Effect of Dietary Supplementations of 17 A-Methyltestosterone and Aromatase Inhibitor (Fadrozole) on Sex Ratio of *Oreochromis Niloticus* Fry

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ABSTRACT

In Egypt, the widespread application of hormonal sex reversal using 17 α-methyltestosterone (17 MT) in the production of all male sex reversed tilapia has led to considerable pollution of the hatchery environment with that hormone and its metabolites accordingly, there is urgent need to reduce the amount used of this potentially carcinogenic hormone.

The aim of the present work is to reduce (as much as possible) the amount of MT needed to produce all-or nearly all male tilapia fry through the combined use of an aromatase inhibitor. A specific non-steroidal aromatase inhibitor "Fadrozole" was added alone or in combination with 17 MT at different proportions to fry feed to find out the most appropriate effective lowest dose of both drugs for sex reversal in *O. niloticus*. Fish fry were fed the treated feed for 30 days after which final weight, survival rate and sex ratio were monitored. Results showed that the best growth was achieved when a combination of 9 mg 17 MT plus 20 mg fadrozole/ kg feed were used. However the best survival rate was observed in fry group fed on 70 mg fadrozole/kg feed. The highest sex reversal (96 % males) along with the lowest dose of 17 MT was observed in fry treated with of 9 mg 17 MT plus 20 mg fadrozole/kg feed. The results of the present work point to the possibility of lowering the commonly used dose of 17 MT (30-60 mg/Kg feed) to 9 mg/Kg feed only through combined addition of fadrozole at 20 mg/Kg feed. However, further studies are needed to reveal the possible health impact of fadrozole.

INTRODUCTION

Unwanted reproduction has been a major limitation in the full development of tilapia culture. A solution is the use of mono-sex populations, but each monosexing method has its complications. The use of sex hormones to induce sex reversal has considerable potential, either by producing mono-sex populations directly or indirectly by breeding sex-reversed individuals (Beardmore *et al.*, 2001). Steroid hormones are commonly applied by oral or immersion treatments (Pandian and Sheela, 1995; Wassermann and Afonso, 2003). Oral administration of the synthetic androgen 17 α-methyltestosterone (MT) and 17 ethynyltestosterone (ET)

are effective in producing all-male populations in tilapia (Guerrero, 1975; Varadaraj and Pandian, 1999).

In recent years, artificial sex change was successfully induced in fish using aromatase inhibitors. Fadrozole is a reversible competitive inhibitor of CYP19 that has been shown to affect estrogen biosynthesis in mammals, birds and fish (Afonso *et al.*, 1999 and 2000). Cytochrome P450 aromatse (CYP19) is responsible for the conversion of C19 androgens to C18 estrogens in brain and gonadal tissues of vertebrates (Callard *et al.*, 1978). Estrogen production is critical for ovarian differentiation in fish and other non mammalian vertebrates (Piferrer, 2001), and the administration of aromatase inhibitors (AI) result in testis differentiation in genetic females (Smith *et al.*, 2003).

In the male Fathead minnow (Pimephales promelas) exposure to fadrozole caused a significant increase in plasma concentrations of both testosterone and 11-ketotestosterone (KT) and resulted in marked accumulation of sperm in the testis (Ankley *et al.*, 2002)

In Egypt, the widespread application of hormonal sex reversal using 17 MT in the production of all male sex reversed tilapia has led to considerable pollution of the hatchery environment with that hormone and its metabolites (El-Hawarry, 2006). Accordingly, there is urgent need to reduce the amount used of this potentially carcinogenic hormone.

The aim of the present work was to reduce (as much as possible) the amount of MT needed to produce all male tilapia fry through the combined use of an aromatase inhibitor. For this goal, a specific non-steroidal aromatase inhibitor "Fadrozole" was used alone or in combination with 17 MT at different proportions, keeping in mind to find out the most appropriate effective lower dose of both drugs to minimize the cost of sex reversal treatment and to avoid excessive use of 17 MT.

MATERIALS AND METHODS

Recently hatched *O. niloticus* fry at the yolk stage were transported in well aerated plastic bags from a private hatchery in Kafr El-Sheikh province Egypt, to the aquarium in the Faculty of Agriculture Saba Pasha, Alexandria University. Fry were allocated into 8 well aerated 80 liter capacity glass aquaria (about 500 fry in each aquarium). Fish fry feed (Table 1) was ground and mixed well. The first fish group was fed such diet without any chemical treatments and considered as a control. The treated groups were fed a diet containing additives for 30 days as shown in Table (2). The appropriate amount of feed was offered 4 times at 20 % of group biomass per day for the first week, 15 % for the second week, 12.5 for the

third week, and 10 % till the end of the experiment (Vera Cruz and Mair, 1994). Water temperature was thermostatically adjusted at 27 ±1 °C. Mortality was recorded in all groups and dead fry were kept frozen until sexed.

Fadrozole was provided as a generous gift from Novartis, Inc. Dr. H.Cooper Echhardt, Summit, N.J.). The neat chemical is soluble in water. Otherwise, the Vitamins and Minerals of Medical Profession for Veterinary Products and Fodders Addition Co. (MUVCO) were used.

Treatment of feeds with 17 α-methyltestosterone (17 MT) was done by the alcohol evaporation method (Guerrero, 1975). A stock solution of 17 Mt was made by dissolving 60 mg of 17 MT in 500 ml ethyl alcohol. Fadrozole was dissolved in distilled water (80 mg in 500 ml water). 17 MT and Fadrozole solutions were sprayed on a thin layer of fry feed at the appropriate dose levels indicated in Table (2) then allowed to dry at room temperature and stored in the refrigerator at 5° C.

Table (1): Composition and proximate chemical analysis of the basal diet used in the experiment.

Ingredient	%	Proximate analysis	%
Fish meal, herring (72 % CP)	30	Dry matter	95.3
Fish meal, menhaden (64 %CP)	23	On dry matter basis	
Commercial diet (20 % CP)	27	Crude protein	42.63
Wheat bran meal (15 %CP)	16	Ether extract	4.92
Yeast	1.5	NFE	39.85
Vitamin premix[1]	0.6	Fiber	4.98
Mineral premix[2]	0.6	Ash	10.6
Biogen	1.2	Total	100
Oxytetracycline	0.1	Gross energy[3] (Kcal/100g DM)	442.44
Total	100	Protein to energy ratio (mg/Kcal)	96.35

[1]Vitamin premix supplied the following in mg/kg premix:
Vitamin A, 6 million IU; Vitamin D^3, 6 million IU; vitamin E, 500 mg; vitamin K, 500 mg; vitamin B^1, 100 mg; vitamin B^2, 100 mg; vitamin B^{12}, 25mg; vitamin C, 10000 mg; Niacin, 2000 mg; Calcium pantothenate, 5000 mg; Folic acid, 1000 mg; BHT, 20000 mg.

[2]Mineral premix/ Kg: 60 gm Manganese, 55 gm Zinc, 30 gm Iron, 4 gm Copper, 1 gm Selenium, 1 gm Cobalt, 3 gm Iodine, 850 gm Calcium carbonate.

[3]Gross energy (Kcal/100g DM), calculated on the basis of 5.64, 4.11 and 9.44 Kcal GE/g protein, NFE and lipid, respectively (NRC, 1993).

At the end of the experiment, fish were weighed to the nearest mg. Around 80 % of the fingerling population were sexed by the aceto-carmine squash method of Guerrero and Shelton (1974) which was validated for Nile tilapia (Wassermann and Afonso, 2002). In this protocol both gonads were examined microscopically after being stained with aceto-carmine solution. The remaining fish were processed for histological examination of their gonads for accurate determination of the sex ratio and the intersex specimens. Longitudinally embedded fry were sectioned at 7 um-thick sections and stained with hematoxylin and eosin. The chemical analysis of the basic experimental diet was performed using standard AOAC (1990) methods. All data were subjected to analysis of variance using SAS/PC statistical software (SAS Institute, 1985) to determine statistical significance. Multiple comparisons among means were made with Duncan's new multiple range test with the same SAS/PC program. The effects with a probability of $P < 0.05$ were considered significant.

RESULTS

Effect on growth:

As evident in Table 2, the fry fed 9 mg MT and 20 mg Fadrozole/kg feed showed the highest final weight (group5) followed by the fry fed 18 MT and 20 mg Fadrozole/kg feed (group 4) compared to the control group (group 1). However, fry fed 30 mg MT/kg feed (group 7) achieved the lowest final weight.

The survival (%) of groups 4 and 5 at the end of the experiment was 82.8 and 80.9 %, respectively which was significantly lower than that of fry in group 1 that fed chemical free diet. Fry of group that fed 70 mg Fadrozole alone (group 8) had the highest survival percent 94.5 vs 91.2 for the control fry (group1).

Effect on sex ratio:

As shown in table (3), fry in the control group (group1) showed nearly the expected 1:1 sex ratio with 0% of intersex individuals. Deviation of sex ratio to the male side started to occur in fry groups fed with MT and Fadrozole. Fry in group 6 (fed 60 mg MT) and those in group 8 (fed 70 mg Fadrozole) had the highest % of males (94.7 and 94.8 % males respectively). The highest percentage of intersex was detected in fry group 7 fed 30 mg MT alone (using squash technique).

Table (2): Effect of dietary supplementation with 17 α-methyltestosterone (MT) and Fadrozole (Fad) on growth rate and survival (%) of *O. niloticus* fry.

Group	Feed treatment (mg/kg feed)		Average initial weigh (mg/fry)	Final weight (mg/fry)	Survival (%)
	MT	Fad			
1	-	-	11.90	152.634^{d}	91.2^{b}
2	30	30	11.90	148.180^{d}	77.5^{f}
3	18	30	10.94	197.825^{b}	80.0^{e}
4	18	20	10.95	204.500^{b}	82.8^{d}
5	9	20	11.82	234.750^{a}	80.9d^{c}
6	60	-	10.89	176.050^{c}	85.5d^{e}
7	30	-	10.90	114.524^{f}	75.9^{f}
8	-	70	11.87	122.784^{e}	94.5^{a}

- 500 fry in duplicate groups in each treatment
- Fry were fed for 30 days
- Values not sharing common superscripts are significantly different (P<0.05).

Table (3): Effect of dietary supplementation with 17 α-methyltestosterone (MT) and Fadrozole (Fad) on sex ratio of *O. niloticus* fry.

Group	Feed treatment (mg/Kg feed)		Mean percentage of fry sex				
			Squash method		Histological sexing		
	MT	Fad	Male	Female	Male	Female	Intersex
1	0	0	56.2^{d}	43.9^{a}	55.0^{e}	45.0^{a}	0.0^{f}
2	30	30	92.5^{ab}	7.5^{cd}	86.5^{d}	10.0^{b}	4.5^{c}
3	18	30	90.6^{bc}	8.6^{c}	91.5^{bc}	2.5^{d}	6.0^{b}
4	18	20	93.6^{a}	6.3^{de}	95.0^{a}	1.0^{e}	4.0^{c}
5	9	20	93.4^{a}	6.3^{de}	96.0^{a}	1.0^{e}	3.0^{d}
6	60	0	94.7^{a}	5.3^{e}	94.0^{ab}	2.0^{d}	4.0^{c}
7	30	0	88.8^{c}	12.2^{b}	90.0^{c}	2.0^{d}	8.0^{a}
8	0	70	94.8^{a}	5.3^{e}	95.0^{a}	4.0^{c}	1.0^{e}

- 500 fry in duplicate groups in each treatment
- Fry were fed the above feed for 30 days
- Values not sharing common superscript are significantly different (P<0.05).

It can be deduced from the present experiment that as low as 9 mg MT can be used in combination to 20 mg Fadrozole (group 5) to induce up to 96 % masculinization in Nile tilapia fry with an 80.9 % survival rate. The percentage of intersex fry were 3% and females were as low as 1 % in this group (using histologicval technique).

The squash technique:

The squash method of Gurrero proved to be effective in differentiating between male and female fry in a very short time and in fry not less than 2.5 cm length. The gonadal cells (spermatogonia and oogonia were clearly visible and easily recognized using the low as well as the high power magnifications of ordinary light microscope. Spermatogonial cells appear as small nucleated cells about 7-10 u diameter (Fig 1) in contrast to the large oogonial cells (about 40 u in diameter) having large central nucleus and deeply stained basophilic cytoplasm.

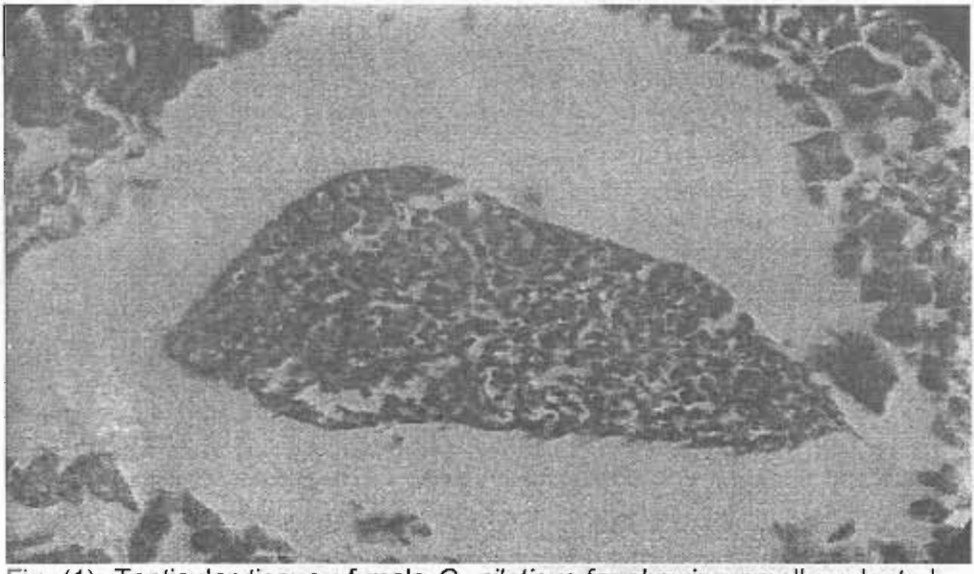

Fig. (1): Testicular tissue of male *O. niloticus* fry showing small nucleated spermatogonial cells (7 – 10 µ) with some stromal cells (X 400). The same cells were observed in control and sex-reversed males.

Histological findings:

Histological sections of fry showed clearly visible triangular testes hanging below the kidney in male fry while female fry had elongated ovarian tissue situated far down the abdominal cavity against the abdominal wall. Testicular tissue composed mostly of small sized spermatogonial cells (about 7-10 u) with the beginning of hiatus formation while the ovarian tissue contained large nucleated oogonial cells (about 40 μ) with deeply basophilic cytoplasm.

Intersex fry showed gonads having both testicular as well as ovarian cell elements in the same gonad (fig. 2).

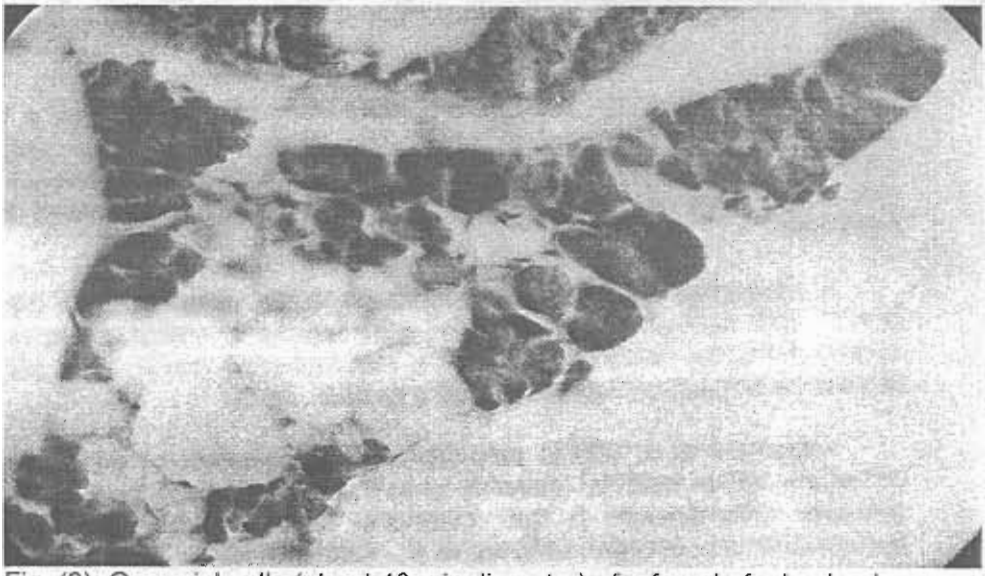

Fig. (2): Oogonial cells (about 40 μ in diameter) of a female fry having large centrally nucleated cells with deeply basophilic cytoplasm.

DISCUSSION

The better performance of MT hormone-treated fish compared to untreated fish is in accordance with observations in other studies. This is due to a combination of factors: faster growth of males (Bardach *et al.*, 1972), improved food conversion efficiency (Hanson *et al.*, 1983) and activation of other endogenous anabolic hormones enhancing growth (Lone and Matty, 1980). It was reported that treatment of tilapia, *Oreochromis*

mossambicus with methyltestosterone stimulates growth through activation of the growth hormone/insulin-like growth factor axis (Riley *et al.*, 2002). However, the suppressed growth observed in some MT treated groups (Group 2, 6 and 7) may be partly attributed to the hepato-toxic effect of MT (Saad and Rezeka, 1999) and explain the improved growth performance of fry treated in groups 3, 4 and 5 that received reduced amount of MT. This suppression is not a serious limitation in mass androgen sex-reversal as this is compensated during the early stages of grow-out in ponds and the survival could be improved by periodic cleaning of hapas and by lowering the fry density after 10-15 days of treatment (Vera Cruz and Mair, 1994). Data on the effect of fadrozole treatment on fish growth are lacking though reports indicate that it do not affect survival rate of treated fish (Kwon *et al.*, 2000)

The present study strongly implicates aromatase activity as a key factor in sexual differentiation in the Nile tilapia. The present results coincide with previous studies of Kobayashi *et al.* (2003) who obtained all male Nile tilapia when 7-14 day fry after hatching were treated with Fadrozole.

A Fadrozole dose-dependant increase in the percentage of males from 0 - 200 mg/Kg feed was obtained when batches of Nile tilapia fry were treated during the first 30 days following yolk resorption (Kwon *et al.*, 2000) and the percentage of males obtained was 92.5 - 96 %.

Nakamura *et al.* (2003) concluded that the absence of endogenous estrogens, rather than the presence of androgens, seems to correlate with testicular differentiation in fish. Fadrozole inhibit the aromatization of testosterone into estrogen (Affonso *et al.*, 1999). Moreover, Fadrozole was successfully used in sex inversion of brood stock of *Epinephelus merra* during the non breeding season (Bhandari *et al.*, 2004) and in the late-pre-breeding season (Alam *et al.*, 2006).

The importance of the present study lies in the present finding that as low as 9 mg 17 MT/Kg feed can be used in obtaining up to 96 % tilapia males which could have an important impact in lowering hatchery environment pollution with the potentially carcinogen 17 MT. However, an impediment to the widespread application of fadrozole in the commercial production of all male tilapia is its high price and restricted availability to the producing Novartis Company. An advantage of fadrozole over 17 MT is its high solubility in water which ensures even distribution of fadrozole on the

fry feed particles. 17 MT is only soluble in fat solvents and easily dissociates from feed particles on water contact which lowers the efficiency of the hormonal treatment and increases the incidence of intersex in 17 MT treated groups. It can be noted from the present results that the fadrozole group (group 8) resulted in the lowest intersex incidence as compared to treatment groups receiving 17 MT combined with fadrozole.

CONCLUSION

The present work points to the possibility of lowering the commonly used dose of 17 MT (30 - 60 mg/Kg feed) to 9 mg/Kg feed only through combined addition of Fadrozole at 20 mg/Kg feed.

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الملخص العربى

أثر إضافة الميثيل تستستيرون ومثبط الأروماتيز (الفادروزول) على النسبة الجنسية

ليرقات البلطى النيلى

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فى مصر تم تطبيق العديد من الطرق الهرمونية لإحداث انعكاس للجنس باستخدام ١٧ ميثيل تستستيرون لإنتاج نسبة عالية من ذكور البلطى، وقد أدى ذلك الى حدوث تلوث جوهرى فى بيئة المفرخات السمكية بذلك الهرمون ونواتج الأيض الخاصة به. وعلى ذلك فإن هناك حاجة ملحة لتقليل الكمية المستخدمة من هذا الهرمون المسرطن.

الهدف من هذا البحث هو محاولة خفض الكمية المستخدمة من الميثيل تستستيرون والتى تلزم لإنتاج نسبة عالية من ذكور يرقات أسماك البلطى النيلى وذلك بالجمع مابين الميثيل تستستيرون ومثبط الأروماتيز (الفادروزول). تم إضافة مثبط الأروماتيز (الفادروزول) بمفرده أو مع الميثيل تستستيرون بنسب مختلفة لعليقة اليرقات للحصول على أقل جرعة مؤثرة من كلا المادتين لإحداث إنقلاب للجنس فى البلطى النيلى. تم تغذية اليرقات على العليقة المعاملة لمدة ٣٠ يوم حيث تم تقدير الوزن النهائى ونسبة الإعاشة والنسبة الجنسية. وقد أشارت النتائج الى أن أحسن معدل نمو كان فى اليرقات المعاملة بـ ٩ ميللجرام ميثيل تستستيرون + ٢٠ ميللجرام من مثبط الأروماتيز لكل كجم من العليقة. وأحسن نسبة إعاشة كان فى اليرقات المعاملة بـ ٧٠ ميللجرام فادروزول/كجم عليقة. أما أعلى نسبة جنسية (٩٦ %) حدثت عند أقل معاملة من الميثيل تستستيرون هى ٩ ميللجرام ميثيل تستستيرون + ٢٠ ميللجرام من مثبط الأروماتيز لكل كجم من العليقة. وقد اقترحت النتائج إمكانية تقليل الجرعة التى تستخدم عادة من الميثيل تستستيرون (٣٠ – ٦٠ميللجرام/كجم عليقة) الى فقط ٩ ميللجرام/كجم عليقة وذلك عن طريق إضافة ٢٠ ميللجرام/كجم عليقة من مثبط الأروماتيز وهناك حاجة الى إجراء دراسة عن إمكانية تأثير الفادروزول على الصحة.