

Optimization of Feminization of *Oreochromis niloticus* L. by Oral Administration of Diethylstilbestrol (DES): The Effects of Stocking Density, Treatment Duration and Environment

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Abstract

The effects of the treatment environment (tank and hapa), duration of treatment, and stocking density of fry on the success of feminization protocols were investigated. In the first of two studies, the fry were hormone-treated in concrete tanks (clear water) and hapa-in-pond environments for three durations: 10, 15 and 20 days. All groups were administered diethylstilbestrol at 1000 mg•kg⁻¹ of feed and stocked at 1000 fry m⁻². In the second study, fry were treated in hapas at three stocking densities: 1000, 2500 and 5000 m⁻². A shorter treatment duration of 10 days was sufficient for effective feminization (>90% phenotypically female populations) of fry held in tanks, whereas 15-20 days was required for effective treatment in hapas. Stocking density greater than 1000 fry m⁻² did not significantly increase the induction of feminization but significantly reduced the growth and survival rate of the fish. The optimum feminization protocol is feeding DES at 1000 mg•kg⁻¹ to fry held at 1000 m⁻² for a treatment duration of 10 days in tanks and 15-20 days in hapas.

Introduction

The problem in the culture of Nile tilapia (*Oreochromis niloticus* L.) is its overpopulation in ponds caused by uncontrolled reproduction. One solution to this problem is the use of monosex male populations that can be produced by a number of means (Mair and Little 1991). One promising method of producing a monosex male population is through manipulation of the predominantly monofactorial sex determining mechanisms of the fish (Scott et al. 1989, Mair et al. 1993). All-male populations can be produced by breeding

homogametic YY males with homogametic XX females. The large-scale production of YY male broodfish involves two stages of feminization of genotypically XY and YY fish during their sexually undifferentiated stage.

The Genetic Manipulations for Improved Tilapia Project (GMIT), established at the Freshwater Aquaculture Center, Philippines, with the thrust of developing a technology for large scale production of genetically all-male tilapia through the production of YY-males, conducted a series of studies to optimize feminization procedures in *O. niloticus* using the synthetic hormone, diethylstilbestrol (DES). Initial studies (Mair and Santiago 1994) indicated that administration of DES at $1000 \text{ mg} \cdot \text{kg}^{-1}$ feed produced the highest rates of feminization of *O. niloticus* fry, held in clear water systems (aquaria and concrete tanks). However, this treatment produced only 75-85% female sex ratios, possibly due to sub-optimal treatment conditions and stocking density, factors known to influence the success of sex reversal treatment (Mair and Little 1991; Vera Cruz and Mair 1994). The experiments presented here examined the effects of environment (tank and hapa), duration of treatment (10, 15 and 20 days) and stocking density ($1000\text{-}5000 \text{ fry} \cdot \text{m}^{-2}$) on the feminization of sexually undifferentiated *O. niloticus* fry using DES.

Materials and Methods

These studies were conducted using the pond and tank facilities of the GMIT Project at the Freshwater Aquaculture Center (FAC), Central Luzon State University (CLSU), Philippines with a locally available strain of *O. niloticus* known to be derived from a number of isolated strains imported into the Philippines over the last two decades.

Study 1 - Feminization in tanks and hapas for different durations

A randomized complete block design (RCBD) was employed using 12 units of 1.8-m^2 outdoor circular concrete tanks and 12 units of $1\text{m} \times 1\text{m} \times 1\text{m}$ hapas suspended in a 200-m^2 fertilized earthen pond. At least 100% of the total water volume in tanks was gradually replaced daily by allowing a continuous supply of water. First feeding fry and fertilized eggs were collected using fine mesh nets 10 days after the stocking of broodfish in 10-m^2 hapas. Fry of similar ages were pooled and divided among the eight treatments that were used in the first block (replicate). Fry from artificially incubated eggs in downwelling systems were used in blocks (replicates) II and III.

First feeding fry with less than 11-mm length were divided into treatment groups as shown in Table 1. Each tank or hapa was provided with a feeding ring. Fry were fed with powdered feed consisting of 30% fishmeal and 70% rice bran. DES-treated feed was prepared using the alcohol evaporation method of Guerrero (1975) at a concentration of $1000 \text{ mg} \cdot \text{kg}^{-1}$ of feed, based on the study of Mair and Santiago (1994). Control feed was prepared in the same manner with the exclusion of hormone.

Table 1. Experimental design for two studies investigating feminization of *O. niloticus* fry.

Treatment	Environment	Stocking density fry m ⁻² (fry m ⁻³)	Duration of treatment (days)	DES treatment mg • kg ⁻¹
STUDY 1				
DES-10T	1.8 m ² tank (0.36 m ³)	1000 (5000)	10	1000
DES-15T	1.8 m ² tank (0.36 m ³)	1000 (5000)	15	1000
DES-20T	1.8 m ² tank (0.36 m ³)	1000 (5000)	20	1000
CT-20T	1.8 m ² tank (0.36 m ³)	1000 (5000)	20	0
DES-10H	1 m ² hapa (0.5 m ³)	1000 (2000)	10	1000
DES-15H	1 m ² hapa (0.5 m ³)	1000 (2000)	15	1000
DES-20H	1 m ² hapa (0.5 m ³)	1000 (2000)	20	1000
CT-20H	1 m ² hapa (0.5 m ³)	1000 (2000)	20	0
STUDY 2				
DES-1000H	1 m ² hapa (0.5 m ³)	1000 (2000)	20	1000
DES-2500H	1 m ² hapa (0.5 m ³)	2500 (5000)	20	1000
DES-5000H	1 m ² hapa (0.5 m ³)	5000 (10000)	20	1000
CT-1000H	1 m ² hapa (0.5 m ³)	1000 (2000)	20	0

Key: DES - diethylstilbestrol treatment; CT - control treatment; T - tank; H - hapa.

Note: Each treatment was replicated three times.

Fry were fed four times a day (0800, 1100, 1400 and 1700 hours) at 20% of the biomass per day during the first two weeks and 15% for the rest of the treatment period. Ten percent fry samples were collected and weighed weekly for the adjustment of the diet ration. Dissolved oxygen and temperature were monitored once a week at 0600 and 1400 hours and 0600 and 1600 hours, respectively. After the specific duration of DES treatment, fry from each treatment-replicate were counted and bulk weighed for the calculation of the survival rate and average weight, after which they were then nursed in 4-m² hapas suspended in earthen pond up to a mean weight of 5.0 g prior to sexing by gonad squash (Guerrero and Shelton 1974).

Study 2 - Feminization in hapas at increasing stocking densities

The methodologies previously described were applied to fry stocked in hapas at increasing stocking densities (1000, 2500 and 5000 m⁻²). Fry were treated for a duration of 20 days. Hapas were replaced after 10 days of DES treatment to reduce fouling of the nylon mesh. The number of surviving fry was counted prior to release in the clean hapas. The experimental design for this follow-up experiment is summarized in the second part of Table 1.

Statistical analyses

In Study 1, data from tanks and hapas were analyzed separately using the analysis of variance (ANOVA) for RCBD. Results of Study 2 were similarly analyzed by ANOVA. Comparison among treatment means was done

using Duncan's Multiple Range Test. Percentage data were arc sine transformed prior to analysis. Chi-square test was used to determine whether the observed sex ratio differed from the expected 1:1. Furthermore, data on DES treatments in tanks and hapas were analyzed using ANOVA for 2x3x3 factorial design with two environments, three durations of DES treatment, and three blocks to determine the effect of environment on feminization and survival.

Results

In the first study, all treated groups in tanks and hapas had significantly ($P < 0.01$) higher proportions of females than the untreated control (Table 2). Chi-square analysis of the control treatments showed that the sex ratio agreed with the expected ratio of 1:1. Duration of treatment was not a significant factor ($P > 0.05$) affecting the percentage of females following treatment in tanks (Table 2). This indicates that a 10-day DES treatment is sufficient to affect high rates of feminization in tanks.

In hapas, duration of treatment was a significant factor ($P < 0.05$). The 10-day treatment had a significantly ($P < 0.05$) lower mean percentage of females than the 15- and 20-day treatments, which were not significantly different.

Environment (tank or hapa) was not a significant factor ($P > 0.05$) in the production of phenotypic female, but it was a more significant factor ($P < 0.001$) than duration of treatment ($P < 0.01$) in affecting survival of fish. Significantly, lower survival was obtained in tanks (62.8-77.2%) compared with that in hapas (94.7-99.5%). Survival of fry in both tanks and hapas agreed with the expectation that shorter treatment duration produces higher survival rates. In both tanks and hapas, no significant difference existed in survival between DES-treated populations (DES-20T and DES-20H) and control populations (CT-20T and CT-20H) treated for similar duration. This indicates that the dosage of hormone used in the study was not toxic to the fish.

Mean water temperature throughout the treatment period ranged from 27.1 - 33.8 °C in tanks and 29.3 - 32.6 °C in hapas (Table 3). The wider daily temperature fluctuation in tanks was due to the small volume and shallow depth (20 cm) of water that was easily influenced by temperature changes in the atmosphere. Dissolved oxygen levels in tanks and hapas were within the desirable range for the survival of the fish. The lower level of early morning dissolved oxygen in hapas compared with those in tanks may be due to the presence of phytoplankton in pond and other fish outside the hapas that both consumed oxygen at night.

In the follow-up experiment, higher stocking density did not significantly affect the induction of feminization. Highest mean proportion of females at 89.2% was obtained at the highest stocking density of 5000 fry m⁻² (DES-5000H) but no significant differences existed among hormone treatments (Table 2). A considerable disadvantage of a stocking density greater

Table 2. Mean ($\pm$ SD) initial weight, harvest weight, survival and percentage phenotypic female of *O. niloticus* fry DES-treated in tanks and hapas at different durations and stocking densities.

Treatment	MEAN*				
	Initial weight mg	Harvest weight** mg	Survival** %	Females %	
STUDY 1					
Tank					
DES-10T	7.7 ^a ± 0.58	20.3 ^b ± 1.52	77.2 ^a ± 2.55	96.2 ^a ± 2.46	
DES-15T	7.7 ^a ± 0.58	27.0 ^b ± 1.00	67.9 ^b ± 4.57	89.5 ^a ± 5.08	
DES-20T	7.7 ^a ± 0.58	37.3 ^a ± 1.15	62.8 ^b ± 6.89	96.6 ^a ± 2.99	
CT-20T	8.0 ^a ± 0.00	42.3 ^a ± 9.90	64.2 ^b ± 4.79	51.7 ^b ± 1.90	
Hapa					
DES-10H	7.0 ^a ± 1.00	48.0 ^c ± 13.53	99.5 ^a ± 0.81	75.6 ^b ± 6.42	
DES-15H	7.0 ^a ± 1.00	82.7 ^b ± 15.31	98.1 ^a ± 0.97	93.3 ^a ± 9.28	
DES-20H	6.7 ^a ± 1.53	99.7 ^{a,b} ± 23.76	94.7 ^a ± 5.49	96.4 ^a ± 4.69	
CT-20H	7.3 ^a ± 1.15	118.0 ^a ± 32.97	97.3 ^a ± 3.92	52.3 ^c ± 2.67	
STUDY 2					
			(10 days)	(20 days)	
DES-1000H	8.0 ^a ± 0.58	88.3 ^b ± 18.15	96.4 ^a ± 2.61	93.7 ^a ± 3.06	83.9 ^a ± 13.05
DES-2500H	8.0 ^a ± 0.58	66.0 ^c ± 21.93	91.7 ^a ± 5.41	81.3 ^b ± 11.45	78.1 ^a ± 0.81
DES-5000H	8.0 ^a ± 0.00	53.5 ^c ± 30.41	75.4 ^b ± 7.20	54.0 ^c ± 4.24	89.2 ^a ± 0.49
CT-1000H	8.3 ^a ± 1.15	105.7 ^a ± 24.83	98.3 ^a ± 1.10	96.0 ^a ± 2.59	50.0 ^b ± 2.31

*Mean of three replicates.
**Immediately following the DES treatment.
Means superscripted with different letters (a, b, c), are significantly different (P<0.05) while those with the same letter are not significantly different (P>0.05).

Table 3. Ranges of dissolved oxygen and temperature readings in tanks and hapas during the duration of DES treatment in Study 1.

Treatment	MEAN*			
	Dissolved oxygen mg.l ⁻¹		Temperature °C	
	0600 hours	1400 hours	0600 hours	1600 hours
Tank				
DES-10T	3.6 $\pm$ 0.59	7.4 $\pm$ 1.50	27.2 $\pm$ 0.76	33.8 $\pm$ 2.25
DES-15T	4.0 $\pm$ 0.81	8.8 $\pm$ 1.84	27.1 $\pm$ 1.01	33.2 $\pm$ 1.44
DES-20T	4.8 $\pm$ 0.42	10.2 $\pm$ 0.61	27.4 $\pm$ 0.65	33.4 $\pm$ 1.53
CT-20T	4.8 $\pm$ 0.78	10.4 $\pm$ 0.78	27.4 $\pm$ 0.51	33.3 $\pm$ 1.38
Hapa				
DES-10H	1.8 $\pm$ 0.47	7.0 $\pm$ 0.99	29.3 $\pm$ 1.10	32.6 $\pm$ 1.15
DES-15H	1.9 $\pm$ 0.44	8.4 $\pm$ 1.55	29.3 $\pm$ 1.10	32.4 $\pm$ 0.85
DES-20H	2.1 $\pm$ 0.26	8.7 $\pm$ 1.11	29.4 $\pm$ 0.64	32.3 $\pm$ 0.55
CT-20H	2.2 $\pm$ 0.23	8.8 $\pm$ 1.17	29.4 $\pm$ 0.64	32.3 $\pm$ 0.55

*Mean of three replicates.

than 1000 m⁻² was the significant reduction in growth and survival rates. Mean gain in weight of fish during the treatment period ranged from 45.5 to 97.4 mg. Mean gain in weight of fry treated at 1000 m⁻² (DES-1000H) was significantly ($P<0.05$) higher than those treated at 2500 (DES-2500H) and 5000 m⁻² (DES-5000H) but significantly ($P<0.05$) lower than that of the untreated control group (CT-1000H). A significant effect of stocking density on the survival of fry was observed as early as 10 days of treatment and was clearly evident after 20 days with DES-5000H having significantly ($P<0.01$) lower survival rate than the DES-2500H treatment, which in turn was significantly ($P<0.05$) lower than the DES and control at 1000 m⁻², which were not significantly different.

Discussion and Conclusions

A key factor in the success of sex reversal treatments is the amount of hormone that is actually ingested by each individual fish during its labile period of sexual differentiation. This in turn can be influenced by a number of factors as outlined by Mair and Little (1991). The periods at which gonadal tissues differentiate vary depending on certain factors such as species, age and size of fish, and temperature (Shelton et al. 1981; Hiott and Phelps 1993). Popma and Green (1990) recommend 14 mm as the minimum harvest size for androgen sex-reversal of *O. niloticus* while Dunham (1990) recommends 12 mm. In *O. mossambicus*, Pandian and Varadaraj (1988) reported a minimum of 11 days of treatment to produce an all-male population. Dutta (1979) reported that gonadal tissue of *O. aureus* differentiated to ovary earlier (14-15 days) at higher temperature (31 °C) with length of 15-17 mm compared with that at lower temperature (21 °C; 24-27 days) with length of 14-17 mm. In *O. niloticus*, Alvendia-Casauay and Carino (1988) reported that gonadal differentiation took place within 30-33 days post-hatch at 25-26 °C while Baroiller et al. (1986) observed it from 28-35 days post-fertilization or approximately 18-25 days after first feeding stage.

In the first study, the first feeding fry used had an initial length of less than 11 mm and 10 days of DES treatment was sufficient to obtain greater than 90% phenotypic female populations in tanks, whereas 15 days was required in hapas. This indicates that using the dose of 1000 mg•kg⁻¹ DES under the adopted feeding regime, 10 days of treatment is sufficient for most fish in the tanks to consume adequate amount of hormones needed for sex reversal. Apparently a longer duration of treatment is required in hapas for the fish to ingest sufficient quantities of hormone. It is possible that sexual differentiation occurs later in fry held in hapas, but given their faster growth rate, this seems unlikely. Little (1989) and Vera Cruz and Mair (1994) mentioned that the availability of phytoplankton as an alternative source of food to fry may have a slightly adverse effect on the efficacy of hormone treatment. In this study, the presence of natural food from primary production in the pond is more likely to be the critical factor, this being consumed together with the artificial diet, thereby reducing the daily rate of

hormone ingestion. This could be compensated by an increase rate of feeding or longer duration of DES treatment. Even though feminization in tanks was achieved in a shorter duration, it was noted, in the near absence of natural food, that following treatment, fry were thin and hollow bellied and their weight was approximately $\frac{1}{3}$ of those reared in hapas. This observation agrees with the findings of Buddle (1984) and Vera Cruz and Mair (1994) that fry treated in hapas grow faster than those treated in tanks (clear water).

It was mentioned earlier that the concentration of DES used was not toxic to the fish because of the absence of significant difference on survival rates between DES-20T and CT-20T. The combined effect of poor feed quality, high stocking density (5000 m^{-2}), absence of natural food and greater diurnal temperature fluctuations (Table 3) may have accounted for the lower survival rate in tanks. It was possible that because of hierarchical interaction among fry on feeding, some fish consumed minimal amount of feed thus suffered mortality brought about by weakness, cannibalism and greater diurnal temperature fluctuations. Vera Cruz (1991) observed a similar effect of wide fluctuation of temperature in tanks on survival during the androgen sex reversal of *O. niloticus*. Rosenstein and Hulata (1994), however, obtained 71-93% survival after a 32-day DES treatment with fry stocked at 4500 m^{-3} but the temperature in the tank was kept at $25 \pm 1^\circ\text{C}$. These studies suggest that sex reversal should be conducted indoor or in shaded areas when using tanks with shallow water to reduce wide variation in temperature.

In relation to duration of treatment, similar results were obtained by Varadaraj (1989), Rosenstein and Hulata (1994) and Mair and Santiago (1994), emphasizing that the effective DES treatment duration in tanks is 10-15 days starting immediately after yolk sac absorption. The results of this study indicate that this should be extended to 15-20 days for treatment in hapas.

Even though this study utilized a high concentration of hormone ($1000 \text{ mg} \cdot \text{kg}^{-1}$) as optimized by Mair and Santiago (1994), maximum rates of feminization were not achieved. This may have been due to problems of unpalatability or insufficiency of DES-feed. The amount of feed was adjusted weekly and it was noted (Table 2) that the weight of fish was more than doubled right after the first week of feminization in tanks. The increase in weight of fish was greater in hapas. This indicates that the amount of DES feed may have become insufficient during the later days of treatment prior to feed amount adjustment. This was evidenced by the condition of fish (thin with hollowed belly) after the treatment in tanks. The stocking density of fish may have also been a factor. Dunham (1990) and Mair and Little (1991) stress the importance of stocking density in minimizing hierarchical interaction among the treated fish which can influence the amount of hormone ingested by some individual fish. The first study utilized a stocking density of 1000 m^{-2} in hapas (equivalent to 2000 per m^3) which is lower than the optimum density of 12 l^{-1} ($12,000 \text{ per m}^3$) recommended for effective masculinization (Mair and Little 1991). There are some studies that were successful in producing an all-female population using lower doses of DES. In *O.*

mossambicus, which appears to be easier to feminize than *O. niloticus*, Basavaraja et al. (1990) reported complete feminization using feeds containing 50 and 100 ppm DES with fry stocked at a low density of 90-150 fry m⁻³. Treatment was administered for 30 days and the fish were fed *ad libitum* for the first 15 days that could have made the total amount of hormone consumed by each fish sufficient for sex reversal prior to the completion of gonadal differentiation. Rosenstein and Hulata (1994), similarly produced all-female groups at doses >10 mg•kg⁻¹ DES when *O. mossambicus* and *O. aureus* were fed *ad libitum* at stocking densities ranging from 3000-5100 fry m⁻³. They attributed the high rate of feminization achieved in their study to the rate of feeding (*ad libitum*), the age of fry at the onset of treatment and the species or strain effects. In *O. niloticus*, Tayamen and Shelton (1978) produced 73-90% females using DES at lower concentrations of 25 and 100 mg•kg⁻¹ diet. They adopted a low feeding rate of 10% of the body weight but the fish were hormone treated for 25-59 days at a density of 650 fry m⁻² in indoor stainless steel troughs. The density was reduced by 1/3 after 25 days and further by 1/2 after another 10 days. Scott et al. (1989) produced up to 48% females in genotypically XY *O. niloticus* fry population, produced from crossing YY males with normal females, using DES at 100 mg•kg⁻¹ diet for 30 days, with a stocking density of 1852 fish m⁻³. Using the same strain of *O. niloticus* used in our study, Mair and Santiago (1994) obtained 68.1% females using 100 mg DES.kg⁻¹ diet for 25 days, with a stocking density of 3600 fish m⁻³. These variable results together with our failure to produce 100% feminization, indicate the effects of other variables such as stocking density, rate of feeding and the species specificity on susceptibility of the fish to sex reversal.

In the follow-up study, the generally lower percentage of females compared to Study 1 may be attributed to the problem of very high seepage in the pond where the hapas were installed, during the first 10 days of treatment. Daily irrigation was performed which increased the turbidity of the water. Turbidity may have lessened the amount of hormone feed consumed by the fish. After 10 days, fry were transferred to new hapas installed in a pond with sufficient natural food. Little (1989) mentioned that the level of natural food present in the pond influences the success of sex reversal in hapas.

The lack of any significant effect of stocking density on treatment efficacy indicates that this factor may not be limiting. The suppressed growth and poor survival of fry treated at high densities (2500 and 5000 m⁻²) support the findings of Rothbard et al. (1983) and Vera Cruz and Mair (1994). The significantly greater mortality rate at a high stocking density of 5000 fry.m⁻² was observed as early as 10 days, during the replacement of the hapas. The low survival rate may be due to poor water quality brought about by inadequate water exchange when hapas become fouled and the high population density of the fish.

In conclusion, the suggested feminization protocols for *O. niloticus* in tanks is feeding DES at 1000 mg•kg⁻¹ of feed given at 20% of the body weight per day for 10-15 days to first-feeding fry stocked at 1000 m⁻². The same DES

concentration and stocking density is recommended for treatment in hapas but for a longer duration of 15-20 days and lower feeding rate (15% BW) during the last week.

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