Hormonal sex reversal in *Oreochromis niloticus* by oral administration of diethylstilbesterole

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Abstract: Induction of sex reversal in *O. niloticus* by diethylstilbesterole (DES) and its effect on growth promotion was studied at two different hormone doses, 50mg and 100mg/kg of feed for two different feeding durations of 25 and 40 days. Treated and control groups were sexed using standard gonadal squash technique and sex ratio was calculated. The basic structure of both testis and ovary at the time of sexing was microscopically studied in semithin sections. The testis was found to be composed of lobules separated by connective tissue septa. Each lobule contained spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids. The ovary displayed the presence of many ovarian follicles at different stages of development in addition to some oogonial cell nests. Both DES levels and feeding durations led to significant increase in female percentage in comparison to control group. The highest female percentage was obtained by using 100mg DES/kg feedfor 40 days feeding duration. In addition, the oral administration of DES variably affected individual fry weight, specific growth rate (SGR) and percentage of survival.

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1. Introduction

Tilapias are of great potential importance in aquaculture in the tropics and subtropics including most of the areas suffering chronically from a lack of animal protein. Pandian and Vradaraj (1990) reported that a major drawback in the world-wide culture of tilapias is the precocious maturation and uncontrolled reproduction resulting in overcrowding and stunted growth.

Many approaches for controlling reproduction and other various purposes have been tried and one of the most promising techniques is the hormonal induction of monosex populations (Varadaraj, 1989 and Ridha and Lone, 1995). Hormonal induction of sex reversal may serve as a valuable tool for understanding the process of sex differentiation and to produce monosex populations for the aquaculture industry (Pandian and Sheela, 1995).

In tilapia, hormone treatment during the period of sexual differentiation can alter the phenotype of the gonads, indicating that endocrine factors can cause gonadal sex reversal (Kuramochi *et al.,* 2011). In fish, the gonadal differentiation takes place well after the fry has hatched and begun feeding (Purdom, 1993). The technology of sex reversal and production of all-females populations provides a new approach to fishery management (Strussman *et al.,*1996). To feminize tilapia, number of estrogens have been used with variable success. The most commonly used estrogens are estrone (Tayamen and Shelton, 1978), 17-β-estradiol (Jensen and Shelton, 1979), diethystilbesterol (Obi and Shelton, 1983) and 17-α-ethynylestradiol (Lahar, 1993 and Rosenstein and Hulata, 1993). The effect of estrogenic hormones such as estrone, estriol, estradiol and diethylstilbesterol on primary and secondary sex characteristics differed widely in various treatments. Sexing according to external features is difficult in young fishes. The standard squash technique is a reliable method for sexing.

The present study was undertaken to determine: (1) the optimal dosage and duration of DES required to produce the highest percentage of females and (2) the effect of the hormone on the growth of tilapia. Such a study may contribute to develop a breeding program for producing monosex male genetic population through super male.

2. Material and Methods

Preparation of treatment diet:

Two treatment feeds (45%protein) were prepared by alcohol evaporation method (Guerro, 1975). Diethylstilbesterol (DES, Sigma chemicals) was added at 50 and 100mg/kg feed. The control feed was prepared in the same manner but without DES.

Treatments:

Fry for treatment (total length range of 9-11mm) were collected from *Oreochromis niloticus* adult females. They were divided into 15 groups, 150 individuals/group. These groups served as control

and the rest for different DES levels and different feeding duration (three for treatment). Each group was stocked in 48L glass aquaria.

Fry were fed at 10% of the body weight daily; the average fry weight was determined by weighing ten fries and the total weight was calculated daily. The feed was divided into three rations given at 8.00, 12.00 and 17.00 O'clock. All control and treated groups were counted and weighed weekly until the end of feeding with treated diet. Specific growth rate (SGR) according to Watanaba *et al.* (1993) and number of survived fry were calculated. The amount of feed was adjusted weekly according to changes in the weight of the fry. All troughs were cleaned every 2-3 days to reduce disease potential. After 40 days all groups were transferred to 128 liters (L) aquaria and allowed to grow until sexing process (after 97 days). Normal levels of temperature; 22-29ºC, Mires (1995), dissolved oxygen; > 3and pH;7-9 (Ross, 2000) were kept throughout the period of experiment.

Evaluation of treatments:

At the end of the experiment, the tilapias were weighed; sexed using the standard squash technique (Guerrero and Sheilton, 1974) the sex ratio was calculated. Testis and ovary samples were collected, fixed in paraformaldehyde-glutraldehyde mixture (Karnovesky, 1965) and processed for semi-thin sectioning (1µm thick) and examined light microscopically.

Statistical analysis:

The obtained results viz., the mean individual fry weight, mean specific growth rate, mean percentage of survived individuals and mean percentage of females were subjected to a two-way analysis of variance. A chi-square test was used to determine if the observed sex ratio differ from the expected 1:1 ratio.

3. Results

The means of individual fry weight (gm), specific growth rate, percentage of survival and percentage of females for the control and treated groups were given in table (1), while analyses of variance are laid out in table (2). As the analysis of variance revealed, there were significant reduction in the mean individual fry weight and the specific growth rate after 40 days of feeding on DES treated diet as compared with the control. At sexing time (97 days of experiment), there were no significant difference in the mean weight and specific growth rate between both 50 and 100mg DES treatments for 40 days and the control group. There were a significantly high percentage of survivals at 25 days feeding on 100mg DES/kg as compared with the control, whereas a comparable reduction was observed at 40 days feeding on 50m DES/kg feed.

With regard with the percentage of females, the obtained results revealed that feeding *Oreochromisniloticus* (tilapia) with DES at 50mg or 100mg levels for either 25 or 40 days, increased the percentage of females significantly than control. However the increase obtained from using the later dose (100mg DES) was greater than that of the former one (50mg DES). Moreover, increasing the feeding duration at both DES levels caused slight increase in the percentage of females. The highest female percentage was obtained after using 100mg DES for 40 days (Fig. 1). A chi-square test employed on the obtained sex ratios (Table 3) indicated significant deviations from the expected 1:1 ratio for all different applied DES treatments.

The differences between male and female gonadal squashes are illustrated in Figs. (2 a & b). Using semi-thin sections, the testis (Fig. 3a) appeared consisting of testicular lobules showing few spermatogonia, many primary spermatocytes, few secondary spermatocytes and numerous spermatids. Spermatogonia had weakly stained cytoplasm and nuclei, the later attained deeper staining as they approached their final forms (spermatids). Few Sertoli cells were hardly differentiated by their irregular nuclei and deeply stained nucleoli. The ovary, however, showed many ovarian follicles at different stages of development in addition to some oogonia forming cell nests. The ovarian follicles were variable in size and structure. Small-sized follicles had only the oocyte with large pale centrally located nucleus and moderately stained cytoplasm. Medium-sized follicles were surrounded by one layer of flat cells and were characterized by the presence of Balbiani's body and few lipid droplets in their cytoplasm. Large follicles had large number of lipid droplets and deeply stained yolk granules. These follicles were surrounded by thin homogenous layer (Zona pellucida) followed by one layer of columnar cells and covered externally by a connective tissue theca

4. Discussion

The observed reductions in specific growth rate with increasing feeding duration supports the findings of Ridha and Lone (1995) who mentioned that estrogens generally have no anabolic effect in most teloest fishes. In the same respect, Tayaman and Shelton (1978) found that *Oreochromis niloticus* fry treated with androgen (17α-methyl testosterone) had faster growth rate than those treated with estrogens (diethyl stilbesterole and estrone) indicating once more that estrogens have no anabolic effect in tilapia species.

Table (1): Effect of feeding *O. niloticus* with different doses of DES for different feeding durations on individual fry weight, specific growth rate, percentage of survivals and percentage of females

Treatment	Mean individual fry weight (gm)		Mean specific growth rate (%bw/day)		Mean percentage of survivals		Mean percentage of females
	At 40 days	At sexing (97 days)	At 40 days	At sexing (97 days)	At 40 days	At sexing (97 days)	
(1) Control	0.236	2.387	0.559	2.447	48.223	22.887	57.073
(2) 50mg DES							
a. 25 days	0.237	1.717*	0.569	1.757*	52.890	29.777	70.290*
b. 40 days	0.153**	2.203	0.364**	2.260	43.997	11.780*	75.863**
(3) 100mg DES							
a. 25 days	0.203	1.290**	0.482	1.323**	57.557	43.333**	81.863**
b. 40 days	0.166**	2.343	0.388**	2.423	50.447	18.667	87.393**
L.S.D. 0.05	0.041	0.664	0.099	0.677	11.431	8.124	9.465
L.S.D. 0.01	0.057	0.944	0.141	0.963	16.250	11.548	13.455

*P<0.05 **P<0.01

Table (2): Analysis of variance for individual fry weight, specific growth rate, percentage of survivals and percentage of females obtained from different DES levels at two different feeding durations (F. duration) in *O. niloyicus*.

Source	Degree of freedom	Mean individual fry weight (gm)				Mean specific growth rate (%bw/day)				Mean percentage of survivals				Mean percentage of females	
		At 40 days		At sexing		At 40 days		At sexing		At 40 days		At sexing			
		MS	F	MS	F	MS	F	MS	F	MS	F	MS	F	MS	F
Replicates	2	0.002	2.21	0.430	1.61	0.013	2.20	0.440	1.60	129.259	1.64	197.621	4.96*	113.297	2.10
DES levels (A)	2	0.004	4.74*	0.527	1.98	0.025	4.11*	0.539	1.95	64.333	0.82	174.754	4.39*	1148.83	21.25**
F. duration (B)	1	0.007	7.94*	1.186	4.46	0.045	7.41*	1.285	4.64	128.053	1.62	910.080	22.86**	61.64	1.14
Interaction (AB)	2	0.003	2.89	0.417	1.57	0.016	2.61	0.455	1.64	33.206	0.42	244.203	6.13*	15.411	0.29
Error	10	0.001		0.266		0.006		0.277		78.828		39.814	54.051		

* P<0.05 ** P<0.01

Table (3): 50 and 100mg/kg diet on sex differentiation of *O. niloticus*.

DES dose (mg/kg diet)	Feeding duration (days)	No. of recovered individuals	Sex ratio			Chi-square values
			Males	Intersex	Females	
0	40	103	44	0	59	2.19
50	25	134	36	4	94	25.88**
	40	53	12	2	39	14.30**
100	25	195	31	5	159	86.24**
	40	84	11	0	73	45.76**

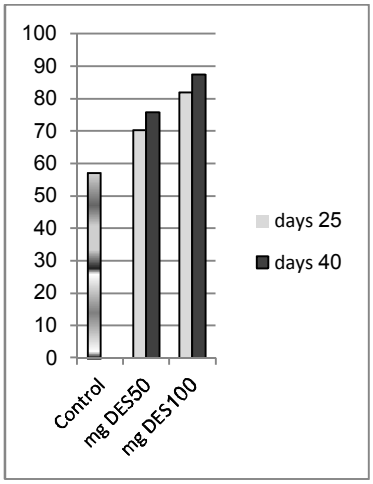

Figure (1): Percentage of females at sexing (97 days) resulting from different DES levels and different feeding durations.

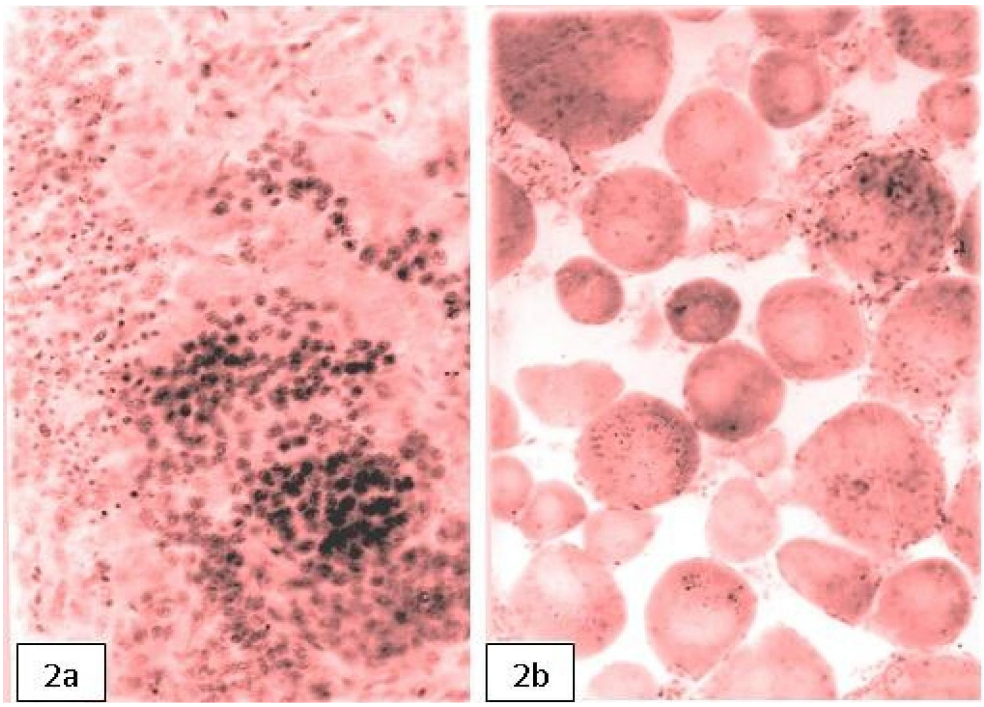

Fig. 2: Gonadal squashes of tilapia stained with acetocarmine. X250.**a:**Testicular squash showing different generations of germ cells.**b:** Ovarian squash showing variably-sized ovarian follicles.

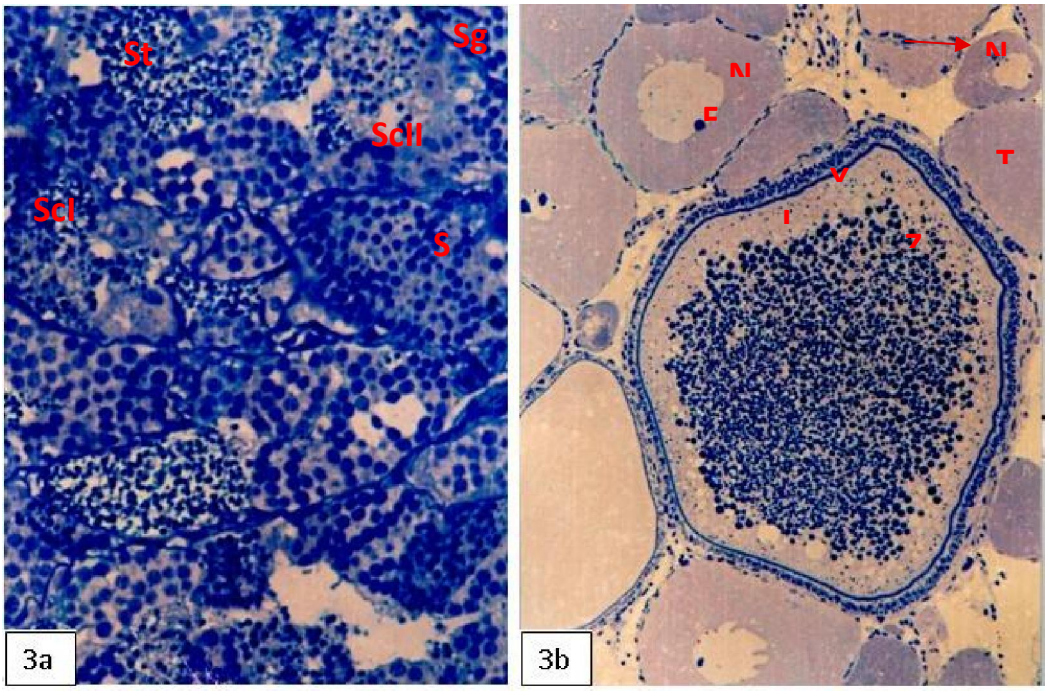

Fig. (3a): A semithin section in the testis of *Oreochromis niloticus*. Notice the presence of all generations of germ cells except → mature sperms. Spermatogonia (Sg), primary spermatocytes (ScI), secondary spermatocytes (ScII), spermatids (St) and Sertoli cells (S). Tolluidine blue, X250.

Fig. (3b): A semithin section in the ovary of *Oreochromis niloticus* showing variably-sized ovarian follicles. Abbreviations: N (nucleus), Ld (lipid droplets). Yg (yolk granules). Zp (zona pelluida). Fc (follicular cells). T (connective tissue theca). Notice Balbiani's body (arrow). Tolluidine blue, X400.

The observed higher values of mean weight after using either 50 or 100mg DES for 40 days as compared with other groups can be attributed to the recorded low survival rate (low rearing density allows fish to grow bigger).

The percentage of survivals at sexing time recorded a significant reduction as the period of DES treatment increased. This supports the suggestion of Purdom (1993) and Karayücel et al (2003) that treating fish with sex steroids during the sexually intermediate phase of life can lead to significant fry mortality.

The organization of the testis in tilapia at the time of sexing (97 days old) resembled the unrestricted or lobular type of other teleost fishes described by Greier *et al.* (1980), Billard (1990) and Mattei *et al.*(1993). No mature sperms have been seen inside the testicular lobules of tilapia at this age. This indicates that the testis was still in the early spermatogenic stage as described in bester by Amiri *et al.*(1996). In this respect, Wahbi and Shalaby (2010) mentioned that the histological characteristic of mature sex reversed males show spermatogenic cysts with cells in early stages of development were predominatingwhile in control testes cysts with predominant spermatozoa were seen.

The ovarian morphology in the present study resembles basically that described in the bass, Ecetrarcus labrax (Mayer *et al.*, 1988) and that of bullhead cat fish;*Ictalorus nebulosus* (Roseneblum *et al.*, 1987). A characteristic Balbiani's body as that described by Beams and Kessel (1973) and Mayer *et al.*(1988) was clearly demonstrated in medium-sized follicles.

With regard to the percentage of females, the present work proved that increasing either the DES level or treatment duration resulted in an increase in the percentage of females. This increase was in all cases significantly higher than control. The highest female percentage (about 87%) was obtained after using 100mg DES for 40 days. In agreement with previous reports, No rate of estrogen administration resulted in a 100% female population. Although complete sex reversal was not achieved in the present study, the obtained 87% females compare well with those reported in the literature. In *Sarotherodon niloticus*, Tayaman and Shelton (1978) achieved 90% female population with DES at 100mg ppm for 25, 35 and 59 days. The sex reversing effect of DES in *Oreochromis mossambicus* (Varadaraj, 1989) was greater than in *Oreochromis aureus* (Hopkins *et al.*, 1979) or *Sarotherodon niloticus* (Tayaman and Shelton, 1978). In the same respect Potts and Phelps (1995) obtained 80% female population in *Oreochromis niloticus* by applying DES 400. Moreover, Singh (2013) mentioned that treatment to fry of *Tilapia putitora* for 60days post fertilization (60dpf) with 17β estradiol (150mg/kg feed) fetched 69.5% female population. It can be concluded that a 100mg DES/kg dose and a long treatment (40 days) can produce a higher monosex female population in *Oreochromis niloticus*.

Unfortunately, estrogen has recently been shown to inhibit the endocrine (liver-derived) and autocrine/paracrine local insulin-like growth factor-I system in fish. It led to lasting impairment of spleen growth and differentiation. Especially, the impairment of spleen and melanomacrophage centres might interfere with the antigen presentation capacity of the immune system and, thus, alter susceptibility to infection (Shved, 2009). This could explain the low survival rate obtained in this experiment and may limit the use of estrogens in sex reversal trials.

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