The Open Access Israeli Journal of Aquaculture – Bamidgeh

As from **January 2010** The Israeli Journal of Aquaculture - Bamidgeh (IJA) will be published exclusively as **an on-line Open Access (OA)** quarterly accessible by all AquacultureHub (http://www.aquaculturehub.org) members and registered individuals and institutions. Please visit our website (http://siamb.org.il) for free registration form, further information and instructions.

This transformation from a subscription printed version to an on-line OA journal, aims at supporting the concept that scientific peer-reviewed publications should be made available to all, including those with limited resources. The OA IJA does not enforce author or subscription fees and will endeavor to obtain alternative sources of income to support this policy for as long as possible.

Published under auspices of
The Society of Israeli Aquaculture and Marine Biotechnology (SIAMB), University of Hawaii at Manoa Library
and
University of Hawaii Aquaculture Program in association with
AquacultureHub
http://www.aquaculturehub.org

ISSN 0792 - 156X

PUBLISHER:
Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

MASCULINIZATION OF GENETIC FEMALES OF THE COMMON CARP *(CYPRINUS CARPIO* L.) BY DIETARY ADMINISTRATION OF AN AROMATASE INHIBITOR

Itai Tzchori[1]*, Tatyana Zak[2] and Ofer Sachs[2]

[1] *Department of Zoology, Tel Aviv University, Tel Aviv 69978, Israel*

[2] *Aquaculture Research Station - Dor, D.N. Hof Hacarmel 30820, Israel*

(Received 2.6.04, Accepted 10.7.04)

Key words: aromatase inhibitor, common carp, methyltestosterone, sex determination, sex inversion

Abstract

In the present study, we demonstrated the ability of Fadrozole, an aromatase inhibitor, to induce sex inversion of genetically female common carp fry during the critical sex differentiation period. Thirty-day-old female fingerlings with a mean initial weight of 4.0 g (experiment I) and 3.5 g (experiment II) were fed a diet containing Fadrozole for 36 or 50 days, respectively. Not a single male was found in the control groups of both experiments. In experiment I, Fadrozole at 200 mg/kg feed resulted in 58.6% males, while fish receiving 17α-methyltestosterone at 50 or 100 mg/kg feed resulted in only 5-10% males. In experiment II, the efficiency of Fadrozole was dose-dependent; the lower dose of 100 mg/kg caused masculinization in 86% of the treated fry while the higher doses of 200 and 400 mg/kg feed increased the percentage of males to 97%. These results confirm the importance of aromatization during the labile period in common carp since low aromatase activity during this period, regardless of genotype, resulted in masculinization.

Introduction

Artificial sex control is an important technique in fish culture. Sex control using steroid hormones has been examined in various fish species (Yamamoto, 1969; Hunter and Donaldson, 1983; Nagahama, 1994; Piferrer, 2001; Devlin and Nagahama, 2002). In general, estrogen treatment causes feminization of genetic males and androgen treatment causes masculinization of genetic females.

A theory for the mechanism that allows hormonal manipulation in sex differentiation was presented by Bogart (1987). According to

* Corresponding author. Current address: 6 Center Drive, National Institutes of Health, Bethesda, Maryland, USA; tel.: +1-301-402-2426; fax: +1-301-402-0543; email: tzchorii@mail.nih.gov

this theory, sex determination in fish is regulated by the ratio of androgen and estrogen levels at the critical time of sex determination, and steroid intervention during this period results in changes in this balance. Several other factors can impact sex differentiation in fish. Temperature, for example, can alter sex determination pathways and influences the sex ratio in fish populations from different habitats (Devlin and Nagahama, 2002).

The common carp *(Cyprinus carpio* L.) has an XX/XY chromosomal genetic sex determination (Nagy et al., 1984). Hormonal sex inversion is required to produce all-female progenies; normal XX females are mated with inverted XX males (Nagy et al., 1981; Gomelsky et al., 1994). Work carried out at the Dor station (Israel) showed that all-female populations of common carp have a higher growth rate (7-8%) than mixed populations, and prevent uncontrolled reproduction (Gomelsky et al., 1994). Androgenic sex inversion in the common carp has been achieved by oral administration of diets containing 17α-methyltestosterone (MT; Nagy et al., 1981; Gomelsky, 1985; Komen et al., 1989, 1993; Gomelsky et al., 1994) or by treatment with testosterone undecanoate (Bharadwaj and Sharma, 2002). Nevertheless, the sex-inversion technique for common carp needs further optimization, mainly to determine the optimal period for androgen treatment under various growth conditions (Gomelsky et al., 1994; Gomelsky, 2003).

Another approach to sex reversal is to reduce estrogen biosynthesis by inhibiting the activity of aromatase, which catalyzes the conversion of androgens to estrogens using nonsteroidal inhibitors such as Fadrozole (CGS 16949A; Steele et al., 1987). Fadrozole has been described as a reversible competitive inhibitor whereby both substrate and inhibitor compete for the same site on the aromatase enzyme (Brodie, 1991). The efficacy of this compound in reducing estrogen biosynthesis has been demonstrated *in vivo* and *in vitro* in mammals (Steele et al., 1987; Schieweck et al., 1988) and chickens (Elbrecht and Smith, 1992). In fish, the potency of Fadrozole was demonstrated in sexual differentiation studies of the chinook salmon *Oncorhynchus tshawytscha* (Walbaum; Piferrer et al., 1994) and Nile tilapia (Kwon et al., 2000, 2002). Fadrozole inhibited estrogen synthesis *in vivo* in maturing female coho salmon *Oncorhynchus kisutch* (Walbaum; Afonso et al., 1999).

In the current study, we treated common carp females (genotype XX) with an aromatase inhibitor (Fadrozole) and an androgen (MT), and compared their ability to induce sex inversion.

Materials and Methods

Two experiments were conducted at the Fish and Aquaculture Research Station at Dor, Israel, during 2001-2002. The genetic sex regulation method (Gomelsky, 2003) was used to generate all-female populations. In experiment I, offspring were obtained by crossing normal XX females of the Dor 70 common carp line with gynogenetic MT sex-reversed males (F1) according to the protocol of Gomelsky et al. (1994; Fig. 1). In experiment II, all-female progenies were obtained by mating Dor 70 XX females with successfully inverted males (‘F1) from experiment I.

Five-day old larvae were fed freshly hatched *Artemia salina* nauplii for 1-2 days and then stocked into earthen ponds for primary nursing soon after the onset of active feeding. After 22 days at an average body weight of 4.0 g, or 30 days at an average body weight of 3.5 g (experiments I and II, respectively), fish samples were collected and transferred indoors for masculinization and control treatments in recirculating water systems. Each system consisted of two 100-l tanks, 50 fish in each, and another 100-l tank that served as a biological filter to which the rearing tanks were connected. The biofilter tanks were filled with a substratum of synthetic fibers (Gomelsky et al., 1994). Water flowed from the rearing tanks to the filter by airlifts and returned through a siphon. The control group contained 25 fish.

Fish were fed 2-mm pellets containing 35% protein, 4.5% fat, and vitamins. Experimental diets containing MT (Sigma, St. Louis) or an aromatase inhibitor (Fadrozole™ CGS 16949A, Novartis, Basel) were prepared by using 96%

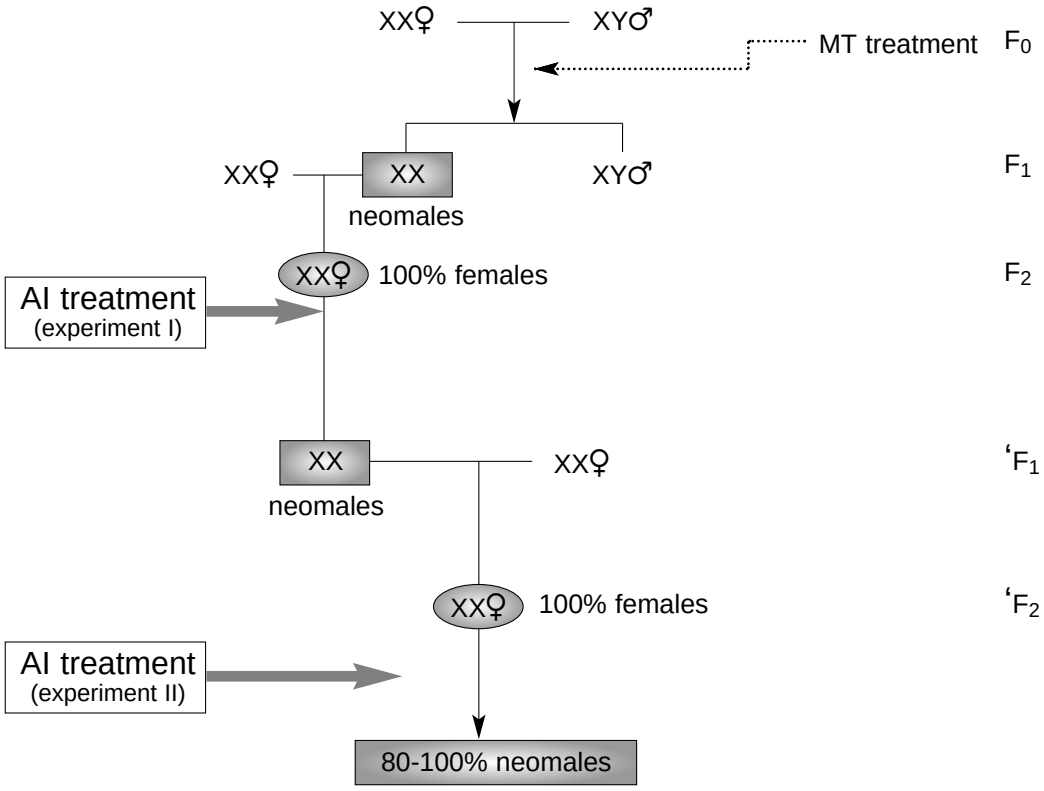

Fig. 1. Matings and endocrine treatments to produce all-female (XX) common carp populations and sex inversions by an aromatase inhibitor to produce XX neomales.

ethanol according to Guerrero (1975). Control diets consisted of 2-mm pellets with no additives. The diets were stored at 4°C and administered within a week after preparation. In experiment I, 1 kg pellets were carefully mixed with 50 or 100 mg/kg of MT, or 200 mg/kg of Fadrozole, and the fish were fed for 40 days in duplicate tanks. For experiment II, Fadrozole was mixed in the diet to a final concentration of 100, 200, or 400 mg/kg. The additives were given for 36 days in four replicates. Twenty-five percent of the fish from each tank within an AI treatment were pooled and allowed to feed an additional 14 days. The number of fish in each tank was counted at the end of treatment period to calculate the survival rate. Water temperature was maintained at 23.5-26.5°C in experiment I, and 26-28.5°C in experiment II, the latter with some fluctuations to 35°C.

The effectiveness of the treatments was determined five and two months after the end of treatment for experiments I and II, respectively. Sperm production after stripping was recorded for 80-90% of the fish in each experimental group. Salmon gonadotropin-releasing hormone analogue (sGnRHa; 10-20 µg/kg) was injected into all the fish that did not release milt, and those releasing milt were identified 20 hours later. Fish that did not release milt after this treatment were sacrificed and sexed by macroscopic inspection of the gonads. The control fish were not injected, and

all of them were sacrificed and sexed. Fish were regarded as sterile when the gonads were undeveloped and sex could not be identified. Statistical significance between the sex ratios in the control and treated groups was determined by the Fisher's Exact Test.

Results

In experiment I, mortality after the MT and Fadrozole treatments was comparable (2-8%; Table 1). The Fadrozole treatment resulted in a significant percentage of males (58.6%). Surprisingly, we found that treatment of fish with the diets containing MT resulted in a lower percentage (5-10%). These results indicate that the aromatase inhibitor was more efficient than the MT treatment in inducing sex inversion.

Fifteen of the inverted males produced in experiment I were used as breeders in experiment II. They produced all-female progenies when mated with normal females. Mortality of the treated fry during the second experiment ranged 24-34%, compared to 30% in the control group (Table 2). A high percentage of males was also observed in experiment II following treatment with the aromatase inhibitor. The efficiency of masculinization was dose-dependent, in that the lower dose of 100 mg/kg Fadrozole caused masculinization of 86% of the treated fry while the higher doses of 200 and 400 mg/kg increased the percentage of males to 97%. The high percentage of males was achieved after a short exposure to treatment of only 36 days. Extending the AI treatment to 50 days did not increase the percentage.

In the control groups, visual inspection of the fish revealed that not a single male was found. Most of the control fish were female, a few were sterile. Three to five months after AI treatment, about 50% of the neomales released milt without sGnRH injections. The testis of the inverted males that did not release milt were somewhat retarded in development, but normal in structure and color.

Discussion

In the present study, we demonstrated the ability of an aromatase inhibitor (Fadrozole) to induce sex inversion in genetic female fry of the common carp during the critical sex differentiation period. Fadrozole is a nonsteroidal, competitive aromatase inhibitor (Steele et al., 1987). This compound induced sex-reversal of genetic females to phenotypic males in fish such as chinook salmon (Piferrer et al., 1994), Japanese flounder (Kitano et al., 2000), and Nile tilapia (Kwon et al., 2000), and in reptiles (Dorizzi et al., 1994; Wibbles and Crews, 1994),

Table 1. Sex ratio of XX genotype *Cyprinus carpio* females treated with Fadrozole, an aromatase inhibitor (AI), or 17α-methyltestosterone, an androgen (MT), for 40 days (experiment I).

Treatment	*Final weight (g)*	*Fish (n)**	*Males (n)*	*Females (n)*	*Sterile (n)*	*Males (%)*	*Survival (%)*
Control	17.4±0.9	25	0	21	4	0[a]	94
MT (50 mg)	18.2±1.2	64	6	27	31	9.4[b]	92
MT (100 mg)	17.6± 0.4	64	3	33	28	4.7[a]	98
AI (200 mg)	23.1±0.5	87	51	3	33	58.6[c]	94

Values with a common superscript do not differ significantly (p>0.05).
* Number of fish whose sex was determined at 5 months.

Table 2. Sex ratio of XX genotype *Cyprinus carpio* treated with an aromatase inhibitor (Fadrozole) at 36 (stage 1) and 50 (stage 2) days (experiment II).

Fadrozole treatment mg/kg dry feed (replicate number)	*Weight at 36 days (g)*	*Weight at 50 days (g)*	*Fish (n)*	*Sterile (n)*	*Males (n)*	*Females (n)*	*Sterile (%)*	*Males (%)*	*Avg males per treatment (%)*	*Survival (%)*
Control	13.5		150	18	0	132	12	0	0[a]	70
100 (1)	7.0		34	6	28	0	17.6	82.4		68
100 (2)	6.7		30	11	19	0	36.7	63.3		60
100 (3)	13.9		38	1	37	0	2.6	97.4		70
100 (4)	13.9		35	1	34	0	2.9	97.1	86±1.0[b]	76
100 (1-4)	6.4	8.0±3.5	45	5	40	0	11.1	88.9	88.9[bc]	
200 (1)	14.9		37	1	36	0	2.7	97.3		74
200 (2)	15.4		33	0	33	0	0	100		66
200 (3)	14.1		34	1	33	0	2.9	97.1		68
200 (4)	14.0		30	2	28	0	6.7	93.3	97±0.1[c]	60
200 (1-4)	13.5	20.6±4.2	47	1	46	0	2.1	97.9	97.9[c]	
400 (1)	14.0		36	0	36	0	0	100		72
400 (2)	13.9		36	1	35	0	2.8	97.2		72
400 (3)	13.6		37	3	34	0	8.1	91.9		74
400 (4)	14.6		38	0	38	0	0	100	97±2.8[c]	76
400 (1-4)	10.7	16.3±3.0	44	4	40	0	9.1	90.9	90.9[bc]	

Values with a common superscript do not differ significantly ($p>0.05$).

and chickens (Elbrecht and Smith, 1992). The mechanism behind the masculinization of the genetically female fry in the present study might be reduction of P450 aromatase activity and decreased level of estrogens.

The lower dose of Fadrozole (100 mg) given for the shorter duration (36 days) resulted in about 97% of males in two of four groups. It would be interesting to examine lower doses for a shorter duration to determine the minimal effective treatment.

The effect of MT treatment on the percentage of males was surprisingly low, less than 10%. This is in contrast to results reported by Nagy et al. (1981), Komen et al. (1989), and Gomelsky et al. (1994) who showed that MT treatment of common carp can masculinize 85-96% of the fry. The difference in the present study might be due to the setup of the experiment and the application of the hormonal treatments. In the experiments of Gomelsky et al. (1994), the fish in only one tank of the experimental system were fed an MT-diet, while the fish in the other two tanks were exposed to the hormone only by sharing water containing soluble MT metabolites. In the present study, fish in all the tanks of the recirculating system of the MT treatment were fed an MT-containing diet. Therefore, the total amount of MT introduced into the system might have been higher than the amount of MT introduced in Gomelsky's experiment. High doses of an androgen can decrease masculinization (Devlin and Nagahama, 2002). On the other hand, our results concur with data of Komen et al. (1993) who showed that MT treatments to gynogenetic clones of common carp resulted in a low percentage of males. Apparently, the response to MT treatment is highly dependent on the environmental and genetic background of the fish. This is in agreement with earlier observations of differences in MT responses between carp strains (Nagy et al., 1981; Komen et al., 1989, 1993).

Comparison of the results of the AI treatments in the two experiments shows that 58.6% males were obtained at 25°C (experiment I) whereas almost complete masculinization of the treated fry occurred at 28°C (experiment II; Table 3). The impact of temperature on sex differentiation has been shown in tilapia (Baroiller et al., 1995) and at least eight families of fish (reviewed by Devlin and Nagahama, 2002). In carp, it was demonstrated that estradiol secretion can range as much as 20-fold in just a 5°C temperature range (Manning and Kime, 1984). However, the effect of temperature on sex determination in carp has not yet been reported. The present study seems to indicate the importance of temperature in sex differentiation during the labile period in common carp.

The fact that the AI treatment caused almost full masculinization whereas the MT treatment was much less successful may be due to the timing of the treatment. It was suggested that the gonads are sensitive to estrogen treatments days before the period in which the gonads are

Table 3. Percentage of males after sex inversion with an aromatase inhibitor in the two experiments.

Experiment	*Duration (days)*	*Temperature (°C; range)*	*Initial weight (g)*	*Final weight (g)*	*Males (%)*
I	40	25 (23.5-26.5)	4.0	23.0	58.6
II	36	27.2 (26-28.5)	3.5	14.5	97.0

sensitive to androgen (Strüssmann and Nakamura, 2002; Ohtani et al., 2003). To induce female differentiation in tilapia, for example, estrogen treatment should start before day 7 post-hatching, whereas successful androgen treatment starts only at day 15 and after (Kwon et al., 2000). This difference in timing can explain the low percentage of males resulting from the MT treatments. Our data indicate that AI treatment, combined with optimal temperature, during the sex determination period results in the highest percentage of males.

Treatment with androgens such as MT probably increases the androgen level in the blood. But since MT can be converted to estrogen by P450 aromatase, it may also increase the estrogen level (Kwon et al., 2000). Treatment with AI does not introduce exogenic steroids to the system. Rather, by blocking the transformation of androgens into estrogens, it increases the androgen/estrogen ratio. Therefore, the efficacy of AI treatment to induce male differentiation may be higher than the efficacy of treatment with aromatizable androgens such as MT.

In conclusion, the present findings demonstrate the importance of aromatization during the labile period in the common carp. Low aromatase activity during this period, regardless of the genotype of the fry, resulted in masculinization.

Acknowledgments

We thank Prof. Zvi Yaron and Dr. Yoav Gothilf for critical reading of the manuscript and helpful comments, and Dr. Sandra Hayes for valuable comments and remarks. This work was supported by the Israeli Ministry of Science, Culture, and Sport (Grant 1296-199). Fadrozole was kindly supplied by Novartis.

References

Afonso L.O.B., Iwama G.K., Smith J. and E.M. Donaldson, 1999. Effects of the aromatase inhibitor Fadrozole on plasma sex steroid secretion and oocyte maturation in female coho salmon *(Oncorhynchus kisutch)* during vitellogenesis. *Fish Physiol. Biochem*., 20:231-241.

Baroiller J.F., Chourrout D., Fosteir A. and B. Jalabert, 1995. Temperature and sex chromosomes govern sex ratios of the mouthbrooding cichlid fish *Oreochromis niloticus*. *J. Exp. Zool*., 273:216-223.

Bharadwaj R. and L.L. Sharma, 2002. Experimental manipulation of sex in *Cyprinus carpio* var. *communis* by oral administration of testosterone undecanoate. *J. Aquaculture Trop*., 17:7-13.

Bogart M.H, 1987. Sex determination: A hypothesis based on steroid ratios. *J. Theor. Biol.*, 128:349-357.

Brodie A., 1991. Aromatase and its inhibitors-an overview. *J. Steroid Biochem. Mol. Biol.,* 40:255-261.

Devlin H. and Y. Nagahama, 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. *Aquaculture*, 208:191-364.

Dorizzi M., Richard-Merceir N., Desvages G. and C. Pieau, 1994. Masculinization of gonads by aromatase inhibitors in a turtle with temperature-dependent sex determination. *Differentiation*, 58:1-8.

Elbrecht A. and R.G. Smith, 1992. Aromatase activity and sex determination in chickens. *Science*, 255:467-470.

Gomelsky B.I., 1985. Hormonal sex inversion in the common carp *(Cyprinus carpio* L.). *Ontogenez*, 16:398-405.

Gomelsky B., 2003. Chromosome set manipulation and sex control in common carp: a review. *Aquat. Living Resour*., 16:408-415.

Gomelsky B., Cherfas N.B., Peretz Y., Ben-Dom N. and G. Hulata, 1994. Hormonal sex inversion in the common carp *(Cyprinus carpio* L.). *Aquaculture*, 126:265-270.

Guerrero R.D., 1975. Use of androgens for the production of all-male *Tilapia aurea* (Steindachner). *Trans. Am. Fish*. *Soc*., 2:342-348.

Hunter G.A. and E.M. Donaldson, 1983. Hormonal sex control and its application to fish culture. pp. 223-303. In: W. S. Hoar, D.J. Randall, E.M. Donaldson (eds.). *Fish Physiology*, Vol. IXB, Academic Press, New York.

Kitano T., Takamune K., Nagahama Y. and S.I. Abe, 2000. Aromatase inhibitor and 17α-

methyltestosterone cause sex-reversal from genetical females to phenotypic males and suppression of P450 gene expression in Japanese flounder *(Paralichthys olivaceus)*. *Mol. Reprod. Dev*., 56:1-5.

Komen J., Lodder P.A.J., Huskens F., Richter C.J.J. and E.A. Huisman, 1989. Effect of oral administration of 17-α methyltestosterone and 17-estradiol on gonadal development in common carp, *Cyprinus carpio* L. *Aquaculture*, 78:349-363.

Komen J., Eding E.H., Boner A.B.J. and C.J.J. Richter, 1993. Gynogenesis in common carp *(Cyprinus carpio)*. Growth, phenotypic variation and gonad differentiation in normal and methyltestosterone-treated homozygous clones and F_1 hybrids. *Aquaculture*, 111: 271-280.

Kwon J.Y., Haghpanah V., Kogson-Hurtado L.M., McAndrew B.J. and D.J. Penman, 2000. Masculinization of genetic female Nile tilapia *(Oreochromis niloticus)* by dietary administration of an aromatase inhibitor during sexual differentiation. *J. Exp. Zool*., 287:46-53.

Kwon J.Y., McAndrew B.J. and D.J. Penman, 2002. Treatment with an aromatase inhibitor suppresses high-temperature feminization of genetic male (YY) Nile tilapia. *J. Fish Biol*., 60: 625-636.

Manning N.J. and D.E. Kime, 1984. Temperature regulation of ovarian steroids production in the common carp, *Cyprinus carpio* L., *in vivo* and *in vitro*. *Gen. Comp. Endocrinol.*, 56:376-388.

Nagahama Y.,1994. Endocrine regulation of gametogenesis in fish. *Int. J. Dev. Biol.*, 38: 217-229.

Nagy A., Bercsenyi M. and V. Csanyi, 1981. Sex reversal in carp *(Cyprinus carpio)* by oral administration of methyltestosterone. *Can. J. Fish Aquat. Sci*., 38:725-728

Nagy A., Csanyi V., Bakos J. and M. Bercsenyi, 1984. Utilization of gynogenesis and sex-reversal in commercial carp breeding: growth of the first gynogenetic hybrid. *Aquaculture Hung*., 4:7-16.

Ohtani H., Miura I. and Y. Ichikawa, 2003. Role of aromatase and androgen receptor expression in gonadal sex differentiation of ZW/ZZ-type frogs, *Rana rugosa*. *Comp. Biochem. Physiol. Part C*, 134:215-225.

Piferrer F, 2001. Endocrine sex control strategies for the feminization of teleost fish. *Aquaculture*, 197:229-281.

Piferrer F., Zanuy S., Carrillo M., Solar I.I., Devkubm R. H. and E.M. Donaldson, 1994. Brief treatment with an aromatase inhibitor during sex differentiation causes chromosomally female salmon to develop as normal, functional males. *J. Exp. Zool.*, 270:255-262.

Schieweck K., Bhatnagar A.S. and A. Matter, 1988. CGS 16949A, a new nonsteroidal aromatase inhibitor: Effects on hormone-dependent and independent tumors *in vivo*. *Cancer Res*., 48:834-838.

Steele R.E., Mellor L.B., Sawyer W.K., Wasvary J.M. and L.J. Browne, 1987. *In vitro* and *in vivo* studies demonstrating potent and selective estrogen inhibition by the nonsteroidal aromatase inhibitor CGS 16949A. *Steroids*, 50:147-161.

Strüssmann C.A. and M. Nakamura, 2002. Morphology, endocrinology, and environmental modulation of gonadal sex differentiation in teleost fishes. *Fish Physiol. Biochem*., 26:13-29.

Wibbles T. and D. Crews, 1994. Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in turtle with temperature-dependent sex determination. *Endocrinology*, 141:295-299.

Yamamoto T., 1969. Sex differentiation. pp. 117-175. In: W. S. Hoar, D. J. Randall (eds.). *Fish Physiology*, Vol. III. Academic Press, New York.