Effect of dietary administration of steroidal and non steroidal compounds on sex ratio of *Oreochromis niloticus*

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Abstract

The present study was undertaken to investigate the effect of dietary administration of 17α methyl testosterone, letrozol and tamoxifen starting with the first exogenous feeding on sex ratio of *Oreochromis niloticus*. The survival rates were significantly decreased among fish treated with letrozol and tamoxifen and showed non significant variations for fish received 17α MT incorporated diet. Growth rates were significantly increased in treated groups compared with controls. The highest growth rate associated with 17α MT treatment. Sex ratio was increased toward male in all treated groups. The high male population was found in 60 mg17α MT treated groups for 30 days (98.93%) followed by those received 40 mg MT and 100 mg letrozol for 30 days (97. 85% and 96.62% respectively). Deformed fish were observed among treated groups with highest percentage in letrozol treated fish (18.5%). Histological analysis of both male and female gonads revealed no pathgnomonic variations. Intersex individuals were mainly recorded in fish treated with lower doses of 17α MT, letrozol and tamoxifen for 15 days. Hormone residues in fish musculature in 17α MT treated fish showed non significant increase than that found in the control groups.

Introduction

Tilapia is considered one of most important cultured species in tropical and subtropical countries, because of their rapid growth, disease resistance and poor water quality tolerance (**27**). In tilapia farming, culture of monosex male population is preferable as it prevents reproduction and stunting of fish in grow-out pond (**21**). All male populations are desirable because males grow faster than females even if reared separately (**17**). Complete sex reversal can be induced in aquaculture practices by exposing of fish to exogenous steroids during gonadal differentiation (**14**). Moreover, other investigators used non steroidal compounds for production of all male tilapias (**2; 13**). Feeding of tilapias on diet incorporated with hormone17 α methyl testosterone effectively produce all male population (**6; 10; 26**) . The safety of this sex reversal technique is of some concern as prolonged consumption of this hormone produce human health hazards (**22**).

Therefore, the objective of this study was to investigate the effect of 17 α Methyl testosterone on sex ratio and to determine their musculature residues. As well as, to explore the effectiveness of non steroidal compounds (letrozol and tamoxifen) on sex ratio of *Oreochromis niloticus*.

Materials and Methods

1-Fish

Batches of eggs and yolk-sac larvae of *Oreochromis niloticus* were obtained from hatchery of Arab fisheries company, Elabassa, Abu hamad, Sharkia. They were transported from the hatchery to the fish diseases and management laboratory Fac. Vet. Med., Moshtohor, under accurate condition (**23**) and were placed in well prepared aquaria at 28 ◦C for about 24h for acclimation and then flushed with potassium permanganates 5 mg/l (Shelton *et al.*, 1981), as a prophylactic measure. They were maintained in these aquaria until absorption of yolk-sac.

2- Diets:

The basal diets containing 45% protein (Joe trade Company) and formulated prepared from local ingredients (**10**) were used.

2.1- Preparation of treated diets

Finely powered fish diets containing 40 or 60 mg 17 α methyl testosterone (Argent laboratory inc. company Philippines) per each kilogram diet were prepared (**26**). Diets, containing 100 or 50 mg /kg letrozol (commercial name, "Femara," Novartis pharma, Switzerland) (**2**) incorporated with 100 or 50 mg /kg tamoxifen (Amryia Pharma ind"Alexandria)(**13**), were prepared. Control diet for each treatment was prepared by the same manner.

3- Fish treatments

A total of 3300 fry were divided into 18 groups (each of two replicate, except control each of one replicate). Fishes were stocked in 0.5×0.5 ×1.0 m aquaria in a rate of 100 fry per each except groups used for 17α methyl testosterone treatment each had 130 fry per aquarium Each aquarium was supplied with air pump to maintain oxygen saturation (Rena, Italy) and heaters (Rena, Italy) to adjust the water temperature along the experimental period at 28 ◦C. At the beginning of exogenous feeding (Table 1).The fry fed on treated food (17 α MT, letrozol, and tamoxifen) while the control groups received the control diet for both treatment durations (two replicate for each treatment except the control had one replicate) . The daily feeding rate was given four times (**32**) during the day light. At the end of the treatment period, fish were fed on the basal diet until aged 8 weeks then the fish received formulated diet tell the end of experiment. The natural photoperiod was 12 L: 12 D throughout the experiment. The excreta and the uneaten food were siphoned twice daily and nearly two thirds of the water were exchanged to maintain proper water quality. Fish number in each group was counted and each fish was weighted at the end of experiment (day 90 post hatching). Only 30 fish from each replicate of 17 α MT treatment and their control groups were separated and reared for up to 180 days post hatching.

Table (1): Exposure times, type of treatment, dose and number of groups

Time of exposure/day	Type of treatment	Dosage/ mg	Group No.
15	Methyltestolsterone (MT)	Control *	1
		40	2
		60	3
	Letrozol	Control *	4
		50	5
		100	6
	Tamoxifen	Control *	7
		50	8
		100	9
30	Methyltestosterone	Control *	10
		40	11
		60	12
	Letrozol	Control *	13
		50	14
		100	15
	Tamoxifen	Control *	16
		50	17
		100	18

*control group each of one replicate for each exposure time

4- Determination of the sex ratio:

To estimate the sex ratio, all fish in treated and control groups were sacrificed on day 90 post hatching. Each was sexed by gross inspection of gonads and actocarmine squash technique (**8**). To confirm the results of squash technique, one gonad from each fish was subjected to histological examination (**7**). Fish showing skeletal deformity were counted and subjected to radiographic examination using x ray at 5MA/40v for 20 sec.

5- Determination of the hormone residue

On day 120 and 180 post hatching fish were sacrificed and pooled muscle samples were collected and stored at -20◦C till used. Extraction of the hormone from musculature was done (**33**) and the residue was determined using testosterone coated tubes radioimmunoassay kits (RIA) kits (diagnostic system laboratories (Inc. Webster, Texas, USA).

Statistical analysis of data was performed by the Statistical Analysis System (**30**) computer program.

Results and Discussion

As presented in Table (2) the survival rates among MT treated groups showed non significant difference with controls. These results came nearly in agreement with those mentioned before (**1; 34**), and in disagreement with others (**19; 25**) indicating that treatments involved synthetic steroids resulted in high mortality of most fish species . Growth rates of the exposed fish showed a significant increase compared with the control but, there was no significant difference between treated groups at different concentrations or exposure times. These results came to support the results obtained before (**4; 20**) and to disagree with others (**19**) indicating that the growth of 17α MT treated fish was lower than the control. Letrozol treated fish showed significantly low survival rates than control groups and the highest significant decrease was recorded in groups received 100 mg/kg while, growth rates of treated fish were significantly increased in exposed groups than in the controls. Moreover, tamoxifen treated fish fed on higher doses showed significant decrease in survival rates than controls. Significant increase in body weight was observed in treated groups compared to controls. Nearly similar results were observed by others (**13**). In all treatments , the groups received treated food showed high growth rates these may be due to increased the number of males .But, the higher growth rates associated with MT hormone may be attributed to its action through activation of other endogenous anabolic hormones and direct effect on gene expression in muscle cells (**16**) . The lower growth rates found in this experiment may attributed to lack of the natural food as the experiments were carried out in aquaria

Regarding to the sex ratio, as given in table 2, results of gross inspection and microscopic examination of fish gonadal tissues revealed that feeding fish with 17α MT at a rate of 40 and 60 mg /kg food showed higher significant increase in male ratio. Intersex individuals were observed with higher significant rate in 40 mg treated groups. Similar observations were recorded in previous reports (**10; 18; 29**). Fish fed on letrozol incorporated diet showed the higher percentage of males (96.62%) in groups received 100 mg for 30 days followed by those treated with 100 mg for 15 days (92.9%). These findings were supported others (**2**). The effect of letrozol on male ratio may be attributed to the aromatase inhibition; it binds reversibly to the enzyme P_{450} aromatase (**3**). Intersex individuals were observed mainly in

fish fed on 50 mg/kg letrozol. Nearly similar observations were recorded previously (**2**). Tamoxifen treated groups revealed a significantly high male percentage in those fed on 100 mg /kg for 30 days and the intersex individuals were observed in a high ratio in 50 mg treated groups. These results came nearly similar with those reported before (**13**). Moreover, the authors explained that, Tamoxifen skewed sex ratios toward males through its binding to estrogen receptors, leading to increase in the androgen levels so the androgen: estrogen ratio is increased causing testicular development.

Skeletal deformity was observed among treated fish. The high percentage of deformity was observed in letrozol (18..9 %) followed by tamoxifen (10%) and MT treated groups (5.5 %). Different forms of malformation observed as vertebral deformity, short operculum, deformed mouth and abnormal pectoral fin (Fig 1). These results came to disagree with others (**13**) indicating that MT produced relatively high incidence of deformity than tamoxifen .The deformities may be attributed to increase the levels of androgen, in MT treatment or reduction of estrogen biosynthesis via aromatase inhibition in letrozol treatment or binding to estrogen receptors by tamoxifen. (**2; 13**).

Table (2): Effect of oral administration of 17α MT, letrozol and tamoxifen on survival, growth rates and sex ratio of *O. niloticus* on day 90 post hatching

Treat. conc. mg/kg	Exp. /day	Fish No.	Survival		Weight (gm)	Male		Female		Inter sex	
			No.	%		No.	%	No.	%	No.	%
17αMT 0	15	130	128	98.4	5.11± 0.30b	62	63.3	36	36.8	0	0
	30	130	125	96.0	5.05 ± 0.25b	60	63.2	35	36.8	0	0
40	15	260	255	98.1	6.66 ± 0.24a	168	86.2	14	7.2	13	6.7
	30	260	246	94.6	6.80 ± 0.24a	182	97.9	3	1.6	1	0.5
60	15	260	252	96.9	7.18 ± 0.23.a	179	93.2	9	4.7	4	2.1
	30	260	247	95.0	7.35. ± 0.25a	185	98.9	2	1.1	0	0.0
Letrozol 0	15	100	96	96.0	4.20 ± 0.23b	62	64.6	34	35.4	0	0.0
	30	100	97	97.0	4.60 ± 0.25b	61	62.9	36	37.1	0	0.0
50	15	200	184	92.0	4.50 ± 0.15b	151	82.1	20	10.9	13	7.1
	30	200	179	89.5	5.15 ± 0.22a	155	86.6	14	7.8	10	5.6
100	15	200	169	84.5	5.29 ± 0.19a	157	2.9	9	5.3	3	1.8
	30	200	148	74.0	5.39 ± 0.22a	143	96.6	4	2.7	1	0.7
Tamoxifen 0	15	100	98	98.0	4.27 ± 0.23b	62	63.3	36	36.7	0	0.0
	30	100	97	97.0	4.30 ± 0.17b	61	62.9	36	37.1	0	0.0
50	15	200	194	97.0	4.63 ± 0.17 b	140	72.2	31	15.9	23	11.9
	30	200	190	95.0	5.56 ± 0.20 a	152	80.0	26	13.7	12	6.3
100	15	200	183	91.5	5.37 ± 0.18a	154	84.2	23	12.6	6	3.3
	30	200	178	89.0	5.33 ± 0.25a	168	94.4	8	4.5	2	1.1

Values with different letters are significantly different ($P<0.05$)

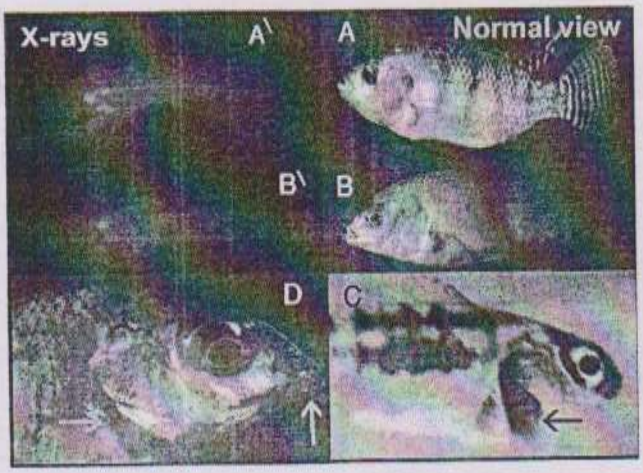


Figure (1): *Oreochromis niloticus* showing normal view (A), x ray of normal fish (A\) , deformed view (B), x ray of deformed fish (B\), short operculum (C) and head deformity (D)

Histological analysis of gonads from control and treated fish with MT, letrozol and tamoxifen, revealed the same pattern. Histological sections of testes showed thin connective tissue capsule with numerous seminephrous tubules each consisted of basal layer of spermatogonium, primary spermatocyte, secondary spermatocyte, spermatid) and large head cells with short thin tail were localized in the center of the seminephrous tubules (Fig.2). Ovary sections revealed fibrous connective tissue capsule filled with different stages of follicular development include; primary oocyte, with centrally located large nucleus and previtellogenic oocyte with large, nearly centrally located nuclei and yolk granules, in addition to yolk vesicles at the periphery of the previtellogenic oocyte (Fig. 3). The intersex fish were directed to the male side. Most of the gonads were consisted of intermingled areas of ovarian and testicular tissues (Fig 4). In other gonads few oocytes were enclosed within the testicular tissue. These results came in close association with that reported before (**2; 9; 10; 12**). Meanwhile, these observation came to support the results obtained before (**5**) indicating that more than 90 % of inter sex gonads were testicular tissue and other types of inter sex in which that testicular tissues was imbedded with single oocytes

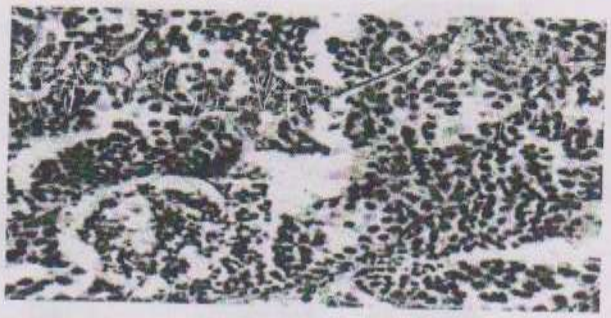


Figure (2) Histological section of testis of *O. niloticus* treated with 17∝MT showing numerous seminferous tubules fild with spermatogonium and spermatocytes. H&E stain ,x100

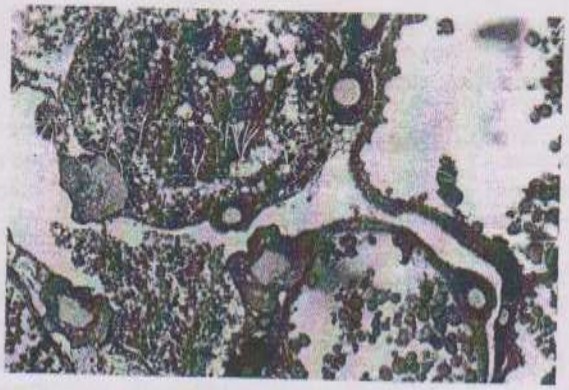
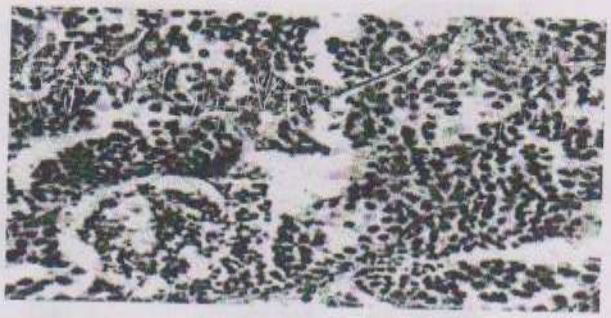


Figure (3): Histological section of ovary of *O. niloticus* treated with 17∝MT showing different stages of folliculler development H&E stain ,x100



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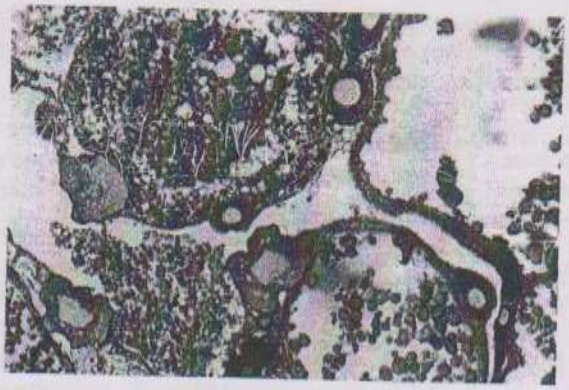

Figure (3): Histological section of ovary of *O. niloticus* treated with 17∝MT showing different stages of folliculler development H&E stain ,x100

Figure(4): Histological section of intersex gonad of *O. niloticus* treated with 17∝MT showing intermingled areas of ovarian (o) and testicular tissues (t) H&E stain, x100

Table (3): Residual values of hormones in fish muscle determined by radioimmunassay (RIA)

Exposure time /day	Treatment / concentration (mg)	Age at sampling /day	Hormone residues (ng/g musculature)
15 and 30	Control	120 180	5.847 6.610
15	MT 40	120 180	6.856 7.356
	MT 60	120 180	5.847 6.049
30	MT 40	120 180	5.696 5.970
	MT 60	120 180	6.865 6.946

As shown in Table 3, the present study revealed that the high level of hormonal residues in pooled muscle samples using radioimmunoassay RIA kits was detected in samples taken at 180 day post hatching from treated fish with 40 (7.356 ng/g) and 60 mg (6.946 ng/g) for 15 and 30 day respectively. While, on day 120 post hatching, the maximum level of hormone residues found in samples from treated fish with 40 (6.856 ng/g) and 60 mg (6.865 ng/g) for 15 and 30 day respectively. The detectable level of hormones in samples from control groups on day 120 and 180 reached 5.865 and 6.946 ng/ g respectively. In the same respect, It has been shown (**28**) that hormone residues in the whole body samples was 7.865 and 7.20 ng/g in control and hormone treated fish but, ten months after last treatment, hormone residues reached 3.753 and 3.207 ng/g in treated and control fish. The hormonal residue in muscular tissue was increased with age in treated and control groups. These findings may be attributed to the endogenous hormones at the sexual maturity of *O. niloticus* which occurs at 120 – 180 days (**15**). In addition, many authors suggested another sources of hormone may share in increasing the hormone residual value in fish tissues such as hormone found in fish meal (**11**) and or polluted water (**24**).

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تأثير التغذية بالعلائق المعالجة بالمركبات الاستيرودية و غير الاستيرودية على نسبة الجنس فى أسماك البلطى النيلى

أمل العسلى - أمانى عباس - عادل شاهين
قسم أمراض ورعاية الاسماك - كلية الطب البيطرى - جامعة بنها

قامت هذه الدراسة بتوضيح تأثير كل من المركبات الاستيرودية (١٧ألفا ميثيل تيستوستيرون) و غير الاستيرودية(الليتروزول و التاموكسافين) على نسبة الجنس فى أسماك البلطى النيلى بتجريعه مع بداية التغذية الخارجية للزريعة .أظهرت النتائج انخفاض نسبى فى معامل الحياة فى الاسماك المعاملة بالليتروزول و التاموكسافين بينما لم يظهر اختلاف نسبى بين الاسماك المجرعة ١٧ألفا ميثيل تيستوستيرون و المجموعة الضابطة . كما أظهرت النتائج وجود ارتفاع نسبى فى معدلات النمو فى المعاملات المختلفة مقارنة بالمجموعات الضابطة ووجدت أعلى نسبة فى الاسماك المعاملة ب ١٧ألفا ميثيل تيستوستيرون.كما أثبتت الدراسة تأثير جميع المعاملات الايجابى على التجنيس وو جدت نسبة الذكور عالية فى كل المجموعات المعالجة و كانت أعلى نسبة فى المجموعات المجرعة ٦٠ مجم١٧ألفا ميثيل تيستوستيرون لمدة ٣٠يوم(٩٨,٩٣%) يليها المجموعات المجرعة ٤٠مجم ١٧ألفا ميثيل تيستوستيرو ن و ١٠٠ مجم ليتروزول امدة٣٠ يوم (٩٧,٨٥% و٩٦,٦٢ % على التوالى). كما وجدت نسبة من الاسماك المشوهة فى كل المجموعات المعالجة وخاصة اليتروزول (١٨,٥%)أوضح الفحص النسيجى للمناسل عدم وجوددتغيرات مميزة.
وقد ظهرت بعض الاسماك بين الجنسية فى المجموعات المعالجة بأقل جرعة لمدة ١٥ يوم.
تقييم متبقيات الهرمون الذكرى فى عضلات الاسماك المعالجة أظهرت وجود زيادة غير نسبية مقارنة بالمجموعات الضابطة.