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Efficacy of exemestane, a new generation of aromatase inhibitor, on sex differentiation in a gonochoristic fish

Sabina Ruksana, Narayan P. Pandit, Masaru Nakamura *

Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Sesoko 3422, Motobu, Okinawa 905-0227, Japan

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ABSTRACT

We report the first use of exemestane (EM), a steroidal aromatase inhibitor (AI) commercially known as aromasin, in studies of sex differentiation in fish. The effectiveness of EM was examined in two different age groups of the gonochoristic fish, Nile tilapia (*Oreochromis niloticus*). Untreated control fish (all female) showed normal ovarian differentiation through 120 days after hatching (dah), whereas fish treated with EM at 1000 and 2000 μg/g of feed from 9 dah through 35 dah, the critical period for sex differentiation, exhibited complete testicular differentiation; all stages of spermatogenic germ cells were evident and well developed efferent ducts were present. Fish treated with EM at 1000 μg/g of feed from 70 dah through 100 dah significantly suppressed plasma estradiol-17β level and increased level of 11-ketotestosterone. Furthermore, untreated control fish showed strong gonadal expression of the steroidogenic enzymes P450 cholesterol-side chain-cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and cytochrome P450 aromatase (P450arom). In contrast, EM-treated fish showed immunopositive reactions against P450scc and 3β-HSD but not against P450arom in interstitial Leydig cells. These results indicate that treatment of tilapia juveniles with EM during sex differentiation leads to the development of testes, apparently by a complete suppression of aromatase activity.

1. Introduction

The hormone estrogen plays a key role in ovarian differentiation in non-mammalian vertebrates, including amphibians (Hayes, 1998; Miyata and Kubo, 2000), reptiles (Pieau and Dorizzi, 2004), birds (Smith and Sinclair, 2004), and fish (Yamamoto, 1969; Nakamura et al., 1998; Devlin and Nagahama, 2002; Nakamura et al., 2003; Kobayashi et al., 2003; Bhandari et al., 2006; Komatsu et al., 2006; Nakamura et al., 2007). Estrogen is synthesized from testosterone by aromatase enzyme (P450arom), an enzyme of the cytochrome P450 super family whose function is to aromatize androgens to form estrogens. P450arom is the key enzyme for biosynthesis of estradiol-17β (E2) from testosterone. Furthermore, the P450arom gene has been shown to be involved in sex differentiation and gonadal development in teleost fish (Fukada et al., 1996; Gen et al., 2001; Ijiri et al., 2003; Deng et al., 2009). Analysis of the role of estrogen in ovarian differentiation therefore is central to understanding the process and mechanism of sex differentiation in fish.

Aromatase inhibitors (AIs) are chemicals that block P450arom activity, leading to reductions in the production of estrogen (Steele et al., 1987). AIs have been used in the treatment of breast and ovarian cancer in postmenopausal women (Howell et al., 2005) and in studies of sex change in a wide range of teleost fish, e.g., genetically female tilapia (Kwon et al., 2000; Afonso et al., 2001; Kobayashi et al., 2003), salmon (Piferrer et al., 1994), Japanese flounder (Kitano et al., 2000), and zebrafish (Fenske and Segner, 2004; Uchida et al., 2004). The ability of AI to induce sex reversal in fish makes this class of chemical a valuable tool for analyzing the role of estrogen in the processes of sex differentiation and sex change.

There are two kinds of AIs, steroidal AI, which bonds irreversibly to the aromatase enzyme complex, and non-steroidal AI, which inhibits aromatase by reversible competition (Mokbel, 2002). Non-steroidal AIs have been widely used in studies of sex change in fish. For example, fadrozole has been used for the masculinization of genetic females in many species of gonochoristic fish (Piferrer et al., 1994; Nakamura et al., 1999; Kitano et al., 2000; Kwon et al., 2000; Afonso et al., 2001; Kwon et al., 2002; Uchida et al., 2004; Komatsu et al., 2006) and sex-changing protogynous species (Kroon and Liley, 2000; Higa et al., 2003; Nakamura et al., 2003; Bhandari et al., 2004; Kroon et al., 2005; Alam et al., 2006). Unfortunately, fadrozole is no longer available commercially.

As alternative, the steroidal AI exemestane (EM) might be useful tools for examining the role of estrogen in sex differentiation. However, little is known about the effectiveness of these compounds. The steroidal AI formestane (4-androsten-4-ol-3, 17-dione), for example, has been used for sex reversal in *Rana rugosa* (Ohtani et al., 2003) and imposex (male sexual character in female) induction in the mollusk *Nucella lapillus* (Santos et al., 2005), but there apparently

* Corresponding author. Tel./fax: +81 980 47 6072.
E-mail address: masaru@lab.u-ryukyu.ac.jp (M. Nakamura).

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are no reports of the use of this or other steroidal AIs on reproduction or sex differentiation in fish.

To address this problem, we tested the effect of EM on sex differentiation as well as on sex change in the gonochoristic fish, *O. niloticus* (Nile tilapia).

2. Materials and methods

2.1. Experimental animals and care

Female juveniles of *O. niloticus* were obtained by mating a pseudo-male (XX)/phenotypic male with a genetic female (XX). A total of 200 individuals at 9 dah were used for the first experiment, with 40 fish in each aquarium. A total of 30 individuals of the same batch at 70 dah were used for the second experiment, with 15 fish in each aquarium. Fish were reared in 50-L glass aquariums in well-aerated, recirculating water maintained at 26 ± 1 °C. The aquariums were cleaned every 7 days throughout the experimental period. Fish were fed twice daily a commercial feed (C-700, 2000 Kyowa Hakko Kogya, Ltd, Tokyo, Japan).

2.2. Feed preparation and EM administration

For the first experiment, to determine the effective dose of EM, four concentrations (100, 500, 1000 and 2000 μg/g diet) were prepared and mixed with the commercial feed. EM (aromasin, 25 mg tablets, Pfizer Japan Inc., Tokyo, Japan) was dissolved in 100% ethanol and added to the feed, which was then dried overnight at room temperature to completely evaporate the ethanol. For experimental treatments, fish were fed EM-containing feed from 9 dah through 35 dah, covering the critical period of sex differentiation (Nakamura et al., 1998), after which they were fed the normal commercial feed through the end of the experiment (120 dah).

For the second experiment, the best effective dose of EM confirmed from the first experiment (1000 μg/g diet) was used for the treatment fish, whereas control fish were fed with normal commercial feed throughout the experiment (70 dah to 100 dah).

2.3. Sampling

As an initial control, 20 fish were sampled at the start of both experiments. Fish of the first experiment were sampled at 35 dah ($n = 20$) and 120 dah ($n = 12$) from each of the five aquariums. For sampling at 35 dah, fish were anaesthetized in ice cold water, decapitated, and then fixed in Bouin's solution overnight at room temperature. For sampling at 120 dah, fish were anaesthetized with 0.05% phenoxyethanol, and the gonads were removed and fixed in Bouin's solution. After overnight fixation, gonads were transferred from Bouin's to 70% ethanol, embedded in paraffin, cross-sectioned at 7 μm, and stained with Delafield's hematoxylin and eosin following routine histological procedures for light microscopy. Fish of the second experiment were sampled 70 dah as initial, 85 dah and 100 dah following the same procedure as the first experiment.

2.4. Immunohistochemistry

Previously prepared antibodies specific for P450scc (Morrey et al., 1998), 3β-HSD and P450arom (Kobayashi et al., 1996) were used for

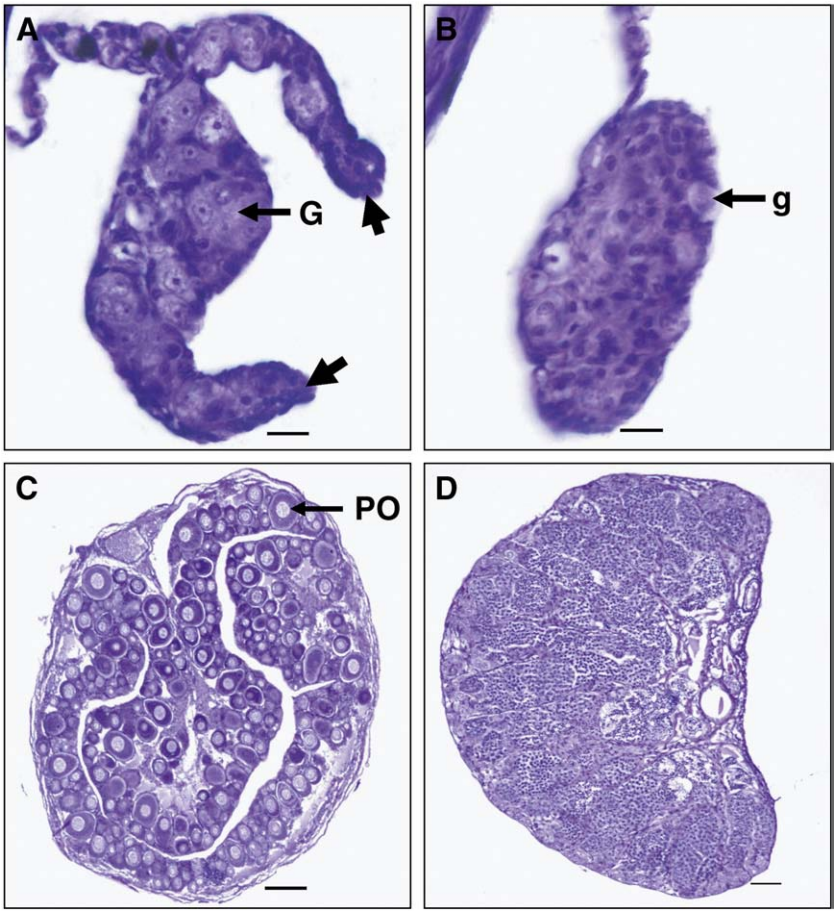

Fig. 1. Histological analysis of EM-induced sex reversal in Nile tilapia *O. niloticus* in the first experiment. (A) Control gonads at 35 dah. Large arrows indicate stromal elongations, a precursor of ovarian cavity formation. (B) EM-treated gonads at 35 dah. (C) Control gonads at 120 dah. (D) EM-treated gonads at 120 dah showing normal testicular differentiation and formation of mature testis. Abbreviations: G, a cyst of meiotic germ cell; g, single gonial germ cell; and PO, primary oocytes. Scale bars = 5 μm.

Table 1
Effects of EM treatment at different doses on sex ratio of Nile tilapia during gonadal sex differentiation.

Groups	Male	Female	Male (%)
Control	0	12	0
EM (100 μg/g diet)	1	11	8.3
EM (500 μg/g diet)	10	2	83.3
EM (1000 μg/g diet)	12	0	100
EM (2000 μg/g diet)	12	0	100

immunohistochemical analyses. Detection of antibody–antigen binding was performed using the Histofine immunohistochemistry kit (Nichirei, Tokyo, Japan) and diaminobenzidine according to the manufacturer's protocol.

2.5. Steroid assay

Blood was collected from the caudal vein using a 1-mL heparinized syringe (Terumo, Japan) and centrifuged at 16,000 × *g* for 10 min to obtain plasma. Plasma was stored at −30 °C until analysis. The plasma levels of estradiol-17β (E2) and 11-ketotestosterone (11-KT) were determined by enzyme-linked immunosorbent assay (ELISA) as described by Asahina et al. (1995).

2.6. Statistical analyses

All data are expressed as mean ± SEM and a P value of <0.05 was considered statistically significant. To identify statistically significant variance, we employed one-way analysis of variance (ANOVA) followed by a Tukey–Kramer honestly significant difference test (JMP Version 4.0, SAS Institute Inc., Cary, NC, USA).

3. Results

In the first experiment, gonads of juveniles at 9 dah were sexually undifferentiated and consisted of large gonial germ cells interspersed among some somatic cells (not shown). At 35 dah, gonads of the control group fish had stromal elongations, a precursor of ovarian cavity formation, and some cysts of meiotic germ cells, consistent with the initiation of oogenesis (Fig. 1A). Similarly, gonads of fish treated with EM 100 μg/g of diet showed ovarian cavity formation and some cysts of meiotic germ cells. However, the gonads of fish at this stage treated with 500, 1000 and 2000 EM μg/g feed consisted of single germ cells surrounded by somatic cells, with no indications of ovarian cavity formation or meiotic germ cells (Fig. 1B).

Results at 120 dah confirmed these observations. All fish in the control group at 120 dah had normal ovaries with late perinucleolar stage oocytes (Fig. 1C). Similarly, eleven out of the twelve examined fish treated with EM 100 μg/g of feed had ovaries with many late perinucleolar oocytes and some degenerated oocytes. One of the twelve fish in this group, however, had well developed testes with active spermatogenic germ cells. In contrast, ten of the twelve examined fish treated with EM 500 μg/g of feed had testes with different stages of spermatogenic germ cells and well developed efferent ducts. The other two individuals in this group had ovaries similar to those of normal females. Furthermore, all of the fish treated with EM 1000 μg/g and 2000 μg/g of feed had well developed testes (Fig. 1D). Sex distribution in each group is shown in Table 1.

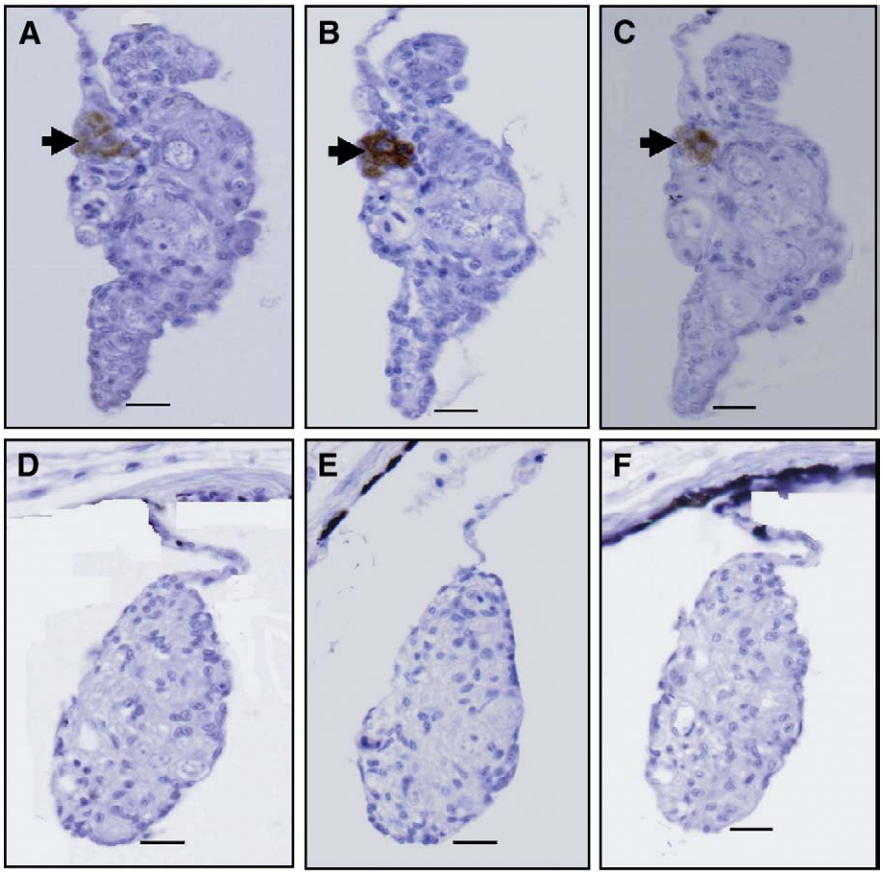

Fig. 2. Immunohistochemical analysis of gonads of *O. niloticus* (Nile tilapia) in the first experiment. Upper panels, control gonads at 35 dah, immuno-stained with (A) P450scc, (B) 3β-HSD, and (C) P450arom. Immunopositive cells are brown in color. Lower panels, EM-treated gonads at 35 dah, immuno-stained with (D) P450scc, (E) 3β-HSD, and (F) P450arom. No immunoreaction is seen. Scale bars = 5 μm.

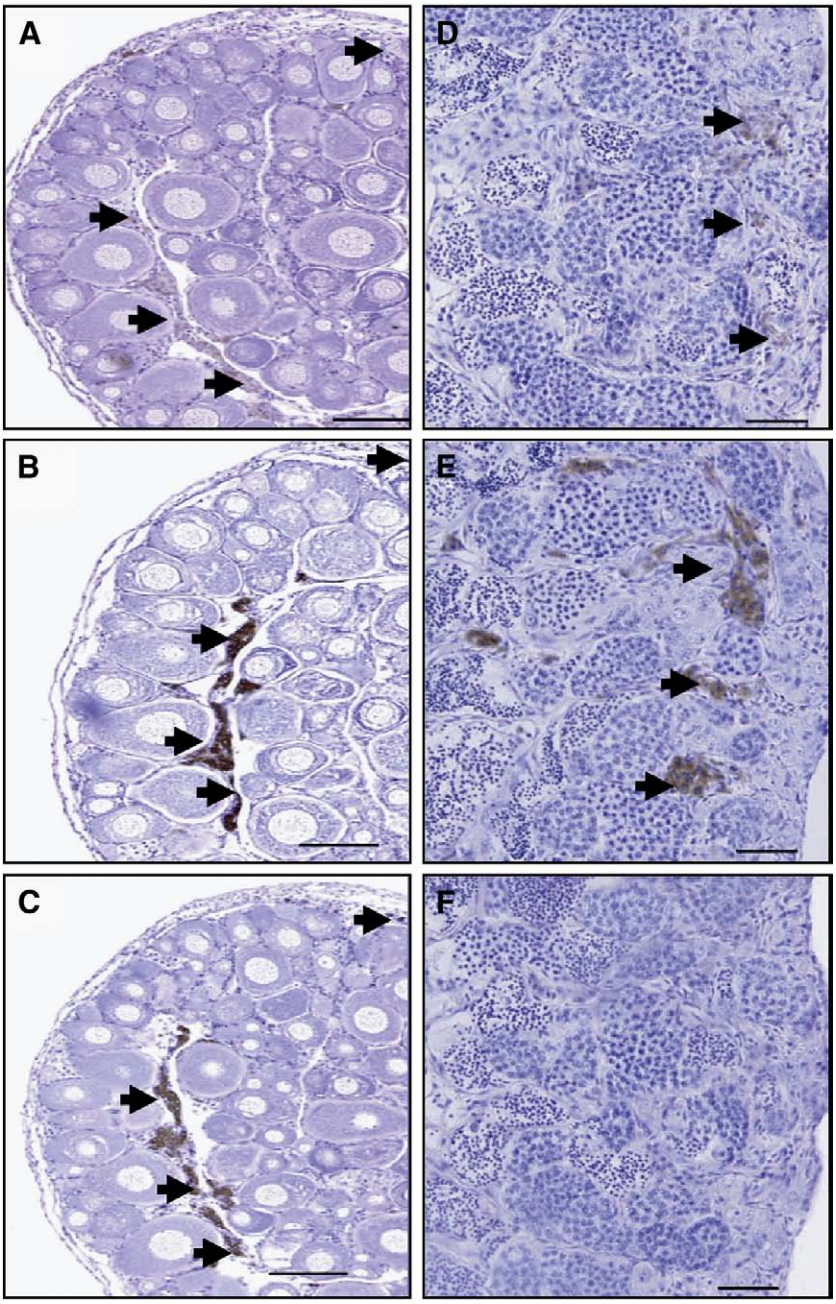

Fig. 3. Immunohistochemical analysis of gonads of *O. niloticus* (Nile tilapia) in the first experiment. Upper panels, control gonads at 120 dah immuno-stained with (A) P450scc, (B) 3β-HSD, and (C) P450arom. Immunopositive cells (brown) are in the interstitial area between the developed oocytes. Lower panels, EM-treated gonads at 120 dah immuno-stained with (D) P450scc, (E) 3β-HSD, and (F) P450arom. Immunopositive reactions in Leydig cells are seen against P450scc and 3β-HSD, but there is no reaction against P450arom in Leydig cells. Scale bars = 20 μm.

Immunohistochemical analyses revealed that at 35 dah, cells with strongly immunopositive responses against P450scc, 3β-HSD and P450arom were evident only in the differentiating ovaries of the control fish and the fish treated with the lowest dose of EM (100 μg/g feed); these cells occurred as clusters in the vicinity of blood vessels (Fig. 2A–C). For fish at 35 dah treated with higher concentrations of EM, no reaction against P450scc, 3β-HSD or P450arom was observed (Fig. 2D–F). At 120 dah, the ovaries of the control fish and the fish treated with the lowest dose of EM showed immunoreactions against all three enzymes in the interstitial area between the developed oocytes (Fig. 3A–C). For fish at 120 dah treated with the higher concentrations of EM (500–2000 μg/g diet), the gonads had transformed into testes with advanced stages of spermatogenic germ cells and had immunopositive reactions against P450scc and 3β-HSD, localized to the Leydig cells (Fig. 3D–E). However, no reaction against P450arom was evident in the testes (Fig. 3F).

In the second experiment, all fish in the initial control group at 70 dah had normal ovaries with perinucleolar stage oocytes (not shown). After 30 days of treatment, the plasma levels of E2 in AI-treated fish (1238 pg/mL) were significantly lower than in the initial (1768 pg/mL) and control fish (3042 pg/mL) (Fig. 4). Similarly, the levels of 11-KT was significantly higher in AI-treated fish (2413 pg/mL) than in the initial (323 pg/mL) and control fish (1267 pg/mL) (Fig.4).

4. Discussion

In the present study, we used EM, a new generation steroidal AI, for studies of the role of estrogen in sex differentiation and

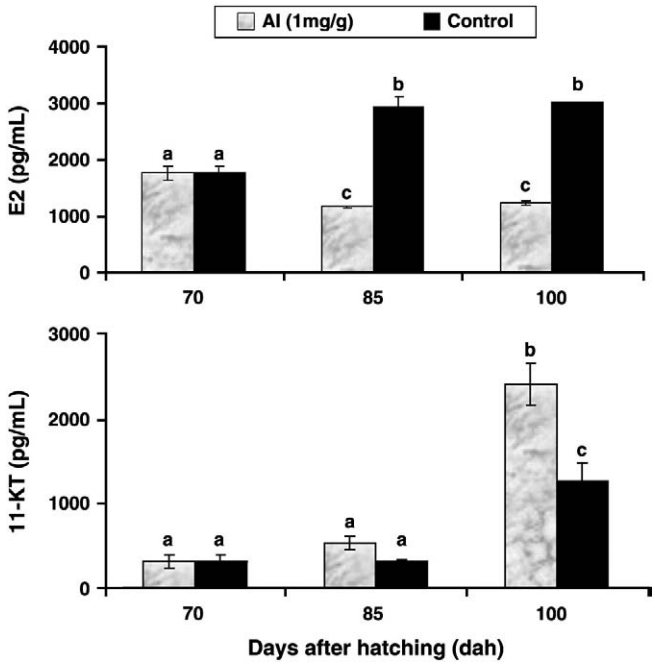

Fig. 4. Effects of EM on *in vivo* production of serum (A) E2 (B) 11-KT in *O. niloticus*. Different superscript letters indicate statistically significant differences ($P<0.05$) from the connected means.

subsequent sex change in tilapia fish. The expression of steroidogenic enzymes, using immunohistochemical methods, was also examined to identify the mechanism by which EM acts to induce testicular differentiation in undifferentiated gonads. In the control group and in fish treated with a low concentration of EM (100 μg/g of feed), the gonads showed normal ovarian differentiation at 35 dah and 120 dah and had cells with strong immunopositive reactions against P450scc, 3β-HSD, and P450arom. These enzymes were detected in the interstitial cells of the developed oocytes. On the other hand, the gonads of fish treated with high doses of EM (500 to 2000 μg/g of feed) exhibited testicular differentiation and showed no expression of P450scc, 3β-HSD, or P450arom at 35 dah. This pattern of enzyme expression was also observed in methyltestosterone-treated masculinized juvenile tilapia (Bhandari et al., 2006). It is likely that during the process of redirection from ovarian differentiation to testicular development, MT treatment initially inactivates the major steroidogenic enzymes.

However, at 120 dah the EM-treated fish (1000–2000 μg/g feed), in which testes were well developed, showed immunopositive reactions against P450scc and 3ß-HSD, but no reaction against aromatase. Immunohistochemical analyses of these enzymes revealed also the process of differentiation of Leydig cells, the site of androgen production during artificial testicular differentiation. The control fish at 120 dah, in contrast, showed immunopositive reactions against all three enzymes in the ovary. These results suggest that, in the newly developed testis, all enzymes except aromatase reappear. Consistent with these results, expression of P450scc and 3ß-HSD was found in Leydig cells of the testis in rainbow trout (Kobayashi et al., 1998).

Furthermore in this study, we found a significantly lower level of E2 and higher level of 11-KT in EM-treated fish. This finding indicates that EM blocked the conversion of testosterone to E2 in gonads and thereby transformed the undifferentiated gonads into functional testes. The depletion of E2 and subsequent sex change using a non-steroidal AI have been observed in gonochoristic (Afonso et al., 1997; Afonso et al., 1999) as well as hermaphrodite fish (Bhandari et al., 2004; Kroon and Liley, 2000; Nozu et al., 2009). In contrast to the steroidal EM, a non-steroidal AI, the fadrozole have been used in a variety of fish species and have been shown to exhibit a wide range of effective doses. For example, significant changes in sex ratio, i.e., increases in the percentage of males, have been observed with fadrozole at concentrations of 40 to 200 μg/g of feed in tilapia fry treated during the critical period of sex differentiation, whereas, no significant changes were observed in response to treatment with fadrozole at concentrations of 200 to 500 μg/g of feed (Kwon et al., 2000). Fadrozole administration at 75 or 100 μg/g of feed to juvenile tilapia resulted in 100% males (Afonso et al., 2001), and fish administered fadrozole at 200 and 500 μg/g of feed from 8 dah to 22 dah had well developed testes (Nakamura et al., 1999). Fadrozole given at 500 and 1000 μg/g of feed from the first feeding to100 dah in fugu (*Takifugu rubripes*) inhibited ovarian cavity formation and led to testicular differentiation (Rashid et al., 2007). In the golden rabbitfish (*Siganus guttatus*), the gonads of fish after 30 days and 90 days administration of fadrozole at 500 μg/g of feed were significantly biased toward testes (Komatsu et al., 2006). Fadrozole at 100 μg/g of feed induced 100% masculinization in genetically female Japanese flounder (Kitano et al., 2000) and zebrafish (Uchida et al., 2004). Our results with EM, a steroidal AI, at doses of 1000-2000 μg/g of feed compare favorably with the results of these studies using fadrozole, previously a commonly used non-steroidal AI that is no longer commercially available.

Thus, in the present study, all females of the Nile tilapia treated with high levels of EM (1000 and 2000 μg/g of feed) during the critical developmental period for sex differentiation, developed testes. These males appeared to be sexually functional, in that they had testes with efferent ducts and with all stages of spermatogenic germ cells, from spermatogonia to spermatozoa. Therefore, EM at 1000 μg/g feed might be the most effective dose for induction of sex reversal. However, since this is the first report of EM uses in sex reversal of fish, we do not know if this dose is exceptionally high or low. We conclude that EM is a highly effective new generation steroidal AI for studies of sex differentiation in fish.

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