Maritime University, Busan 606-791, Korea

This study examined the effects of an injection of 17β-estradiol (E_2) on the expression of estrogen receptor (ER) α and β subtypes in the olive flounder (*Paralichthys olivaceus*). Time- and dose-related effects of E_2 on ERα and ERβ mRNA expression were determined by RT-PCR. In the liver of males, the two ER transcripts were up-regulated at 24 h. In the liver of females, ERα and ERβ were up-regulated at 36 h with the highest dose. After E_2 treatment, increases in ERα and ERβ expression were observed in the testis and ovary at 36 h; RT-PCR analysis showed that this increase was dose-dependent. After E_2 treatment, the brain tissue of males showed lower levels of ERα and ERβ compared with the untreated control group, whereas the brain tissue of females showed no significant difference compared with controls. The results confirm the hypothesis that ER regulation is tissue-specific and may be involved in E_2-mediated regulation of reproduction in the olive flounder.

Key words: estrogen receptor α, estrogen receptor β, dose response, olive flounder, RT-PCR, time course, tissue distribution

INTRODUCTION

Estrogen plays important roles in vertebrate physiological processes, including the regulation of oogenesis and vitellogenesis. The actions of estrogen are mediated via estrogen receptors (ERs) (Nilsson *et al*., 2001). ERs belong to a large nuclear receptor superfamily of ligand-activated transcription factors, which also includes receptors for other steroid hormones, thyroid hormone, and vitamin D as well as several orphan receptors (Mangelsdorf *et al*., 1995).

Two isoforms of ER, designated ERα and ERβ, have been described in vertebrates, and recent sequence alignments have shown the existence of ERα and ERβ subtypes in fish (Chang *et al*., 1999; Tchoudakova *et al*., 1999; Ma *et al*., 2000; Choi and Habibi, 2003). Also, a third type, ERγ, expressed in reproductive organs was found to be present in the Atlantic croaker, *Micopogonias undulates* (Hawkins *et al*., 2000) and largemouth bass, *Micropterus salmoides* (Sabo-Attwood *et al*., 2004). Many studies have shown that the expression of ER subtypes differs according to developmental stage and reproductive season (Pakdel *et al*., 1991; Mosconi *et al*., 2002; Sabo-Attwood *et al*., 2004). Furthermore, exposure to 17β-estradiol (E_2) up-regulated ERα expression in the liver in various fish species (Pakdel *et al*., 1991; MacKay *et al*., 1996; Mosconi *et al*., 2002). Nevertheless, much is unknown about ER regulation in lower vertebrates and fish.

According to Sabo-Attwood *et al.* (2004), different ER subtypes were expressed in the largemouth bass (*Micopterus salmoides*), depending on the time of year. It was reported that the ERβ level was not affected by E_2 stimulation, although the ERα level increased. Similarly, it was also reported that E_2 increased the generation of ERα, decreased ERβI levels, and did not affect ERβII (Menuet *et al*., 2004). In mammalian systems, ERβ tended to counteract the effects of ERα at the estrogen response element (ERE) (Hall and McDonnell, 1999; Weihua *et al*., 2000).

Differential responses of ERs to pharmacological agents such as tamoxifen have been well studied in mammals (Hanstein *et al*., 2004). Whether the ERα and ERβ subtypes function in a redundant fashion or play different roles is still unclear. ER subtypes are expressed in the brain, gonads, and liver of both sexes in fish.

In this study, we investigated the in vivo effects of E_2 stimulation on ER subtype expression as a step to towards understanding the molecular mechanisms of ER action in the brain, gonads, and liver of male and female olive flounder.

MATRIALS AND METHODS

Fish

Cultured olive flounder (*Paralichthys olivaceus*) purchased from fishermen in the Gijang area (Busan, Korea) ranged from 22 to 25 cm in length. The fish were maintained in recirculation tanks with a 12:12 h light/dark cycle at a temperature of 15–17°C. Experimental fish were killed, and then separated by sex. There were at least five males and five females. The gonads were weighed for calculation of the gonadosomatic index (GSI=gonad weight/body weight×100). Gonadal tissues were removed from males (GSI 0.6–0.8) and females at early stages of gonadal recrudescence (GSI 1.6–2.1), immediately frozen, and stored at –80°C until use. No food was provided during the experimental period.

* Corresponding author. Phone: +82-51-410-4756;
Fax : +82-51-404-3988;
E-mail: choic@hhu.ac.kr
doi:10.2108/zsj.24.824

Time-course experiment

Olive flounders were lightly anesthetized with a 200 mg/L solution of tricaine methane sulphonate (MS-222). 17β-estradiol was initially dissolved in ethanol and was then diluted in physiological saline. Each fish was given an injection of 17β-estradiol (5 μg/g body weight (BW)). At 12, 24, or 36 h post-injection, fish were anesthetized and killed. The brain, liver, and gonads were removed and stored at –80°C until analysis by reverse transcriptase-polymerase chain reaction (RT-PCR).

Dose-response experiment

Olive flounders were injected intraperitoneally with 17β-estradiol at 0.05, 0.5, or 5 μg/g BW, as described above for the time-course experiment. At 36 h post-injection, the fish were separated by sex and killed, and the tissues were removed and frozen at –80°C for further analysis by RT-PCR.

Validation of semi-quantitative RT-PCR

Total RNA, extracted using TRIzol reagent (Invitrogen, Carlsbad,

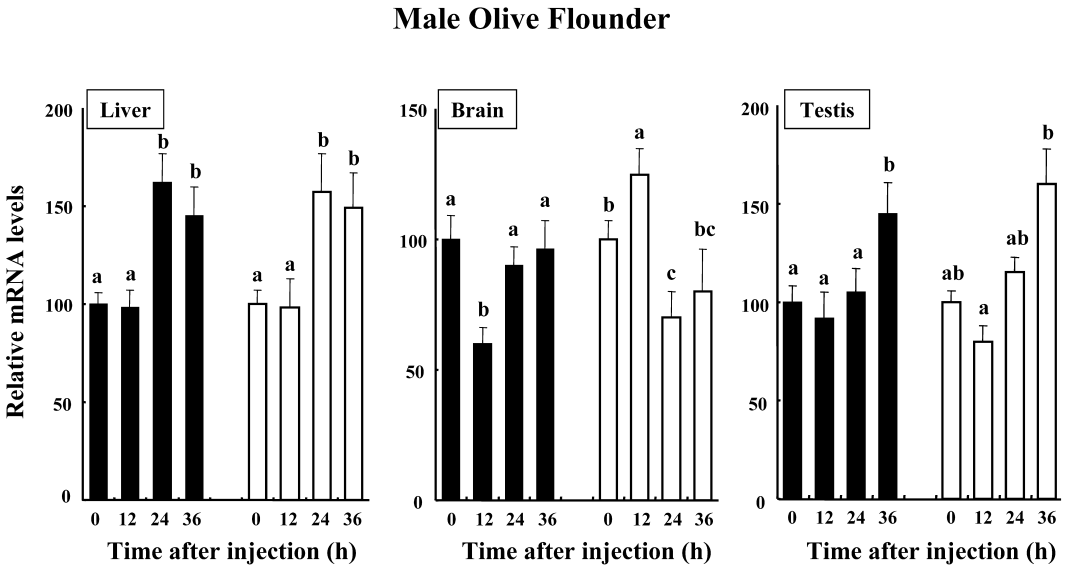

Fig. 1. Time-related effects of 17β-estradiol (E_2) on ERα (■) and ERβ (□) mRNA expression in the liver, brain, and testis of male olive flounders, as determined by RT-PCR. The fish were treated with E_2 (5 μg/g) in a time-course experiment. Control fish were injected with saline. The expression of β-actin mRNA was evaluated in each RT reaction as a loading control. The expression level in each tissue is expressed relative to the β-actin expression level (mean±SEM). Values indicated by dissimilar letters are significantly different ($P<0.05$). Each experimental group consisted of five olive flounders.

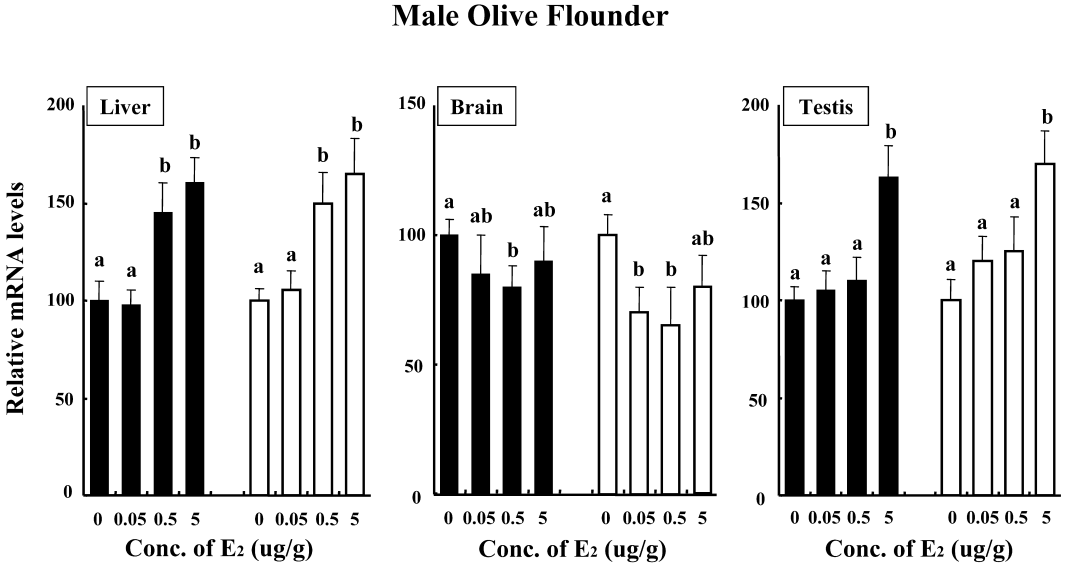

Fig. 2. Dose-related effects of 17β-estradiol (E_2) on ERα (■) and ERβ (□) mRNA expression in the liver, brain, and testis of male olive flounders, as determined by RT-PCR. The fish were treated with E_2 at concentrations of 0.05, 0.5, and 5 μg/g for 36 h. The expression of β-actin mRNA was evaluated in each RT reaction as a loading control. The expression level of each tissue is expressed relative to the β-actin expression level (mean±SEM). Values indicated by dissimilar letters are significantly different ($P<0.05$). Each experimental group consisted of five olive flounders.

CA, USA) according to the manufacturer's protocol, was reverse transcribed (1 μg) with an oligo (dT) primer and SuperScript reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

ERα-specific primers for RT-PCR were 5'-TGGCTGAGATCTTCGACATGC-3' and 5'-TGTCCTGAACTGGCTGAAGA-3'; ERβ-specific primers were 5'-AAGTGCTACGAAGTCGGCAT-3' and 5'-AAGCAACTTGGAGCGACTGT-3'. These primers were based on the sequences of olive flounder ERα (accession number AB070629) and ERβ (accession number AB070630).

Amplication of cDNA was carried out at cycling conditions of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min. To optimize the cycle number used for semi-quantitative PCR analysis, RT reaction products (1 μl) from the brain, liver, ovary or testis were used as templates for PCR amplification.

β-actin mRNA was amplified in each RT reaction as a loading control. β-actin primers were 5'-TCGAGCGTATTGTGACC-3' for the forward primer and 5'-ACGGAACCTCTCATTGCCGA-3' for the reverse primer.

The PCR products from different numbers of amplification

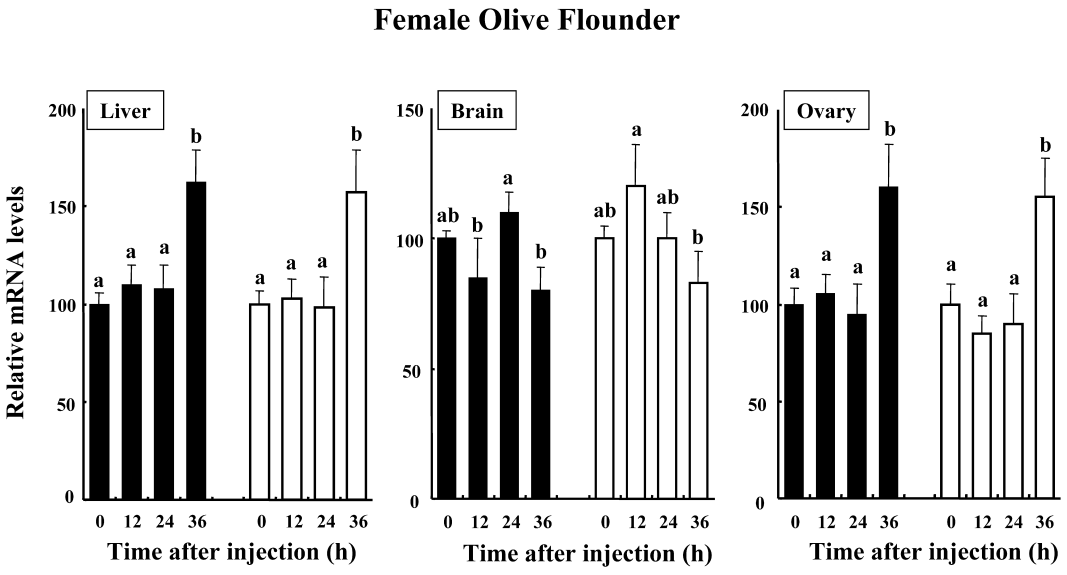

Fig. 3. Time-related effects of 17β-estradiol (E_2) on ERα (■) and ERβ (□) mRNA expression in the liver, brain, and ovary of female olive flounders, as determined by RT-PCR. The fish were treated with E_2 (5 μg/g) in a time-course experiment. Control fish were injected with saline. The expression of β-actin mRNA was evaluated in each RT reaction as a loading control. The expression level in each tissue is expressed relative to the β-actin expression level (mean±SEM). Values indicated by dissimilar letters are significantly different ($P<0.05$). Each experimental group consisted of five olive flounders.

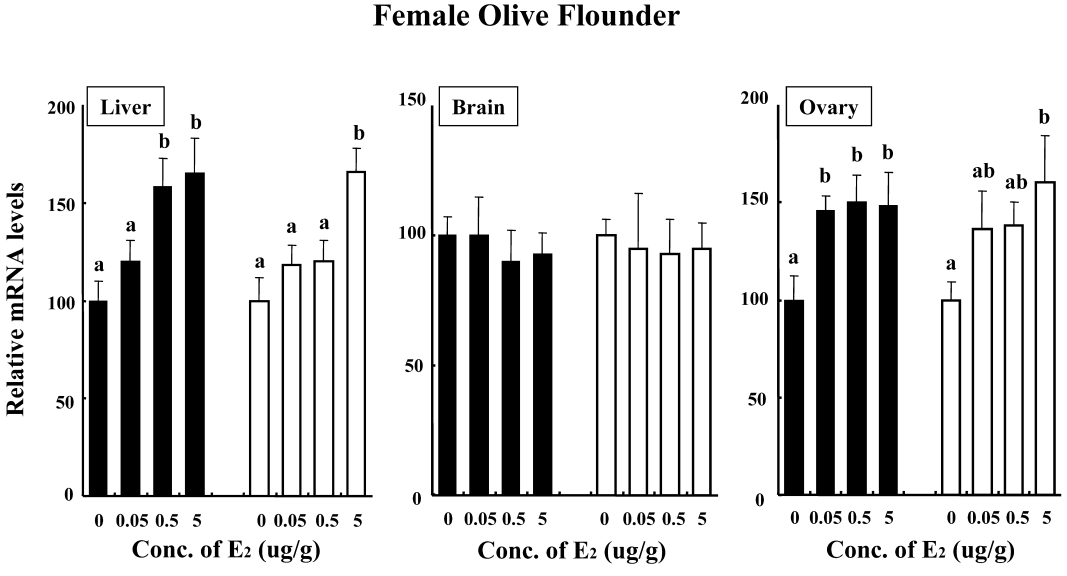

Fig. 4. Dose-related effects of 17β-estradiol (E_2) on ERα (■) and ERβ (□) mRNA expression in the liver, brain, and ovary of female olive flounders, as determined by RT-PCR. The fish were treated with E_2 at concentrations of 0.05, 0.5, and 5 μg/g for 36 h. The expression of β-actin mRNA was evaluated in each RT reaction as a loading control. The expression level of each tissue is expressed relative to the β-actin expression level (mean±SEM). Values indicated by dissimilar letters are significantly different ($P<0.05$). Each experimental group consisted of five olive flounders.

cycles were visualized on a UV-transilluminator after electrophoresis on 1.0% agarose gel containing ethidium bromide, and the signal intensity was quantitated with the Gel-Doc System and Gelpro 3.1 Software (KBT, Korea). The cycle numbers that generated half-maximal amplification were used for subsequent semi-quantitative analysis of gene expression; cycle numbers were 30 cycles for ERα and ERβ, and 27 cycles for β-actin.

One-way ANOVA followed by a post-hoc multiple comparison test (Tukey's test; Zar, 1984) was used for the analysis of differences in expression levels.

RESULTS

In male flounders, expression of the two ER subtypes increased substantially in the liver by 24 h after E_2 treatment and further increased by 36 h (Fig. 1). The expression of the two ER subtypes also increased significantly in the testis by 36 h (Fig. 1). In the dose-response experiment, RT-PCR revealed significant increases in ERα and ERβ transcript levels in the liver and testis of males after a 5 μg/g BW dose of E_2 (Fig. 2). Expression of ERα and ERβ mRNA decreased in the brain of males in response to E_2 doses of 0.05 and 0.5 μg/g BW (Fig. 2).

In females, expression of ERα and ERβ mRNA significantly increased in the liver and ovary by 36 h after E_2 treatment; expression of ERβ mRNA increased in the brain by 12 h but was normal at 36 h after treatment with E_2 (Fig. 3). In the dose-response experiment, the liver and ovary showed significantly increased transcript levels for both ER subtypes in response to E_2 at 5 μg/g BW (Fig. 4). In contrast, the ERα and ERβ transcript levels were not changed significantly in the brain at 36 h after any dose tested (Fig. 4). Thus, the dose-response results from RT-PCR were generally consistent with the findings of the time-course experiment.

DISCUSSION

In this study, I investigated the expression of two ER subtypes in response to doses of E_2 and found that the response was sex- and tissue-specific.

In male and female olive flounders, ERα and ERβ mRNA levels increased in the liver and gonads by 24 and 36 h after E_2 treatment (Figs. 1, 3). This finding is consistent with a previous report (Laurenzana *et al*., 2002) that the ERα mRNA level increased in the liver of male rats in response to an oral dose of the estrogenic compound ethynyl estradiol.

The expression of ERs after E_2 treatment has been examined in the liver of various fish species, including rainbow trout (Pakdel *et al.*, 1989), sea bream (Mosconi *et al.*, 2002), zebrafish (Menuet *et al.*, 2004), and largemouth bass (Sabo-Attwood *et al*., 2004). However, most of these studies looked only at ERα or failed to differentiate between the ER subtypes. Sabo-Attwood *et al.* (2004) demonstrated that the expression of ERα and ERγ increased, whereas ERβ was not up-regulated, in male largemouth bass after E_2 treatment. Menuet *et al.* (2004) reported that E_2 stimulated ERα expression, down-regulated ERβI, and had no effect on ERβII in zebrafish.

An estrogen response element (ERE) has been documented in the promoter of the ERα gene in rainbow trout (Le Drean *et al*., 1995) and zebrafish (Menuet *et al*., 2002). In zebrafish, AP-1 and AP-4 sites were located in the promoter region of the ERα gene (Menuet *et al*., 2004). In the olive flounder, it is believed that an increase in ERα mRNA is caused by an ER-dependent mechanism mediated through ERE or AP sites, although this remains untested.

The expression of ERα and ERβ increased in males by 24 h after E_2 treatment and increased in the liver of females by 36 h, depending on the dose (Figs. 1, 3). It is possible that *in vivo*, E_2 interacts with different EREs or interacts with other components of the endocrine system that regulate expression without involving an ERE of the ER gene promoter. Alternatively, it may be that E_2 up-regulates other transcription factors or even down-regulates RNAses. All of these mechanisms may play roles in the specific regulation of ER subtypes.

In the olive flounder testis and ovary, there was significant and dose-dependent up-regulation of the transcripts for the two ER subtypes following an E_2 dose of 5 μg/g BW (Figs. 2, 4). E_2 is involved in the functioning and maintenance of the testis through processes such as androgen activation via the androgen receptor and aromatization of androgen to E_2 (Thomas and Benjamin, 1988; Chowen *et al*., 1990). The physiological significance of ER up-regulation in the testis is as yet undetermined, although it may be involved in amplifying the estrogenic response before the reproductive season. It would be interesting to monitor the expression of ER subtypes in the testis, under the regulation of androgens.

In both the testes and ovaries, the increases in ERα and ERβ mRNA were generally preceded by a slight decrease in expression (Figs. 1, 3). This finding is consistent with the report of Ihionkhan *et al.* (2002) that ERα decreased in rat uterus and sheep endothelium.

The ER subtypes remained relatively unchanged in the olive flounder brain of either sex after E_2 treatment, with ERα and ERβ mRNA levels initially decreasing and then recovering (Figs. 1, 3). The small down-regulations of the ERα transcript observed in the male and female olive flounder brain were time- and dose-dependent. Whereas whole brain was taken for these experiments, future studies of specific brain areas may reveal that the effects of E_2 vary among brain regions, as has been suggested in previous reports. Greco *et al.* (2001) reported decreases in ERα mRNA expression in the medial amygdala and ERβ expression in the periventricular preoptic area of the forebrain of rats treated with E_2. Rune *et al.* (2002) reported that only ERα was generated in rat hippocampus treated with E_2.

Although injection of E_2 into the flounders differs from natural secretion, E_2 injection did not result in any differences between males and females, except in the brain. In the time-course experiment, there was a difference in the expression of the ERs in the brain of both sexes by 36 h after E_2 treatment. However, as the fish were in the early stages of gonadal recrudescence, it is possible that low concentrations of E_2 present in the pituitary already differed between the sexes. Further studies will be required to investigate the effects of E_2 treatment in both sexes in the period after 36 h post-injection.

The present study demonstrated that the ERα and ERβ subtypes are both affected by E_2. To our knowledge, this is the first study in fish to simultaneously examine the regulation of ER isoforms across three tissues. Although the precise regulatory mechanism(s) and physiological context

remain to be investigated, this study provides additional evidence for E_2-mediated regulation of reproduction in fish.

REFERENCES

Chang X, Kobayashi T, Todo T, Ikeuchi T, Yoshiura M, Kajiura-Kobayashi H, Morrey C, Nagahama Y (1999) Molecular cloning of estrogen receptor α and β in the ovary of a teleost fish, the tilapia (*Oreochromis niloticus*). Zool Sci 16: 653–658

Choi CY, Habibi HR (2003) Molecular cloning of estrogen receptor alpha and expression pattern of estrogen receptor subtypes in male and female goldfish. Mol Cell Endocrinology 204: 169–177

Chowen JA, Argente J, Vician L, Clifton DK, Steiner RA (1990) Pro-opiomelanocortin messenger RNA in hypothalamic neurons is increased by testosterone through aromatization to estradiol. Neuroendocrinology 52: 581–588

Greco B, Allegretto EA, Tetel MJ, Blaustein JD (2001) Co-expression of ER beta with ER alpha and progestin receptor proteins in the female rat forebrain: effects of estradiol treatment. Endocrinology 142: 5172–5181

Hawkins MB, Thornton JW, Crews D, Skipper JK, Dotte A, Thomas P (2000) Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. Proc Natl Acad Sci USA 97: 10751–10756

Hall JM, McDonnell DP (1999) The estrogen receptor beta-isoform (ER beta) of the human estrogen receptor modulates ER alpha transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140: 5565–5578

Hanstein B, Djahansouzi S, Dall P, Beckmann MW, Bender HG (2004) Insights into the molecular biology of the estrogen receptor define novel therapeutic targets for breast cancer. Eur J Endocrinol 150: 243–255

Ihionkhan CE, Chambliss KL, Gibson LL, Hahner LD, Mendelsohn ME, Shaul PW (2002) Estrogen causes dynamic alterations in endothelial estrogen receptor expression. Circ Res 91: 814–820

Ma CH, Dong KW, Yu KL (2000) cDNA cloning and expression of a novel estrogen receptor beta-subtype in goldfish (*Carassius auratus*). Biochim Biophys Acta 1490: 45–152

MacKay ME, Raelson J, Lazier CB (1996) Up-regulation of estrogen receptor mRNA and estrogen receptor activity by estradiol in liver of rainbow trout and other teleostean fish. Comp Biochem Physiol 115: 201–209

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM (1995) The nuclear receptor superfamily: the second decade. Cell 83: 835–839

Menuet A, Le Page Y, Torres O, Kern L, Kah O, Pakdel F (2004) Analysis of the estrogen regulation of the zebrafish estrogen receptor (ER) reveals distinct effects of ER alpha, ER beta1 and ER beta2. J Mol Endocrinol 32: 975–986

Mosconi G, Carnevali O, Habibi HR, Sanyal R, Polzonetti-Magni AM (2002) Hormonal mechanisms regulating hepatic vitellogenin synthesis in the gilthead sea bream, *Sparus aurata*. Am J Physiol Cell Physiol 283: C673–678

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson JA (2001) Mechanisms of estrogen action. Physiol Rev 81: 1535–1565

Laurenzana EM, Weis CC, Bryant CW, Newbold R, Delclos KB (2002) Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hephatic testosterone metabolism, cytochrome P-450 enzymes, and estrogen receptor alpha expression. Food Chem Toxicol 40: 53–63

Le Drean Y, Lazennec G, Kern L, Saligaut D, Pakdel F, Valotaire Y (1995) Characterization of an estrogen-responsive element implicated in regulation of the rainbow trout estrogen receptor gene. J Mol Endocrinol 15: 37–47

Pakdel F, Le Guellec C, Vaillant C, Le Roux MG, Valotaire Y (1989) Identification and estrogen induction of two estrogen receptor (ER) messenger ribonucleic acids in the rainbow trout liver: sequence homology with other ERs. Mol Endocrinol 3: 44–51

Pakdel F, Feon S, Le Gac F, Le Menn F, Valotaire Y (1991) *In vivo* estrogen induction of hepatic estrogen receptor mRNA and correlation with vitellogenin mRNA in rainbow trout. Mol Cell Endocrinol 75: 205–212

Rune GM, Wehrenberg U, Prange-Kiel J, Zhou L, Adelmann G, Frotscher M (2002) Estrogen up-regulates estrogen receptor alpha and synaptophysin in slice cultures of rat hippocampus. Neuroscience 113: 167–175

Sabo-Attwood T, Kroll KJ, Denslow ND (2004) Differential expression of largemouth bass (*Micropterus salmoides*) estrogen receptor isotypes alpha, beta, and gamma by estradiol. Mol Cell Endocrinol 218: 107–118

Tchoudakova A, Pathak S, Callard GV (1999) Molecular cloning of an estrogen receptor beta subtype from the goldfish, *Carassius auratus*. Gen Comp Endocrinol 113: 388–400

Thomas WT, Benjamin JD (1988) Developmental changes in and hormonal regulation of estrogen and androgen receptors present in the rabbit epididymis. Biol Reprod 39: 818–828

Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M, Gustafsson JA (2000) Estrogen receptor (ER) beta, a modulator of ER alpha in the uterus. Proc Natl Acad Sci USA 97: 5936–5941

Zar JH (1984) Biostatistical Analysis. 2nd ed, Prentice-Hall, Engleawood Cliffs, NJ, pp 186–190

(Received October 14, 2006 / Accepted March 6, 2007)