International Journal of Aquatic Biology (2013) 1(6): 273-280
ISSN: 2322-5270
Journal homepage: www.NPAJournals.com

Original Article

Effects of the aromatase inhibitore Letrozole on serum immunoglobulin and lysozyme levels in immunized rainbow trout (*Oncorhynchus mykiss* Walbaum) females

Paria Akbary[1], Alireza Mirvaghefi*[1], Mostafa Akhlaghi[2], Bagher Majazi Amiri[1], Mohammad Saeid Fereidouni[2]

[1]Department of Fisheries and Environmental Sciences, Faculty of Natural Resources, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran.
[2]Department of Aquatic Animal Health Unit, School of Veterinary Medicine, Shiraz University, Shiraz, Iran.

Abstract: Letrozole is a synthetic aromatase inhibitor and interfere in the committed step in the synthesis of endogenous estrogens from androgens. Also estrogens regulate the immune system in teleost. Changes of 17- β- esrtradiol (E_2), serum immunoglobulin and lysozyme levels were measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus*, enzyme-linked immunosorbent assay (ELISA) and ELISA respectively. Twelve broodstocks were injected weekly with 2.5 mg kg^{-1} letrozole (an endocrine disrupter component) two months before spawning season and vaccinated intraperitoneally (i.p) with a bacterin (inactivated *L. garviae*) one month before spawning. Twelve broodstocks for vaccination and twelve female rainbow trout as control group were also immiunised (i.p) with the bacterin and injected (i.p) with PBS, respectively. In the group received 2.5 mg AI kg^{-1} per week, serum E_2 levels were significantly lower than that of other groups. Total immunoglobulin level and lysozyme activity were significantly higher in the parents received 2.5 mg kg^{-1} per week and were immunized with 10^{-9} cells ml^{-1} *Lactococcus garvieae* compared to the group which immunized with *L. garvieae* and the control (non- immunized). The present study, suggests that aromatase inhibitors such as letrozole may be a potential tool to regulate the synthesis of E_2, is involved in the hormone- immune system interaction in rainbow trout.

Article history:
Received 1 November 2013
Accepted 12 December 2013
Available online 25 December 2013

Keywords:
Letrozole
Lysozyme
17-β- estradiol (E_2)
Immunoglobulin
Oncorhynchus mykiss

Introduction

Letrozole (CGS 20264), with commonly used brand name Femara, is a non-steroidal trizole derivation and one of the most potent aromatase inhibitors yet developed (Smith, 1999). As such, aromatase inhibitors (AIs) have been used to treat metastatic breast cancer for over 25 years (Howell and Buzdar, 2005). Letrozole has a potential to prevent the conversion of androgenic steroids to estrogens (Haynes et al., 2003; Smith, 1999). Letrozole is capable of inhibiting aromatase 98-99% and reducing serum concentrations of estrone and E_2 beyond the limit of detection in patients (Smith, 1999). The potential of aromatase inhibitors to adversely affect sexual differentiation and reproduction in fish was demonstrated in a study by Piferrer et al. (1994), and similar results have been reported by Afonso et al. (1999, 2000), Ankley et al. (2002) and Panter et al. (2004).

During the last decade, a number of studies have shown that in addition to their classically described reproductive functions, estrogens and androgens also regulate the immune system in teleosts (Swain and Nayak, 2009).

In aquatic environments, fishes are often used as bioindicators for environmental contaminants. Many environmental contaminants (for example Letrozole), interfere with hormonal regulation in fish and are therefore known as endocrine disruptors (Shilling et al., 1999). The studies on reproductive-

* Corresponding author: Alireza Mirvaghefi
E-mail address: avaghefi@ut.ac.ir

immune interactions in fishes indicate that sexual maturation (i.e., gonadal maturity and reproductive activities) can potentially influence both the innate and adaptive immune responses (Harris and Bird, 2000; Milla et al., 2011).

The reproductive cycle of fishes is marked by seasonal variation in plasma sex-steroids and changes in immune parameters which render them more sensitive to the presence of pathogens (Hou, 1998). Furthermore, immune biomarkers may prove useful for the identification of contamination in the wild (Milla et al., 2011)

Some studies, have shown that the increase of 17-β-estradiol during the spawning season affects the lymphocyte proliferation and IgM level, and antibody- producing cells in several fish species like *Oncorhynchus mykiss*, *Salmo trutta*, Chinook salmon, *Oncorhynchus tshawytscha*, gold fish, *Carassius auratus* (Hou, 1998; Slater and Schreck, 1993; Suzuki et al., 1997; Thilagam et al., 2009). For example, in *O. mykiss* (Hou et al., 1999; Lei et al., 2010) and gilthead seabream (Cuasco et al., 2008), the rise of sex hormones can suppress the plasma IgM and IgM secreting cells during spawning period leading to immunosuppressive condition. However the suppressive effects of E_2 on IgM production are not observed in carp (Saha et al., 2004) and zebra fish (*Danio rerio*) (Lei et al., 2010).

Elevation of sex steroids can make fish more vulnerable to several microbial infections during the spawning season and subsequent mortality following the spawning phase. Therefore the overall health and immune status of brood fish is very important not only for breeding performances but also for the health condition of offspring (Lei et al., 2010). In this paper, we investigated the effects of endocrine disrupter component (EDC) letrozole on lysozyme and IgM levels after female immunization of rainbow trout.

Materials and methods

Letrozole: The non-steroidal aromatase inhibitor letrozole (CGS 20267) [1H]-4-Androstron-3,17-dione, obtained from Iran Hormone Venture Pharmaceutical Technology Development Co., Ltd., Iran and was dissolved in the vehicle ethanol (Shilling et al., 1999) Stock solution containing 2.5 mg of AI mL^{-1} was prepared.

Bacteria and bacterin (vaccine) preparation: *Lactococcus gar*vieae (EU727199) was isolated from diseased fish showing symptoms of lactococcosis which were collected from rainbow trout farms in Fars Province (obtained from the Shiraz University, Shiraz, Iran). This strain was selected on the basis of its antigenic characteristics that were determined in a previous work (Sharifiyazdi et al., 2010). The strain was routinely grown onto Columbia sheep blood agar (Oxid, Madrid, Spain) plates at 25 °C for 24-48 h. Stock cultures were maintained fozen at -80 °C in tryptone soy broth (Difco, Madrid, Spain) with 15% glycerol. A formaline-killed vaccine was prepared as previously described (Toranzo et al., 1995; Romalde et al., 2004). The selected strain of *L. garvieae* was grown in Trypticase Soy Broth (TSB, Difco) for 48 h. Bacterial cells were killed by addition of formalin to achieve a final concentration of 0.3% and incubated for 3h at 25 °C and then 4 °C overnight. Bacterial cells were collected by centrifugation at 6500 ×g for 30 min at 4 °C and washed three times with phosphate buffered saline (PBS: pH 7.4) and then were re-suspended in PBS at a final concentration of 1.2×10^8 cells mL^{-1}.

Fish and rearing conditions: Thirty six adult rainbow trout (*O. mykiss*) females were obtained in mid-September 2011 from Dalkhan rainbow trout hatchery farm (Sepidan, west of Shiraz, Iran) and held outdoor in a 10 m^2 concrete pond in a flow-through water system. The specimens were kept in well-aerated water at 15.8 ± 0.5 °C, dissolved oxygen 5.5 ± 0.1 ppm and pH 7.8 ± 0.18 (mean ± SD). Fish were initially weighed (854 ± 0.1 g) under anesthesia (150 ppm clove oil). After 7 days of acclimation to the condition, they were randomly divided in three groups and each group was kept in a 2 m^2 concrete pond with a water depth of 50 cm in

the same flow-through water system. During the experiment, the specimens were fed with commercial salmon food (Beyza 121 Feed Mill (BFM) Co., Ltd., Iran).

Letrozole injection of brood fish: These procedures were carried out at least 3 days after females had been transferred to two m^2 concrete ponds. In mid-August 2011, twelve specimens were weekly injected as itraperitonially injection (i.p) with 2.5 mg kg^{-1} letrozole at the base of the right ventral fin using individual 4 mL syringes fitted with an 18.5 gauge needle. Twenty four fish were injected with the vehicle ethanol only (1.0 mL kg^{-1} body weight).

Vaccination of brood fish: In mid-September, letrozole injected parents, were immunized with 1.0 mL of formalin (3%) inactivated *L. garvieae* (10^9 cells mL^{-1}) (n=12). Also twenty four fish injected only with ethanol were divided into two groups. Control group (n=12) was immunized (i.p) with 1.0 mL sterile PBS (phosphate- buffered saline 0.1 M, pH 7.2) and group immunized with 1.0 mL of formalin (3%) inactivated *L. garvieae* (10^9 cells mL^{-1}. Before any handling procedure, fish were anaesthetized using a solution of freshly powdered clove oil with concentration of 150 ppm.

Serum sampling: After injection of letrozole, blood samples (3 mL) were collected from the caudal vein at 1, 2, 4, 6, 8, 12, 16, 20, 22 days after injection with AI. Serum samples were obtained from six fish per pound. After immunization blood was collected from the caudal vein of fish prior to immunization and every ten day post-injection, random serum samples were obtained from six fish per pond (non-lethal bleeding) of the immunized (the group only immunized and the group injected with 2.5 mg kg^{-1} letrozole and immunized) and control fish (non-immunized), and allowed to clot at room temperature for 1-2 h and then at 4 °C overnight. Serum was collected and divided into ependroff tubes and stored at -20 °C (Salamat et al., 2012).

Blood was collected from the caudal vein of fish prior to immunization and every ten day post-injection, random serum samples were obtained from six fish per pond (non-lethal bleeding) of the immunized and control fish, and allowed to clot at room temperature for 1-2 h and then at 4 °C overnight. Sera were collected and stored at -20 °C.

Serum E_2 levels: For steroid analysis, serum samples were extracted with alcohol ice-cold methanol, added to the serum (6:1 v/v), shaken and centrifuged (3000 g, 15 min, 4 °C). The pellet was re-extracted twice with 200 µl of methanol. Supernatants were pooled, dried and reconstituted in 120 µL of potassium phosphate buffer (0.1 M, pH 7.4), then stored at -20 °C for analysis. Serum E_2, levels were measured by enzyme linked imunosorbent assays described by Navas and segner (2000) and Guzmán et al. (2008) with slight modification. In the steroid ELISA, the microtiter plate provided in this kit has been pre coated with a goat anti-rabbit antibody (Cusabio Biotech Co., Ltd). Fifty µL standards or samples were then added to the appropriate microtiter plate wells with a HRP-conjugated (E_2) and antibody preparation specific for steroid and incubated. Then substrate solutions were added to each well. The enzyme-substrate reaction was terminated by the addition of a sulphuric acid solution and the color density was measured spectrophotometrically at a wavelength of 450 ± 2 nm. The concentration of steroid in the samples was then determined using comparison of the optical density (O.D) of the samples to the standard curve. All samples were placed in triplicate on the plates. Data were expressed as ng mL^{-1} for serum steroid levels (Navas and Segner, 2000; Guzmán et al., 2008).

Lysozyme activity: The lysozyme activity of samples was measured using a method based on the ability of lysozyme to lyse the bacterium *Micrococcus lysodeikticus* (Ellis, 1990). In a 96-well microplate, 250 µL of samples in four twofold serial dilutions in PBS (0.05M, PH 7.2) were mixed with 250 µL of 0.7 mg mL^{-1} suspension of *M. lysodeikticus* (Sigma) in phosphate buffer (175 mL). The microplate was incubated at 24 °C and O.D. was read at 450 nm at 15 and 30 min. For a

positive and a control, serum was replaced by the hen egg white lysozyme (serial dilutions starting at 1.6 μg mL^{-1}) and buffer, respectively. A unit of lysozyme activity was defined as the amount of serum causing a decrease in the O.D. reading of 0.001 min.

Total IgM: The serum samples from weekly letrozole injected immunized, only immunized and non-immunized were assessed by ELISA to measure the total IgM using the method of Hanif et al. (2004) with slight modifications. Samples were washed three times with PBS containing 0.05% Tween 20 (PBS-Tween) between steps. Each well of microplate (Falcon, USA), which was coated with 50 μL of rabbit anti-rainbow trout IgM (6.6 μg Ml^{-1}) (Sigma, St. Louis, U.S.A), was incubated for 2 h at 37 °C. After washing, 50 μL of serum samples, serially diluted standards from 800 to 3.2 ng mL^{-1} and were added to the wells, and the plate was incubated overnight at 4 °C. After washing, 50 μL of goat anti-trout antibody which was conjugated with horse radish peroxidase (Sigma, St. Louis, MO), and diluted in PBS-Tween, was added to the plate, and incubated for 2 h at 37 °C. After washing, peroxidase activity was measured by adding 50 μL of substrate solution which contained o- phenylenediamine dihydrochloride (1 mg mL^{-1}, BRL, USA) and 0.04 % H_2O_2 in 0.1 M citrate/ 0.2 M phosphate buffer (pH 5.5). After incubation for 30 min at room temperature, the enzyme-substrate reaction was stopped by adding 25 μL of 2M H_2SO_4 to each well and the color change was measured spectrophotometrically at a wavelength of 450 ± 2 nm. All samples were placed in triplicate on the plates and the mean and standard error (SE) were calculated for each sample IgM concentration. Data were expressed either as mg mL^{-1}.

Statistical analysis: ANOVA was used to detect variations in IgM and lysozyme parameters and all data were shown as mean ± standard error (SE). Unpaired t-test was also used to compare the group injected with letrozole and the control, and data were shown as mean ± standard error (SE). A significant difference among the different treatments was detected by ANOVA following by all pairwise multiple comparisons using the Duncan test. Statistically significant differences were determined at α=0.05 using SPSS for windows version 11.5 (SPSS, Chicago, USA).

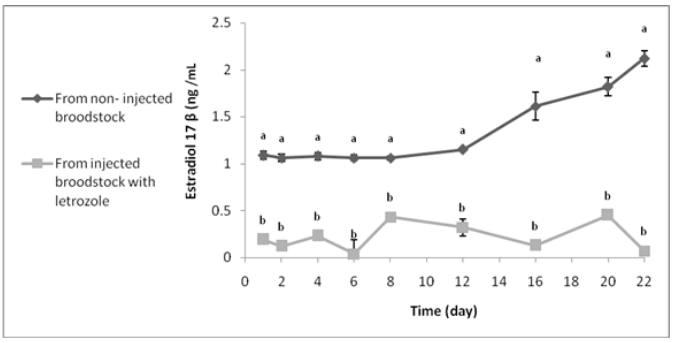

Figure 1. Serum concentration of 17β- estradiol in female rainbow trout injected or not with Letrozole (AI). Each data point represents the mean (± SE) of six fish (n=6).

Results

The injection procedure of letrozole was started in mid-September 2011 while all females were in pre-pubertal stage (two months before spawning). In the control group injected with the ethanol vehicle, serum E_2 levels 22 days after injection increased (2.12 ± 0.08 ng mL^{-1}) significantly in relation to the first blood collection. In the group injected weekly with 2.5 mg kg^{-1} letrozole, declined significantly one day after injection, and remained low throughout the experimental period (0.07 ± 0 ng mL^{-1}) (Fig. 1).

The lysozyme activity (μg mL^{-1}) in sera of the injected with letrozole and immunized group was higher (293.31 ± 2.78 μg mL^{-1}) than the immunized (288.27 ± 2.86 μg mL^{-1}) and the non-immunized (182.27 ± 1.07 μg mL^{-1}) broodstocks (Fig. 2). There was a significant difference among the groups. The immunized groups expressed more lysozyme activity than the non-immunized group.

The total immunoglobulin level in the sera of the injected with letrozole and immunized broodstocks (11.06 ± 0.03mg mL^{-1}) was higher the immunized (10.47 ± 0.29 mg mL^{-1}) and the non-immunized (7.91 ± 0.23 mg mL^{-1}) broodstocks (Fig. 3). The

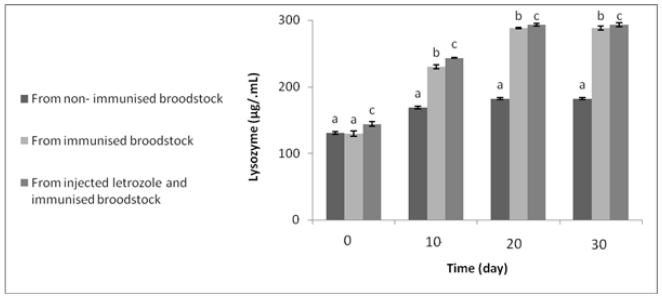

Figure 2. Total lysozyme level changes after immunization with *L. garvieae* (10^{-9} cells mL^{-1}) of the rainbow trout broodstock serum (µg mL^{-1}) .Each data point represents the mean (± SE.) with three replications. A significant difference among the groups was identified by the different superscript letter (P>0.05).

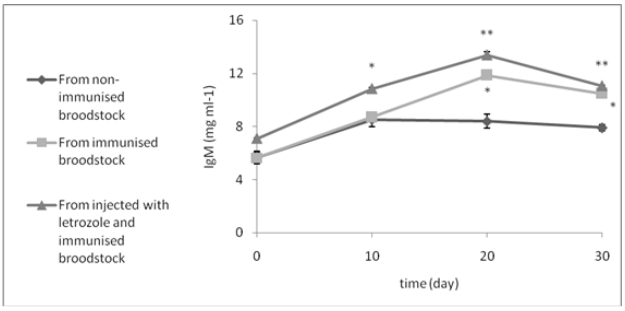

Figure 3. Total IgM level changes after immunization with *L. garvieae* (10^{-9} cells mL^{-1}) of the rainbow trout broodstock serum (µg mL^{-1}) .Each data point represents the mean (± SE.) with three replications. A significant difference among the groups was identified by the symbols * and ** (P<0.05).

difference was significant among the groups. The immunized groups expressed more IgM level than the non- immunized group.

Discussion

The interactions between hypothalamic-pituitary adrenal axis and immune system in fishes are yet to be fully understood (Lutton and Callard, 2006). Also aquaculture industry is recently facing a serious setback due to infectious diseases leading to sever economic loss (Sugita et al., 2002; Swain et al., 2006). Lactococcosis is a bacterial disease, caused by *L. garvieae* that occurs in both fresh water and seawater and has been a source of major economic losses since the early seventies for the rainbow trout industry (Vendrell et al., 2006; Hosseini et al., 2011). The reproductive–immune interactions studied in fishes indicates that sexual maturation (i.e., gonadal maturity and reproductive activities) can potentially affect both the innate and adaptive immune responses (Milla et al., 2011). Therefore, reliable methods to control the onset of puberty are required (Afonso et al., 1999). The present study aimed to investigate as an approach to control E_2 level and gonadal maturity by a sharp decrease in aromatase activity and effects of letrozole on serum lysozyme and immunoglobulin levels in rainbow trout.

In the present study, basal E_2 levels of were twice as high non-injected females as injected females from 1 to 2 days after injection with letrozole and serum E_2 levels were significantly lower in females injected weekly with 2.5 mg kg^{-1} letrozole. This suggests that letrozole, non-steroidal inhibitor, inhibits aromatase which catalyzes the conversion of androgens, androstendione and testosterone via three hydroxylation steps to estrone and estradiol (Sun et al., 2007). Similar results were obtained by Kelloff et al. (1998) who showed a dose responsive increase in blood serum steroid in trout injected with 50 mg Fadrozole kg^{-1} per day. Shilling et al (1999) used letrozole (CGS20267) and aminoglotethimide (AG) as non-steroid and examined in vitro for activity in trout ovarian microsomes. They showed that letrozole reduced aromatase activity a maximum of 90% in dose-dependent manner. But letrozole and clorimazole fed to juvenile rainbow trout at doses up to 1000 ppm for 2 weeks were not effective in suppressing 17 β-estradiol levels (Shilling et al., 1999). Our in vivo data showed that the mechanism and efficiency of inhibition of letrozole are different. Also the study of Afonso et al. (1999) provided the first evidence that injection of the fadrozole was effective in vivo in lowering plasma E_2 levels (Afonso et al., 1999). Sun et al. (2007) showed that letrozole for 21 days affected the reproductive, gonadal development and vitellogenin production of Japanese medaka (*Oryzias latipes*) females (Sun et al., 2007).

The total immunoglobulin level in the sera of the injected with letrozole and immunized broodstocks (11.06 ± 0.03mg mL^{-1}) was higher than those of the immunized (10.47 ± 0.29 mg mL^{-1}) and the non-

immunized (7.91 ± 0.23 mg mL^{-1}) broodstocks. This finding is in agreement with observation in rainbow trout (Hou et al., 1999), gilthead seabream (Cuesta et al., 2008). Sex hormones are usually elevated during spawning season of fishes and the rise of sex hormones can severely affect the immunity of fishes (Harris and Bird, 2000; Slater and Schreck, 1993). The rise of estradiol-17 β is reported to affect IgM level, and antibody- producing cells in several fish species like *Oncorhynchus mykiss*, *Salmo trutta*, Chinook salmon, *Oncorhynchus tshawytscha*, gold fish, *Carassius auratus* (Hou, 1998; Slater and Schreck, 1993; Suzuki et al., 1997; Thilagam et al., 2009). For example, in *O. mykiss* (Lei et al., 2010; Hou. Y. et al., 1999) and gilthead seabream (Cuasco et al., 2008), the rise of sex hormones can suppress the plasma IgM and IgM secreting cells during the spawning period leading to immunosuppressive condition. The elevation of sex steroids can prone fishes more vulnerable to several microbial infections during the spawning season and subsequent mortality following the spawning phase. We used the endocrine disrupter component (EDC) letrozole for rising lysozyme and IgM levels in immunized females.

The significant rise in lysozyme IgM levels of the broodstocks injected with letrozole and immunized and only immunized, one month before breeding was found. A set of genes related to innate immunity in vertebrates, including chemotoxin, poly saccharide-binding protein1, and antimicrobial peptide hepcidin were down- regulated after exposure to estrogen (Williams et al., 2007; Wang et al., 2009; Roberson et al., 2009). Sin et al. (1994) reported antibody against *Ichthyophinus multifilis* in *Oreochromis aureus* and Hanif et al (2004) in *Sparus aurata* against *Photobacterium damsella* by immunizing one moth prior to spawning.

Finally, the injection of letrozole with the vaccination of brood fish through bacteria (*L. garvieae*) has not only enhanced the specific immunity (IgM) but also the non-specific factor (lysozyme). Further studies are required to elucidate the modulation of different non- specific factors following injection of Aromatase inhibitors and specific immunization. Hence, strategies should be taken to inject with letrozole and immunized broodstocks of rainbow trout to breeding for better health management of females.

Acknowledgments

The authors thank the Ministry of Science, Research and Technology of Iran, for financial support.

References

Afonso L.O.B., Iwama G.K., Smith J., Donaldson E.M. (1999). Effects of the aromatase inhibitor fadrozole on plasma sex steroid secretion and ovulation rate in female coho salmon, *Oncorhynchus kisutch*, close to final maturation. General Comparative Endocrinology, 113: 221-229.

Afonso L.O.B., Iwama G.K., Smith J., Donaldson E.M. (2000). Effects of the aromatase inhibitor fadrozole on reproductive steroids and spermation in male coho salmon (*Oncorhynchus kistuch*) during sexual maturation. Aquaculture, 188:175-187.

Ankley G.T., Kahl M.D., Jensen K.M., Hornung M.W., Korte J.J., Makynen E.A., Leino R.L. (2002). Evaluation of the aromatase inhibitor fadrozole in a short- term reproduction assay with the fathead minnow (*Pimephales promelas*). Toxicological Sciences, 67: 121- 130.

Bakopoulos V., Peric Z., Rodger H., Adamms A., Richards R. (1997). First report of fish *Pasteurellosis* from Malta. Journal of Aquatic Animal Health, 9: 26-33.

Cuasco B., Gucco M., Malacarne G., Ottonelli R., Tanvez A. (2008). Yolk testosterone levels and dietary carotenoids influence growth and immunity of grey partridge chicks. General Comparative Endocrinology, 156: 418-425.

Ellis A.E. (1990). Lysozyme Assays: In Stolen J.S, Fletcher T.C, Anderson D.P, Roberson B.S, Van Muiswinkel W.B. (Ed.). Techniques in Fish Immunol. Fair Haven, N.J: SOS Publications, 101-103.

Guzmán J.M., Norberg B., Ramos J., Mylonas C.C., Mañanós E.L. (2008). Vitellogenin, steroid plasma levels and spawning performance of cultured female Senegalese sole (*Solea senegalensis*). General Comparative Endocrinology, 156: 285-297.

Harris J., Bird D.J. (2000). Modulation of the fish immune system by hormones. Veterinary Immunology and Immunopathology, 77: 163-176.

Haynes B.P., Dowsett M., Miller W.R., Dixon J.M., Bhatnagar A.S. (2003). The pharmacology of letrozole. Journal of Steroid Biochemistry, 87: 35-47.

Hosseini M.H., Akhlaghi M., Moazzeni Jula G. (2011). Experimental vaccine against *lactococcosis* in cultured rainbow trout (*Oncorhynchus mykiss*). Achieves of Razi Institute, 66: 51-57.

Hou Y.Y. (1998). Endocrinological aspects of reduced immunocompetence with gonadal maturation in rainbow trout. Ph.D. thesis. Tokyo: The University of Tokyo.

Hou Y., Suzuki Y.Y., Aida K. (1999). Effects of steroid hormones on immunoglobulin M (IgM) in rainbow trout, *Oncorhynchus mykiss*. Fish Physiology and Biochemistry, 20: 155-162.

Howell A., Buzdar A. (2005). Are aromatase inhibitors superior to antiestrogens? Journal of Steroid Biochemistry, 93: 237-247.

Kelloff G.J., Lubet R.A., Lieberman R., Eisenhauer K., Steele V.E., Crowell J.A., Hawk E.T., Boone C.W., Sigman C.C. (1998). Aromatase inhibitors as potential cancer chemopreventives. Cancer Epidemiology Biomarkers and prevention, 7: 65-78.

Lei L., Zhang Sh., Tongo Z., Liu J. (2010). In vivo effect of 17-β estradiol on plasma immunoglobulin levels and leukocyte density in zebrafish *Danio rerio*. Journal of Oceanology and Limnology, 28: 527-532.

Lutton B., Callard I. (2006). Evolution of reproductive- immune interactions. Integrative and Comparative Biology, 46: 1060-1071.

Milla S., Depiereus S., Kestemond P. (2011). The effects of estrogenic and androgenic endocrine disruptors on the immune system of fish: a review. Ecotoxicology, 20: 305-319.

Navas J.M., Segner H. (2000). Antiestrogenicity of beta-naphthoflavone and PAHs in cultured rainbow trout hepatocytes: evidence for a role of the arylhydrocarbon receptor. Aquatic Toxicology, 51: 79-92.

Panter G.H., Hutchinson T.H., Hurd K.S., Sherrenb A., Stanleya R.D., Tylerc C.R. (2004). Successful detection of (anti-)androgenic and aromatase inhibitors in pre-spawning adult fathead minnows (*Pimephales promelas*) using easily measured endpoints of sexual development. Aquatic Toxicology, 18: 11-21.

Piferrer F., Zanuy S., Carrillo M., Solar I.I., Devlin R.H., Donaldson E.M. (1994). Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. Journal of Experimental Zoology, Part A, 270: 244-262.

Romalde J.L., Alvarez A.L., Ravelo C., Toranzo A.E., Mendez J.B. (2004). Oral immunization using alginate microparticles as a useful strategy for booster vaccination agaist fish *lactoccocosis*. Aquaculture, 236: 119-129.

Saha N.R., Usami T., Suzuki Y. (2004). In vitro effect of steroid hormones on IgM- secreting cells and IgM secretion in common carp (*Cyprinus carpio*). Fish and Shellfish Immunology, 17: 149-158.

Salamat N., Havasi M., Earfani Majd N., Savari A. (2012). Seasonal change of thyroid histomorphological structure and hormone production in yellowfin seabream (*Acanthopagrus latus*) in the Persian Gulf. Iranian Journal of Fisheries Science, 11: 840-848.

Sharifiyazdi H., Akhlaghi M., Tabatabaei M., Mostafavi Zadeh S.M. (2010). Isolation and characterization of *Lactococcus garvieae* from diseased rainbow trout (*Oncorhynchus mykiss*, Walbaum) cultured in Iran. Iranian Journal of Veterinary Research, Shiraz University, 11: 342-350.

Shilling A.D., Carlson D.B., Williams D.E. (1999). Rainbow trout, *Oncorhynchus mykiss*, as a model for

aromatase inhibition. Journal of Steroid. Biochemistry, 70: 89-95.

Sin, Y.M., Ling K.H., Lam T.J. (1994). Passive transfer of protective immunity against *ichthophthiriasis* from vaccinated mother to fry in tilapias, *Oreochromis aureus*. Aquaculture, 120: 229-237.

Slater C.H., Schreck C.B. (1993). Testosterone alters the immune response of Chinook salmon, *Oncorhynchus ishawytscha*. General of Comparative Endocrinology, 89: 291-298.

Smith L. E. (1999). Aromatase inhibitors: a dose-response effect? Endocrine Related Cancer 6: 245-249.

Sugita H., Okano R., Suzuki Y., Iwai D., Mizukami M., Akiyama N. (2002). Antibacterial abilities of intestinal bacteria from larvae and juvenile Japanese flounder against fish pathogens. Fisheries Science, 2: 1004-1011.

Sun L., Zha J., Spear P.A., Wang Z. (2007). Toxicity of the aromatase inhibitor letrozole to Japanese medaka (*Oryzias latipes*) eggs, larvae and breeding adults. Comparative Biochemistry and Physiology, Part C, 145: 533-541.

Suzuki Y., Otaka T., Sato S., Hou Y.Y., Aida K. (1997). Reproduction related immunoglobulin changes in rainbow trout. Fish Physiology and Biochemistry, 17: 415-421.

Swain P., Dash S., Bal J., Routray P., Sahoo P.K., Sahoo S.K., Saurabh S., Gupta S.D., Meher P.K. (2006). Passive transfer of maternal antibodies and their existence in eggs, larvae and fry of Indian major carp, *Labeo rohita* (Han). Fish and Shellfish Immunology, 20: 519-527.

Swain P., Nayak S.K. (2009). Role of maternally derived immunity in fish. Fish & Shellfish. Immunology, 27: 89-99.

Thilagam H., Gopalakrishnan S., Bo J., Wang K.J. (2009). Effect of 17 beta estradiol on the immunocompetence of Japanese sea bass (*Lateolabrax japonicus*). Environmental Toxicology Chemistry, 28: 1722-1731.

Toranzo A.E., Devesa S., Romalde J.L., Lamas J., Riaza A., Leiro J., Barja J.L.M. (1995). Efficacy of intra- peritoneal and immersion vaccination against Enterococcus sp. infection in turbot. Aquaculture, 134: 17-27.

Vendrell D., Balcázar J.L., Ruiz-Zarzuela I., Blas I.D., Gironés O., Mùzquiz J.L. (2006). *Lactococcus garvieae* in fish: A review. Comparative Immunology. Diagnostic Microbiology and Infectious Disease, 29: 177-198.

Wang K.J., Cai J.J., Qu H.D., Yang M., Zhan M. (2009). Cloning and expression of a hepcidin gene from a marine fish (*Pseudosciaena crocea*) and the antimicrobial activity of its synthetic peptide. Peptides, 30: 638-646.

Williams T.D., Amer M.D., George S.G., Sabine V., Chipman J.K. (2007). Gene expression responses of European flounder (*Platichtys flesus*) to 17- beta estradiol. Toxicology Letters, 168: 236-248.