Kasetsart J. (Nat. Sci.) 46 : 80 - 90 (2012)

Effects of Pharmaceutical Mestranol on Estrogen Receptor *β* mRNA Expression Levels and Morphometry in the Anal Fins of Adult Thai Ricefish (*Oryzias minutillus*)

Arin Ngamniyom[1], Kun Silprasit[1] and Busaba Panyarachun[2]*

ABSTRACT

This study aimed to examine the estrogen receptor (*ER*) *β* mRNA expression levels and morphometry of the anal fin of adult Thai ricefish (*Oryzias minutillus*) after treatment with pharmaceutical mestranol. Time- and concentration-related courses of mestranol on *ERβ* expression were investigated in anal fins by semi-quantitative reverse transcription polymerase chain reaction. In the time course experiments, *ERβ* expression levels in male anal fins were increased at 30 d after treatment with 10 μg.mL^{-1} of mestranol. *ERβ* levels in female anal fins were highly expressed at 21, 30 and 60 d after mestranol treatment. In the concentration-response experiment, *ERβ* levels were increased in males after treatment with 10 μg.mL^{-1} of mestranol for 21 d and in females treated with 0.1, 1, or 10 μg.mL^{-1} mestranol for 21 d. Morphometrical analysis showed that the proportion of anal fin length to standard length was low in males after 10 μg.mL^{-1} mestranol treatments for 30 and 60 d. In contrast, there was no morphometrical difference in females after exposure to mestranol in any of the treatments analyzed. The results suggested that mestranol regulates the sex-dependent morphology of anal fin in development at least in part by mediating *ERβ* expression and may lead to the disruption of endocrine activities in fish.

Keywords: estrogen receptor (*ER*) *β*, anal fin, Thai ricefish, mestranol

INTRODUCTION

It is well known that in all vertebrates, the effect of natural estrogens plays a crucial role in the development and maintenance of the female phenotype (Nimrod and Benson, 1998). However, the combination of this hormone with progesterone suppresses the hypothalamic-pituitary system causing a reduction in the secretion of gonadotropin-releasing hormones (Daniels and Berga, 1997; Cheon *et al*., 2000). Estrogen works on its target cells *via* estrogen receptors (Nilsson *et al.*, 2001). The estrogen receptor (*ER*) belongs to a member of the steroid/thyroid hormone nuclear receptor superfamily of ligand-inducible transcription factors (Choi, 2007). Three isoforms of *ERs*, designated as *ERα*, *ERβ*, and *ERγ*, have been reported in vertebrates (Chang *et al*., 1999; Sabo-Attwood *et al*., 2004). Especially, in teleosts, *ERβ* is highly expressed in several tissues, such as those in the gonads, brain, liver, gastro-intestinal tract, cartilage and

[1] Faculty of Environmental Culture and Ecotourism, Srinakharinwirot University, Bangkok 10110, Thailand.
[2] Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok, 10110, Thailand.
* Corresponding author, e-mail: ngamniyom.a@gmail.com

Received date : 30/08/11 Accepted date : 28/12/11

bone (Socorro *et al*., 2000; Menuet *et al*., 2002; Hawkins and Thomas, 2004). This suggests that *ERβ* is the main receptor among *ERs*.

In aquatic animals, however, estrogen activity is interfered with by endocrine disruptors, including xenoestrogens (Vom Saal and Hughes, 2005) and pharmaceutical estrogens (Isidori *et al.*, 2009). These disrupting compounds lead to abnormal endocrine functions in reproductive and developmental processes (Min *et al.*, 2003; Yamaguchi *et al.*, 2005).

Mestranol ((17*β*)-17-ethynyl-3-methoxyestra-1, 3, 5(10)-trien-17-ol) is a known pharmaceutical estrogen that is frequently used in combination with a progestin in oral contraceptives (Faigle and Schenkel, 1998). Mestranol is the 3-methyl ether of ethinylestradiol, a prodrug which is converted to ethinylestradiol in the liver by demethylation (Schmider *et al.*, 1997). Muechler and Kohler (1980) reported that the effects of ethinylestradiol on *ER* in human oviducts were greater than those of mestranol. In wild animals, however, the effects of estrogenic mestraol have been poorly studied and remain to be investigated. A report by Nimrod and Benson (1997) suggested that mestranol has the ability to induce *ER* levels in the hepatic tissues of channel catfish (*Ictalurus punctatus*).

Fish species of the genus *Oryzias* are commonly used in laboratory studies of vertebrates in fields such as embryology, toxicology and endocrinology (Iwamatsu *et al.*, 2003; Kiparissis *et al.*, 2003; Han *et al.*, 2010). Thai ricefish (*Oryzias minutillus*), the smallest species of the genus *Oryzias*, is widely distributed in Thailand (Magtoon and Uwa, 1985). The anal fins of males are usually longer than those of females. This species has many advantageous characteristics as an experimental animal: it is easy to maintain in an aquarium, its secondary sex characteristics are clear, and its organs are sensitive in response to exogenous sex hormones (Ngamniyom *et al.*, 2007).

The aim of this study was to examine the changes in the mRNA expression levels of estrogen receptor (*ER*) *β* and the morphometrical changes in the anal fins of adult Thai ricefish (*Oryzias minutillus*) exposed to concentrations of pharmaceutical mestranol. Furthermore, the possible role of this fish was investigated as an alternative bio-indicator for screening a mestranol activity *in vivo* by monitoring the mRNA expression levels of *ER* through the anal fin tissues.

MATERIALS AND METHODS

Fish sample collection

Adult Thai ricefish were collected from ponds and ditches in the suburbs of Bangkok, Thailand, in November 2009. Their standard length was 12–15 mm. The average water temperature in the areas where the fish were captured was 26 ± 1ºC. For acclimation, males and females were kept in separate aquaria under a controlled 14:10 hr light/dark cycle at 26 ± 1ºC for 1 wk and fed *ad libitum* with TetraMin (Tokyo, Japan).

Preparation of chemical solutions

In the study, dimethyl sulfoxide (DMSO) solutions (Wako, Osaka) were prepared following González-Doncel *et al*. (2008) at suitable concentrations of non-lethal toxicity levels for *Oryzias* spp.

For the mestranol preparation, 1 mg of mestranol ((17*β*)-17-ethynyl-3-methoxyestra-1, 3, 5(10)-trien-17-ol) (Wako, Osaka) was dissolved in 0.1 mL of DMSO to make a stock solution (10 $mg.mL^{-1}$). For the experiments on mestranol concentrations, 0.001, 0.01, 0.1, and 1 mL of the stock solution was diluted with 1,000 mL of aquarium water to make concentrations of 0.01, 0.1, 1, or 10 $\mu g.mL^{-1}$, respectively. In the time-course studies, 30 males and 40 females were treated with 10 $\mu g.mL^{-1}$ of mestranol for 14, 21,

30, or 60 d under the above conditions. In the dose-response studies, 30 males and 40 females were placed in aquarium water containing 0.01, 0.1, 1 or 10 µg.mL^{-1} of mestranol for 21 d. In the control groups, males or females were treated with 1 mL of DMSO in 1,000 mL of aquarium water without mestranol solutions for 60 d. In each experiment, 3 L of the chemical solution in test aquariums was changed with the same volume of this solution every 3 d. As shown by the mortality data (Table 1), concentrations of mestranol seem to be non-lethal and exhibit non-acute toxicity for Thai ricefish.

Preparation of fins

Adult males and females were anesthetized with 10 mg.L^{-1} of an ethyl-3-aminobenzoate methanesulfonate (MS-222) solution (Sigma, St. Louis, MO) and placed in a Petri dish. Each anal fin from 30 males and 40 females was removed with a clean scalpel. Three or four pieces of the fin samples were randomly taken at each time point and pooled in a tube for the gene expression analysis.

Semi-quantitative reverse transcription (RT) polymerase chain reaction (PCR)

Total RNA was extracted using an RNeasy Fibrous Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol, and 1 µg of RNA was treated with 1 µg of DNaseI (1U.µL^{-1}) (Takara, Tokyo, Japan) for 30 min at 37 ºC. The total RNA (100 ng) was reverse-transcribed with AMV reverse transcriptase XL (Takara, Tokyo, Japan) according to the manufacturer's instructions. The first strand cDNA solution (0.5 µL) was used as a PCR template. Primers were designed based on previous data from Japanese ricefish (*Oryzias latipes*) and were then used in the analysis of the Thai ricefish. The primers used to amplify *ERβ* were 5′-CTGTTAGATGCCTCGGACCTT-3′ and 5′-GATTGGCTGGTTTCGTG-3′ (Inui *et al.*, 2003). As a loading control and reference, *β-actin* mRNA was amplified for each RT reaction; the primers used were 5′-AGGGAGAAGATGACC-3′ and 5′-CGCAGGACGCCATACCAA-3′ (Scholz *et al*., 2004). The PCR conditions for the amplification of cDNA were: 95 ºC for 30 s for denaturation; 64 ºC (*ERβ*) or 58 ºC (*β-actin*) for 45 s for annealing; and 72 ºC for 1 min for extension. The RT-PCRs of linear phases were determined on 16–26 cycles for *β-actin* and 26–36 cycles for *ERβ* to allow semi-quantitative comparisons of cDNAs which were developed for the optimum reactions according to the previous methods of Ngamniyom *et al*. (2009). Therefore, the number of cycles for *ERβ* was 30 cycles, and for *β-actin* was 20 cycles. The PCR

Table 1 Effects of various durations and concentrations of mestranol on survival percentages of adult Thai ricefish.

Group	Time of treatment (day)	Concentration of mestranol (µg.mL^{-1})	Number of treated individuals	Survival (%)
Control	60	0	20	100
Mestranol treatment	14	10	20	95
	21	10	20	100
	30	10	20	100
	60	10	20	90
	21	0.01	20	100
	21	0.1	20	85
	21	1	20	100

products were electrophoresed on a 2% agarose gel, stained with ethidium bromide and visualized under a UV transilluminator. The density of the amplified band was quantified using Scion Image Software for Windows (Scion, Maryland, USA). The *ERβ* density for each sample of the amplified bands was divided by the corresponding *β-actin* density to obtain the relative expression levels.

Morphometrical analysis of anal fin

Samples of 30 males and 40 females were contained in separate aquaria under the above conditions. The measurement of the fins was conducted according to the method of Khan *et al.* (2002). To evaluate the fin lengths of the males and females, the anal fin length (AFL) was divided by the standard length (SL) and multiplied by 100. The resulting value was defined as AFL/SL % (Figure 1).

Statistical analysis

One-way ANOVA with Tukey's multiple comparison test was employed to determine whether differences were statistically significant ($P < 0.05$ and $P < 0.01$). The data were analyzed using the Statistical Package for the Social Sciences (SPSS) for Windows (version 13, SPSS, Chicago, USA).

RESULTS

Time-course experiment of *ERβ* mRNA expression levels in the anal fins of adult Thai ricefish after mestranol treatment

In the males, the *ERβ* mRNA expression levels had increased significantly in anal fins at 30 d after treatment with 10 $\mu g.mL^{-1}$ of mestranol and had decreased to the normal level at 60 d after treatments (one-way ANOVA test, $P < 0.05$), as shown in Figure 2a. In the females, the *ERβ* mRNA expression levels were significantly higher at 21 ($P < 0.05$), 30 ($P < 0.01$) and 60 ($P < 0.05$) d after treatment with mestranol than the levels in the control group and at 14 d after treatment (Figure 2b).

Concentration-course analysis of *ERβ* mRNA expression levels in the anal fins of Thai ricefish after mestranol treatment

In the males, the *ERβ* mRNA expression levels were significantly ($P < 0.01$) higher after treatment with 10 $\mu g.mL^{-1}$ of mestranol for 21 d, as shown in Figure 2c. In the females, significantly higher expression of *ERβ* mRNA was found after treatment with 0.1 ($P < 0.05$), 1 ($P < 0.05$) or 10 ($P < 0.01$) $\mu g.mL^{-1}$ of mestranol for 21 d (Figure 2d).

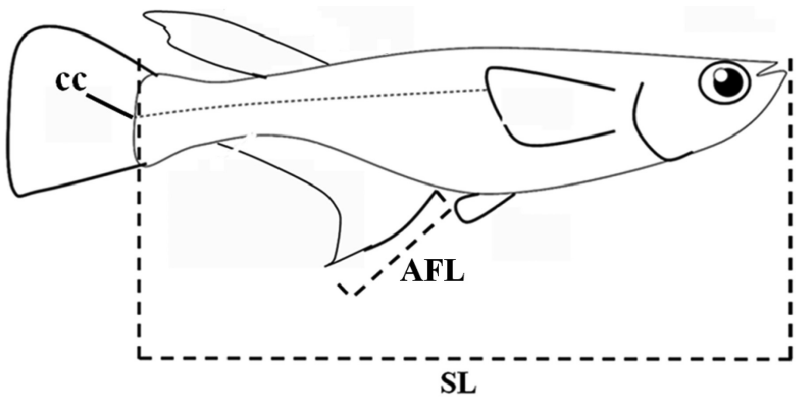

Figure 1 Diagrammatic illustration of measurement of length of anal fin (AFL) and standard length (SL). CC = Caudal peduncle.

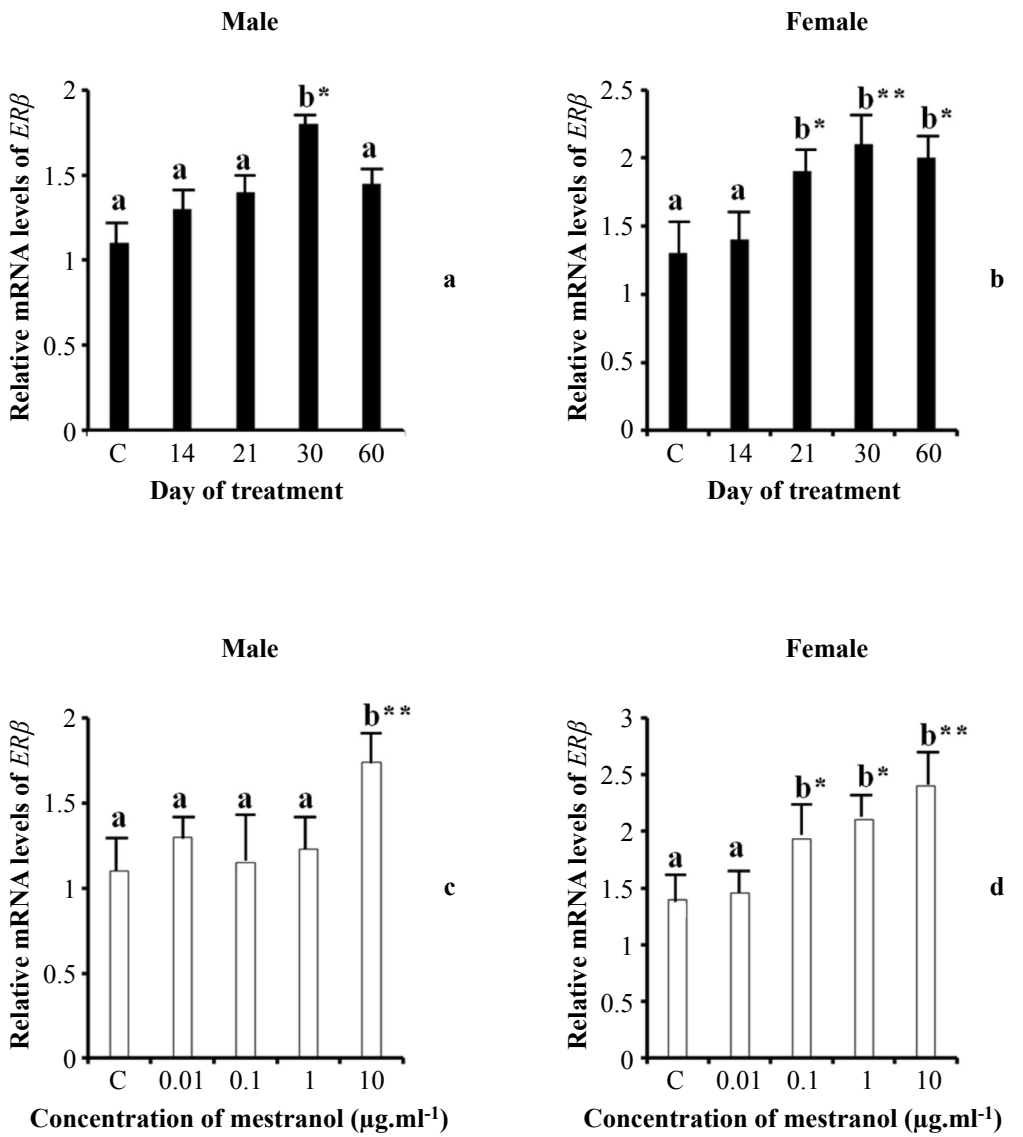

Figure 2 Time-related effects (a and b) and concentration-related effect (c and d) of mestranol on estrogen receptor (*ER*) β mRNA expression level in anal fins of Thai ricefish, as examined by semi-quantitative reverse transcription polymerase chain reaction. Control groups (C) were treated with dimethyl sulfoxide. The expression levels in each fin are relative values compared to those of β-actin mRNA (mean + SE bar shown). Single- and double-asterisk symbols indicate significant differences (one-way ANOVA with Tukey's multiple comparison test; * = $P < 0.05$ and ** = $P < 0.01$, respectively) between values, which are marked by different letters.

Morphometrical changes in the anal fins of Thai ricefish after mestranol treatment

In the males, the values of AFL/SL% were significantly decreased at 30 ($P < 0.05$) and 60 ($P < 0.01$) d after treatment with 10 μg.mL^{-1} of mestranol compared with the untreated control group and with the group treated for 14 and 21 d (Table 2). In contrast, no significant differences in the values of AFL/SL% were found between the untreated control group and treated groups of females.

DISCUSSION

This study investigated the *in vivo* effects of pharmaceutical mestranol on the *ERβ* expression levels in Thai ricefish anal fins, a typical secondary sex characteristic of this species. The *ERβ* expression levels indicated that the response in the anal fin of this gene to various concentrations of mestranol was different between males and females. In the male and female adult Thai ricefish, the *ERβ* levels were up-regulated in both the time-and concentration-dependent treatments of mestranol. These finding were consistent with a report by Hayashi *et al.* (2007) that the mRNA expression levels of *ER* increased in the anal fins of Japanese ricefish in response to concentrations of the estrogenic bisphenol A. It is also similar to the finding in a study by Islinger *et al.* (2000), who showed that the pharmaceutical ethinylestradiol can induce *ER* mRNA expression levels in the testes of zebrafish (*Danio rerio*).

In the adult male anal fins, the *ERβ* levels were initially altered at 30 d after treatment with mestranol. However, in the adult female anal fins, the *ERβ* levels were affected at 21 d after treatment. Furthermore, the *ERβ* levels in the males were found to be significantly altered only by the highest concentration of mestranol. In contrast, the *ERβ* levels in the females were significantly changed in response to the low concentration. Therefore, the female response to pharmaceutical mestranol may be stronger than that in males. In vertebrates, androgen is known to biologically suppress the action of estrogenic chemicals (Poulin *et al.*, 1989; Brenner *et al.*, 2003; Sun *et al.*, 2009). Therefore, endogenous androgen may suppress mestranol regulation in male anal fins, causing the more delayed response in *ERβ* expression levels to mestranol. After up-regulation, *ERβ* down-regulation was observed from 30 to 60 d with mestranol treatment in the males. It may be that *in vivo*, this effect of mestranol is resuppressed by the endogenous-androgenic hormones in the male fish. In the female anal fins, when the *ERβ* expression levels were increased by mestranol effects, there remained a high level at 21, 30 and 60 d in the time-related treatments. This result corresponds to a report by Choi (2007), in which 17β-estradiol caused the *ER* expression rates to be physiologically increased and maintained in ovarian tissues of the olive flounder (*Paralichthys olivaceus*).

Estrogen can be rapidly activated by mediating with *ERs* on the surface of the

Table 2 Fin measurement of adult Thai ricefish after treatment with mestranol (10 μg.mL^{-1}).

Measument	Days of Treatment				
	C	14	21	30	60
Male-AFL/SL% (Mean ± SE)	21.6±0.7^{a}	21.4±0.5^{a}	21.1±0.6^{a}	19.2±0.3^{b*}	18.1±0.4^{b**}
Female-AFL/SL% (Mean ± SE)	16.6±0.3	16.1±0.5	17.1±0.9	15.9±0.6	16.0±0.8

AFL = Anal fin length; SL = Standard length of fish.

Mean values in the same row superscripted by different letters are significantly different (One-way ANOVA with Tukey's multiple comparison test; * = $P < 0.05$ and ** = $P < 0.01$); C = Control group.

cell membrane (Zivadinovic *et al.*, 2005). An exogenous-estrogenic mestranol may act on the dorsal and anal fins by associating through the membrane estrogen receptor in the rapid-response effects and play an important role *via* the nuclear estrogen receptor in the late-response effects. Therefore, the current study was only concerned with regulation of the latter issue.

It should be noted that in teleost fish, the quantitative real-time PCR has efficacy for analyzing gene expressions in a significant response to the low concentrations of estrogenic substances between treatment groups (Le Page *et al*., 2006; Zhang *et al*., 2008). However, in the current study, the semi-quantitative RT-PCR analysis was sufficient for the investigation of significant differences of the mestranol effect on the *ERβ* expression levels in the experimental groups, since the mestranol concentrations were 0.01–10 $\mu g.mL^{-1}$.

The pharmaceutical effects after exposure to estrogenic chemicals have been examined in various species of vertebrates, including humans (Kustera *et al.*, 2008; Wright-Walters and Volz, 2009). Tavassoli *et al.* (1988) showed the potential of mestranol and ethynerone to induce carcinoma cells in the human breast to a moderate degree. Li *et al.* (1998) reported that administration of diethylstilbestrol and moxestrol caused estrogen-induced carcinogenesis in the hamster kidney. Conversely, certain estrogenic drugs can reduce cancer risk by acting as selective estrogen receptor modulators; examples include tamoxifen (Howell *et al.*, 2004), bazedoxifene (Archer *et al.*, 2009) and raloxifene (Chin *et al.*, 2005). Hagino *et al.* (2001) demonstrated that sex reversal in Japanese ricefish was caused by estrogenic ethinylestradiol and diethylstilbestrol treatment. In the current study of male Thai ricefish, the long anal fins were shortened by a mestranol-induced effect, making the fins more feminized. It is known that in male *Oryzias*, the length of the anal fin is related to mating and appears to be important in stimulating the female into oviposition, despite the fact that the anal fin is not a copulatory organ (Koseki *et al.*, 2000). It is possible that pharmaceutical estrogens function on their target tissues with species-specific differences. The action of mestranol disrupts the reproductive endocrine system causing feminization of male anal fins, which may retard the fertilization success rate in adult Thai ricefish.

Recently, Thai ricefish have been tested as a bio-indicator for monitoring the environmental pollution of freshwater (Ngamniyom and Panyarachun, 2011). Ngamniyom *et al*. (2007) reported that the sex ratio (male to female) of Thai ricefish collected from an unpolluted environment was almost 1:1. Conversely, in polluted water, abnormal sex ratios (3:1) were found with the occurrence of many sex-undeterminable individuals. In these individuals, the morphometry of the dorsal anal fins was intermediate between that of males and females. Ngamniyom *et al*. (2009) showed that the *ERβ* expression levels in the dorsal and anal fins of sex-undeterminable individuals were intermediate between those of normal males and females, suggesting that some chemicals might disturb the function of estrogen alone *via ERβ* in those fins. Therefore, the previous reports support the potential of Thai ricefish as a bio-indicator for screening the chemical activities *in vivo* by monitoring the morphological changes and gene expressions in the fins.

CONCLUSION

The current study was the first to demonstrate that *ERβ* mRNA expression levels are affected by pharmaceutical estrogenic mestranol in the anal fins of fish. This chemical interferes with the endocrine systems and leads to some feminization in the fin morphometry of males. The current study has increased the understanding of the effects of pharmaceutical estrogen on freshwater fish, although the mechanism of the actions remains to be evaluated.

ACKNOWLEDGEMENTS

The authors thank the Department of Anatomy, Faculty of Medicine, Srinakharinwirot University, Bangkok, Thailand for providing the molecular analytical instruments.

LITERATURE CITED

Archer, D.F., J.V. Pinkerton, W.H. Utian, J.C. Menegoci, T.J. de Villiers, C.K. Yuen, A.B. Levine, A.A. Chines and G.D. Constantine. 2009. Bazedoxifene, a selective estrogen receptor modulator: Effects on the endometrium, ovaries, and breast from a randomized controlled trial in osteoporotic postmenopausal women. **Menopause** 16(6): 1109–1115.

Brenner, R., O. Slayden, N. Nayak, D. Baird and H. Critchley. 2003. A role for the androgen receptor in the endometrial antiproliferative effects of progesterone antagonists. **Steroids** 68(10): 1033–1039.

Chang, X., T. Kobayashi, T. Todo, T. Ikeuchi, M. Yoshiura, H. Kajiura-Kobayashi, C. Morrey and Y. Nagahama. 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.

Cheon, M., D. Park, Y. Park, K. Kam, S.D. Park and K. Ryu. 2000. Progesterone together with estrogen attenuates homologous upregulation of gonadotropin-releasing hormone receptor mRNA in primary cultured rat pituitary cells. **Endocrin** 13(3) 379–384.

Choi, C.Y. 2007. Effects of 17beta-estradiol on estrogen receptor alpha and beta mRNA expression in tissues of the olive flounder (*Paralichthys olivaceus*). **Zoolog. Sci.** 24(8): 824–828.

Chin, M., M. Isono, K. Isshiki, S. Araki, T. Sugimoto, B. Guo, H. Sato, M. Haneda, A. Kashiwagi and D. Koya. 2005. Estrogen and raloxifene, a selective estrogen receptor modulator, ameliorate renal damage in db/db mice. **Am. J. Pathol.** 166(6): 1629–1636.

Daniels, T.L. and S.L. Berga. 1997. Resistance of gonadotropin releasing hormone drive to sex steroid-induced suppression in hyperandrogenic anovulation. **J. Clin. Endocrinol. Metab.** 82(12): 4179–4183.

Faigle, W. and L. Schenkel. 1998. Pharmacokinetics of estrogens and progestogens, pp. 273–294. *In* J.W. Fraser and S. Ian, (eds.). **Estrogens and Progestogens in Clinical Practice.** Churchill Livingstone Press, London.

González-Doncel, M., M.S. Okihiro, C. Torija, J.V. Tarazona and D.E. Hinton. 2008. An artificial fertilization method with the Japanese medaka: Implications in early life stage bioassays and solvent toxicity. **Ecotoxicol. Environ. Saf.** 69: 95–103.

Hagino, S., M. Kagoshima and S. Ashida. 2001. Effects of ethinylestradiol, diethylstilbestrol, 4-t-pentylphenol, 17beta- estradiol, methyltestosterone and flutamide on sex reversal in S-rR strain medaka (*Oryzias latipes*). **Environ. Sci.** 8: 75–87.

Han, S., K. Choi, J. Kim, K. Ji, S. Kim, B. Ahn, J. Yun, K. Choi, J.S. Khim, X. Zhang and J.P. Giesy. 2010. Endocrine disruption and consequences of chronic exposure to ibuprofen in Japanese medaka (*Oryzias latipes*) and freshwater cladocerans Daphnia magna and Moina macrocopa. **Aquat. Toxicol.** 98(3): 256–264.

Hawkins, M.B. and P. Thomas. 2004. The unusual binding properties of the third distinct teleost estrogen receptor subtype ERbeta are accompanied by highly conserved amino acid changes in the ligand binding domain. **Endocrinology** 145: 2968–2977.

Hayashi, H., A. Nishimoto, N. Oshima and S. Iwamuro. 2007. Expression of the estrogen receptor alpha gene in the anal fin of Japanese medaka, *Oryzias latipes*, by environmental

concentrations of bisphenol A. **J. Toxicol. Sci.** 32(1): 91–96.

Howell, S.J., S.R. Johnston and A. Howell. 2004. The use of selective estrogen receptor modulators and selective estrogen receptor down-regulators in breast cancer. **Best Pract. Res. Clin. Endocrinol. Metab.** 18(1): 47–66.

Inui, M., T. Adachi, S. Takenaka, H. Inui, M. Nakazawa, M. Ueda, H. Watanabe, C. Mori, T. Iguchi and K. Miyatake. 2003. Effect of UV screens and preservatives on vitellogenin and choriogenin production in male medaka (*Oryzias latipes*). **Toxicology** 194: 43–50.

Isidori, M., M. Bellotta, M. A. Cangiano and A. Parrella. 2009. Estrogenic activity of pharmaceuticals in the aquatic environment. **Environ. Int.** 35(5): 826–829.

Islinger, M., D. Willimski, A. Völkl and T. Braunbeck. 2000. Effects of 17a-ethinylestradiol on the expression of three estrogen-responsive genes and cellular ultrastructure of liver and testes in male zebrafish. **Aquat. Toxicol.** 62(2): 85–103.

Iwamatsu, T., H. Nakamura, K. Ozato and Y. Wakamatsu. 2003. Normal growth of the "see-through" medaka. **Zoolog. Sci.** 20(5): 607–615.

Khan, M., A. Cleveland and F. Mollah. 2002. A comparative study of morphology between F1 hybrid Magur (*Clarias*) and their parents. **J. Biological. Sci.** 2(10): 699–702.

Kiparissis, Y., G.C. Balch, T.L. Metcalfe and C.D. Metcalfe. 2003. Effects of the isoflavones genistein and equol on the gonadal development of Japanese medaka *Oryzias latipes*. **Environ. Health Perspect.** 111(9): 1158–1163.

Koseki, Y., K. Takata and K. Maekawa. 2000. The role of the anal fin in fertilization success in male medaka, *Oryzias latipes*. **Fish. Sci.** 66(4): 633–635.

Kustera, M., M.J. Aldaa, M.D. Hernandob, M. Petrovica, J. Martín-Alonsod and D. Barceló a. 2008. Analysis and occurrence of pharmaceuticals, estrogens, progestogens and polar pesticides in sewage treatment plant effluents, river water and drinking water in the Llobregat river basin (Barcelona, Spain). **J. Hydrol.** 358(1): 112–123.

Le Page, Y., M. Scholze, O. Kah and F. Pakdel. 2006. Assessment of xenoestrogens using three distinct estrogen receptors and the zebrafish brain aromatase gene in a highly responsive glial cell system. **Environ. Health. Perspect.** 114(5): 752–758.

Li, J.J., X. Hou, J. Bentel, E.M. Yazlovitskaya and S.A. Li. 1998. Prevention of estrogen carcinogenesis in the hamster kidney by ethinylestradiol: Some unique properties of a synthetic estrogen. **Carcinogenesis** 19(3): 471–477.

Magtoon, W. and H. Uwa. 1985. Karyotype evolution and relationship of a small rice fish (*Oryzias minutillus*) from Thailand. **Proc. Japan Acad.** 61: 157–160.

Menuet, A., E. Pellegrini, I. Anglade, O. Blaise, V. Laudet, O. Kah and F. Pakdel. 2002. Molecular characterization of three estrogen receptor forms in zebrafish: Binding characteristics, transactivation properties, and tissue distributions. **Biol. Reprod.** 66: 1881–1892.

Min, J., S. Lee and M. Gu. 2003. Effects of endocrine disrupting chemicals on distinct expression patterns of estrogen receptor, cytochrome P450 aromatase and p53 genes in *Oryzias latipes* liver. **J. Biochem. Mol. Toxicol.** 17(5): 272–277.

Muechler, E. and D. Kohler. 1980. Properties of the estrogen receptor in the human oviduct and its interaction with ethinylestradiol and mestranol in vitro. **J. Clin. Endocrinol. Metab.** 51(5): 962–967.

Nimrod, A.C. and W.H. Benson. 1997. Xenobiotic interaction with and alteration of channel catfish estrogen receptor. **Toxicol. Appl.**

Pharmacol. 147(2): 381–390.

Nimrod, A.C. and W.H. Benson. 1998. Reproduction and development of Japanese medaka following an early life stage exposure to xenoestrogens. **Aquat. Toxicol.** 44(1): 141–156.

Ngamniyom, A., W. Magtoon, Y. Nagahama and Y. Sasayama, 2007. A study of the sex ratio and fin morphometry of the Thai medaka (*Oryzias minutillus*) inhabiting suburbs of Bangkok, Thailand. **Fish Biol. J. Medaka.** 11: 17–21.

Ngamniyom, A., W. Magtoon, Y. Nagahama and Y. Sasayama, 2009. Expression levels of hormone receptors and bone morphogenic protein in fins of medaka. **Zool. Sci.** 26: 74–79.

Ngamniyom, A. and B. Panyarachun, 2011. Expression levels of hormone receptor and vitellogenin mRNAs in livers of Thai medaka, *Oryzias minutillus*, inhabiting the suburbs of Bangkok, Thailand. **J. Fish Aquat. Sci.** 6: 438–446.

Nilsson, S., S. Mäkelä, E. Treuter, M. Tujague, J. Thomsen, G. Andersson, E. Enmark, K. Pettersson, M. Warner and J. Gustafsson. 2001. Mechanisms of estrogen action. **Physiol. Rev.** 81(4): 1535–1565.

Poulin, R., J. Simard, C. Labrie, L. Petitclerc, M. Dumont, L. Lagace and F. Labrie. 1989. Down-regulation of estrogen receptors by androgens in the ZR-75-1 human breast cancer cell line. **Endocrinology** 125(1): 392–399.

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

Schmider, J., D. Greenblatt, L. von Moltke, D. Karsov, R. Vena, H. Friedman and R. Shader. 1997. Biotransformation of mestranol to ethinyl estradiol *in vitro*: The role of cytochrome P-450 2C9 and metabolic inhibitors. **J. Clin. Pharmacol.** 37(3): 193–200.

Scholz, S., C. Kordes, J. Hamann and H.O. Gutzeit. 2004. Induction of vitellogenin *in vivo* and *in vitro* in the model teleost medaka (*Oryzias latipes*): Comparison of gene expression and protein levels. **Mar. Environ. Res.** 57: 235–244.

Socorro, S. D.M. Power, P.E. Olsson and A.V. Canario. 2000. Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization and tissue distribution. **J. Endocrinol.** 166: 293–306.

Sun, L., J. Zha and Z. Wang. 2009. Effects of binary mixtures of estrogen and antiestrogens on Japanese medaka (*Oryzias latipes*). **Aquat. Toxicol.** 93(1): 83-9.

Tavassoli, F., H. Casey and H. Norris. 1988. The morphologic effects of synthetic reproductive steroids on the mammary gland of rhesus monkeys. Mestranol, ethynerone, mestranol-ethynerone, chloroethynyl norgestrel-mestranol, and anagestone acetate-mestranol combinations. **Am. J. Pathol.** 131(2): 213–234.

Vom Saal, S. and C. Hughes. 2005. An extensive new literature concerning low-dose effects of bisphenol A shows the need for a new risk assessment. **Environ. Health Perspect.** 113(8): 926–933.

Yamaguchi, A., H. Ishibashi, S. Kohra, K. Arizono and N. Tominaga. 2005. Short-term effects of endocrine-disrupting chemicals on the expression of estrogen-responsive genes in male medaka (*Oryzias latipes*). **Aquat. Toxicol.** 30;72(3): 239–249.

Wright-Walters, M. and C. Volz. 2009. Municipal wastewater concentrations of pharmaceutical and xeno-estrogens: Wildlife and human health implications. *In* **Proceedings of the National Conference on Environmental Science and Technology** 3: 103–113.

Zivadinovic, D, B. Gametchu and C.S. Watson. 2005. Membrane estrogen receptor-alpha levels in MCF-7 breast cancer cells predict cAMP and proliferation responses. **Breast Cancer Res.** 7(1): 101–112.

Zhang, Z., J. Hu, S. Sai, Y. Zhao, C. Huang and X. Tian. 2008. Gene cloning, sequence analysis and tissue expression of estrogen-related receptor alpha (Erralpha) in Japanese medaka and its transcriptional responses after differential EDCs exposure. **Huan Jing Ke Xue** 29(11): 3153–3158.