PROGENY TESTING OF CONVERTED MATERNAL (XY) GENOTYPE AFTER SEX REVERSE TO PRODUCE SUPER MALE (YY) IN *OREOCHROMIS NILOTICUS* EXPERIMENTALLY

MOHAMED E. FARAG[1], GAMAL A. MOHAMED[2], HUSSEIN A. ELGHOBASHY[1] AND AHMED M. KHATER [2]

1- *Genetics Department Central Laboratory for Aquaculture Research, Abbassa, Sharkia, Egypt*
2- *Reproductive physiology and hatchery Departement Central Laboratory for Aquaculture Research, Abbassa, Sharkia, Egypt*

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Abstract

The present study is continuation to the first step in a comprehensive program for producing "supermales" (YY) of *Oreochromis niloticus* in Egypt to produce all male tilapia for increasing fish production and prevent unwanted reproduction in ponds. Sexually undifferentiated fry after yolk sac absorption period were feminized using 17-β ethynylestradiol incorporated into the feed. Fry were reared in hapas suspended in an earthen pond and fed for three time durations, 3, 4 and 5 weeks on three different dosages of the feminizing stimulating agent (FSA) 50, 100 and 150 mg/kg/ feed. Growth of fry was affected by the dosages used. Average weight of fry that were fed on higher doses of 17-β ethynylestradiol supplemented feed was significantly higher than those fed on lesser amounts for the same period. The effect of 17-β ethynylestradiol as a (FSA) was highly detectable among *O. niloticus* fry (1st generation) that were fed on 150 mg/kg (FSA) - treated feed for 5 weeks. Progeny test was adopted to estimate the mean percentage of phenotypic females MPF that reached 92.0 ± 2.3% when fry were fed for 5 weeks on 150 mg/kg (FSA) treated - feed. MPF decreased with decreasing the quantity of (FSA) in the supplementary feed. The length of feeding period when interacted with the (FSA) dose in the supplementary feed had an appreciable effect on sex reverse among progenies of *O. niloticus*. After rearing of the sex reversed fry to sexual maturity and crossing normal males (xy) to the undifferentiated pseudo- and normal females (C-xy and xx), twenty four converted pseudofemales with female phenotype and xy male genotype could be differentiated by progeny testing of their offspring (2nd generation) that consisted male percentage varying from 69 to 79% males exceeding the normal ratio. Hypothetically, one third of these males should be super males of the "YY" genotype and two thirds should be normal males. Further research by crossing these males after sexual maturation to normal females and testing their progeny will prove or disprove this hypothesis.

Key words: Sex reversal, *Oreochromis niloticus*, feminization

INTRODUCTION

The most difficult problem associated with tilapia culture is the early sexual maturation and uncontrolled reproduction in ponds. A great deal of work has been done looking for solutions such as manual sexing, predator stocking, hybridization, gynogenesis and steroid-sex reversal to produce all male tilapia (YY super males). Mair and Little (1991) evaluated these solutions, specially in the developing countries, and reported that all the traditional techniques have no longer been adopted widely in aquaculture. Manual sexing is laboreus and requires skill. The major disadvantages of this method are human error in sexing and the wastage of females. Interspecific and intergenic hybridizationare known to produce all-male progeny. However difficulty in maintaining pure parental stocks that consistently produce 100% male offspring, poor spawning success and incompatibility of breeders resulting in low fertility. Therefore, studies on the genetic basis of sex determination of *Oreochromis niloticus* and other *Oreochromis* species have been developed by Mair *et al.* (1991) to provide an alternative and effectual monosex breeding program for producing all - male offspring (Desprez *et al.,* (2003b) and for generating YY-male broodstock that could be considered safe and friendly as no hormones are applied to fish that are consumed. The steps of this program have been described by Mohamed *et al.*, (2004). Desperz *et al.,* (2003a) carried out sex determination system on blue tilapia, *Oreochromis aureus* using pseudofemale populations. Their data reported that sex reversal of fry with estradiol resulted in the production of some functional sex-reversed fish with a female phenotype and ZZ male genotype, known as pseudofemales. Ramos *et al.*, (2003) carried out some gynogenetic studies on *O. mossambicus* to produce YY and XY males.

The first step which is feminization of sexually undifferentiated tilapia progeny from normal crosses has been achieved by many authors (Rosenstein & Hulata, 1994, Mair and Santiago, 1994 and Mohamed *et al.*, 2004 in *Oreochromis niloticus* and in a number of other *Oreochromis* species. The successful feminization of the xy genotype is a vital step in the development of the YY technique. Identification of the converted female C-xy, (C-xy) refers to converted genotypic male into functional phenotypic female, by progeny testing them in crosses with normal xy males is the second step because the morphology and behavior of C-xy is indistinguishable from that of their genetically female siblings. This has been reported by Mair *et al.* (1991). Sex reversal of tilapia either by feminization or masculinization must begin before the gonadal tissue of young genetic males or females has differentiated into testes or ovaries. Functional sex reversal is most easily achieved through oral application of estrogens or androgen incorporated into the feed and administered during the period of sex

differentiation which is known to be 30 days according to Alvendia-Casauay and Carino (1988) and 14 days according to Srisakultiew (1993). However, Popma and Green (1985) reported that fry can be effectively sex reversed in 20 days, but occasionally only 95% of the fry develop as phenotypic males. They also stated that the labile period sex reversal success is very wide ranging from 25 to 59 days for *O. niloticus*. In Egypt, the present study is a trial to carry out the second step in such Central Laboratory for Aquaculture Research, Abbassa, research program number 2003020507 to produce supermale (YY), after feminization of sexually undifferentiated progeny from normal crosses of *Oreochromis niloticus*, to differentiate the converted tilapia after maturation through subsequent crossing with normal male. Progeny sex ratio approximating 3:1 is indicative of a maternal (XY) genotype.

The objective of this study was to produce supermale (YY) through the feminize sexually undifferentiated progeny of *Oreochromis niloticus* from normal crosses using 17-β ethynylestradiol for sex reversal as a feminizing stimulating agent as well as rcognizing the target converted maternal (XY) genotype.

MATERIALS AND METHODS

The steps followed in this experiment are illustrated in figure 1. A number of 48 broodstock females and 24 of active males of *Oreochromis niloticus* were accommodated separately at a sex ratio of 2:1 in 3 hapas (net cages) installed in an earthen pond each of which containing 16 females and 8 males. Dimension of hapa was 350 x 200 x 150 cm. Average body weight of *O. niloticus* females was138.5 ± 12.6 g. Fish were fed 2% of the total biomass daily with pelleted feed (25 % crude protein). Hapas were cleaned every day from uneaten food and feces. Females were checked regularly by opening their mouths gently. When females spawned, eggs were extruded from the buccal cavity of the female and incubated in Macdonald jars until hatching. The first batch of hatched larvae were collected and stocked in 27 hapas, installed in the same pond, at a density of 100 larvae/ hapa. The twenty seven hapas were randomly assigned into nine groups representing nine treatments with three replicates each. After the yolk sac absorption period, the same feminizing agent (FSA), 17-β ethynylestradiol for Aquaculture (Argent Laboratory Inc. 255 Salcedo St. Legaspi Village. Makati City, Philippines) and the same methodology as in Mohamed *et al.*, 2004 were used for feminization of the fry, however fry of the nine treatments were fed 6 days a week for three time durations, 3, 4 and 5 weeks on feminizing agent – treated feed with three different dosages of 17-β ethynylestradiol 50, 100 and 150 mg/kg feed, respectively. The daily feed ration schedule of Popma and Green, 1985 was used. At the end of the experiment feeding was suspended and measurements of

weight of fry of each hapa were taken. After sex reversal, the gonad squash technique reported in Guerrero and Shelton (1974) was adopted for identification of the phenotypic sex of 50 fish from each Hapa using microscopic examination of the gonads when the fish reached 2-3 cm length. The remaining fry of the treatment that had the highest percentage of mixed females (converted and normal) were transferred to a concrete tank inside a green house and reared to maturation. Throughout the rearing period, the water temperature ranged from 22 to 28 °C, dissolved oxygen varied from 5 - 6.2 ppm while pH ranged from 7.2 to 8.65. After maturation the males were gotten red of. One hundred mixed females were distributed over 100 hapas, one female per hapa, and crossed with one normal male (xy). The rest of females were stocked in an isolated hapa to substitute the mortality. The resulting siblings were isolated and reared separately in marked hapas and when reached 2-3 cm length, a representative sample of 50 fry were taken from each hapa and the gonads were examined microscopically using the squash method to estimate sex ratio. The actual converted females C-xy and the normal ones could be determined as the first should hypothetically generate progeny including 25% females and 75% mixed males of which one third should presumably be super males "yy" and two thirds normal males "xy". The identified converted females and its fry were kept separately in concrete tanks inside the green house for further research.

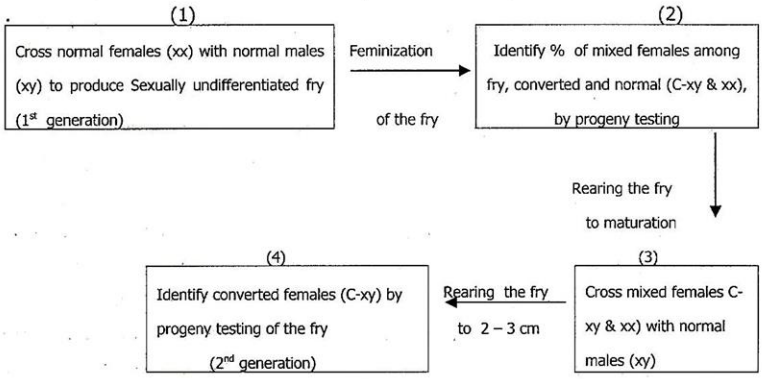

Figure 1. Diagram showing the steps followed in the present study.

RESULTS

As demonstrated in table 1, after 3 weeks feeding with feminizing agent treated-feed 50, 100 and 150 mg/kg, fry reached an average body weight of 45.3 ± 3.7, 51.5 ± 5.4 and 62..2 ± 5.6 mg/fry at survival rate of 84 ± 3, 82 ± 4 and 80 ± 2%, respectively. While fry that were fed for 4 weeks attained an average body weight of

49 ± 3.5, 54 ± 3.3 and 64.4 ± 5.0 mg/fry at survival rate of 79 ± 4, 75 ± 5 and 70 ± 5 %, for the same hormone doses, respectively. Feeding the fry with the same feed for 5 weeks resulted in fry of 48 ± 6.5, 50 ± 5.2 and 71.9 ± 4.9 mg/fry average body weight and mean survival rate reached 73 ± 4, 70 ± 6 and 66 ± 5 %, respectively.

Table 1. Average body weight of *Oreochromis nilotocus* fry fed 50, 100 and 150 mg/kg feminizing agent –treated feed 3, 4 and 5 weeks.

Feminizing agent	After 3 weeks feeding		After 4 weeks feeding		After 5 weeks feeding	
	Wt./fry (mg)	Survival %	Wt./fry (mg)	Survival %	Wt./fry (mg)	Survival %
50 mg/kg	86.4 ± 3.7	84 ± 3	90.2 ± 3.1	79 ± 4	112.9 ± 8.0	73 ± 4
100 mg/kg	108.5 ± 8.1	82 ± 4	116.4 ± 6.7	75 ± 5	130.7 ± 4.1	70 ± 6
150 mg/kg	130.4 ± 5.0	80 ± 2	145.0 ± 4.0	70 ± 5	167.1 ± 5.4	66 ± 5

After sex reversal with 17-β ethynylestradiol treated-feed 50, 100 and 150 mg/kg for 3 weeks feeding period, mean percentage of phenotypic females in *Oreochromis niloticus* fry recorded 57.5 ± 3.2, 72.0 ± 4.3 and 77.0 ± 3.1% for the three doses of feminizing agent, respectively, as shown in figure 2 and table 2. However it reached 54.8 ± 5.4, 61.7 ± 5.7 and 78.4 ± 2.5% when the three doses of feminizing agent were used for 4 weeks feeding period.

When the fry were fed on the three treated feed for 5 weeks, the mean percentage of phenotypic females among fry was 48.0 ± 4.0, 59.3 ± 4.2 and 92.0 ± 2.3%, respectively.

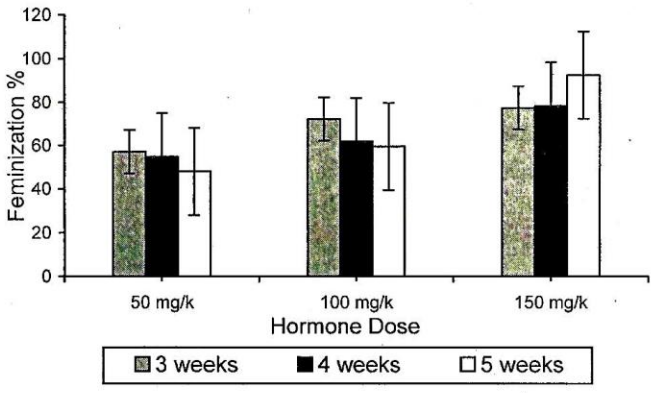

Figure 2. Percentage of females (phenotypic and genotypic) among *Oreochromis niloticus* fry fed 17-β ethynylestradiol - treated feed with different dosages for 3, 4 and 5 weeks.

Table 2. Mean percentage of phenotypic females (converted C-xy and normal xx) in *Oreochromis niloticus* fry fed feminizing agent treated-feed 50, 100 and 150 mg/kg for 3, 4 and 5 weeks.

Dose / Time	50 mg/kg	100 mg/kg	150 mg/kg
3 weeks	57.5 ± 3.2	72.0 ± 4.3	77.0 ± 3.1
4 weeks	54.8 ± 5.4	61.7 ± 5.7	78.4 ± 2.5
5 weeks	48.0 ± 4.0	59.3 ± 4.2	92.0 ± 2.3

Tables 3, 4 and 5 shows the analysis of data by ANOVA and Duncan's Multiple Range test at significant level of 0.05.

Table 3. Two way ANOVA test of the feminization percentage under the effect of feminizing agent dose and time.

Source	Df	MS	F	P	
Main effects					
Dose	2	172.63	72.7369	0.0000	***
Time	2	78.843	3.33078	0.0588	ns
Interaction					
dose x time	4	21.7183	9.15539	0.0003	***
Error	18	23.6711			

Table 4. Duncan's Multiple Range Test of means of feminization percentage when fry were fed on three different doses of feminizing agent 50, 100 and 150 mg/kg (at significance level of 0.05)

Feminizing agent dose	Mean	n	Significant range
Treatment (3) 150 mg/kg	82.44	9	a
Treatment (2) 100 mg/kg	64.34	9	b
Treatment (1) 50 mg/kg	55.27	9	c

Table 5. Duncan's Multiple Range Test of means of feminization percentage when fry were fed for three different feeding durations 3, 4 and 5 weeks (at significance level of 0.05)

Feeding time	Mean	n	Significant range
Treatment (1) 3 weeks	70.66	9	a
Treatment (2) 4 weeks	66.43	9	a b
Treatment (3) 5 weeks	64.96	9	b

At the end of the rearing period, the survival rate reached 25% among fry of the 1st generation that had the highest feminization percentage and reared to maturation in the second season. Number of males that were gotten red of was 36. Number of mature mixed females remaining was 176.

After crossing 100 mixed females (converted and normal), separately with normal males in hapas, progeny testing of the resulting fry revealed that there were 24 converted females (C-xy) among the mixed females as their production contained 70 – 79% males as shown in figure (3)

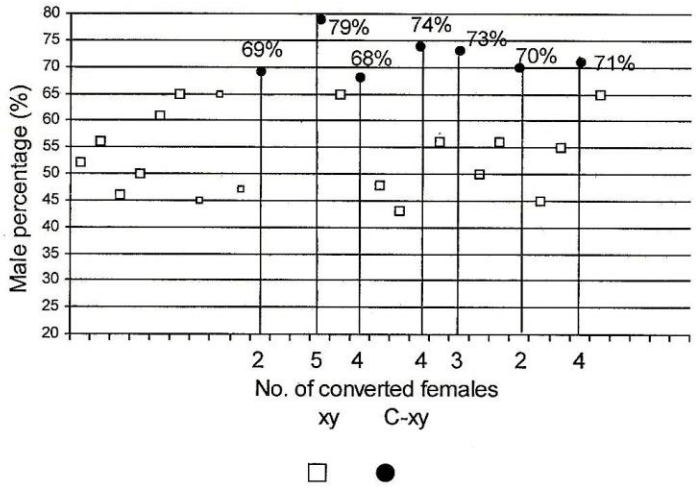

Figure 3. Number of phenotypic converted females (C-xy) and male percentage in their progeny (xy).

DISCUSSION

The technology of producing "yy" male provides a reliable solution to the serious and widespread problem of early sexual maturation, unwanted reproduction and overpopulation in tilapia culture (Mair *et al.* 1997). Accordingly, this species has been the focus of considerable biological research with genetics receiving much emphasis. Several authors have proposed a breeding program through which "yy" males could be generated in *O. niloticus* (Baroiller and Jalbert 1989).

Growth of fry that were fed 17-β ethynylestradiol treated-feed was affected by the dosages used. Upon termination of the experiment, average weight of fry that were fed on higher doses of ethynylestradiol supplemented feed was significantly

higher than those fed on lesser amounts for the same period. This result is in agreement with that of Mohamed *et al.*, 2004 who assured the effectiveness of the feminizing agent for enhancement of growth of fry reared in glass aquaria.

The effect of ethynylestradiol as a feminizing stimulating agent was highly detectable among *O. niloticus* fry that were reared in hapas installed in an earthen pond and orally fed on 150 mg/kg feminizing agent -treated feed for 5 weeks. Mean percentage of phenotypic females MPF was 92.0 ± 2.3%. MPF decreased with decreasing the quantity of ethynylestradiol in the supplementary feed. The same observation was reported by Mohamed *et al.*, 2004 using the same feminizing agent however with lower doses to produce nearly the same feminization percentage. This may be attributed to:-

1- Difference in quality of the feminizing agent as it was brought from different source.
2- Wastage of some of the feminizing agent by diffusion in water of the earthen pond through the mesh of the net cages, the availability of natural food.
3- The different physicochemical and biological conditions of water outdoor.

The feeding period that resulted in the highest feminization percentage in the present study was different from Mair *et al.* (1997) who used diethylstibestrol as a feminizing stimulating agent for 20 days oral feeding. It was also different from that reported by Srisakultiew (1993), Alvendia-Casauay and Carino (1998). This may be attributed to type, dose and effectiveness of the feminizing stimulating agent used.

The length of feeding period when interacted with the feminizing agent dose in the supplementary feed had an appreciable effect on sex reverse among fry of *O. niloticus*. This result coincides also with that of Mohamed *et al.*, (2004).

When the sex reversed fry were reared to sexual maturity stage, the pseudo-females (C-xy) could be differentiated form the normal ones (xx) using pair-mating of normal genotypic males to the mixed females with normal males separately in isolated. hapas. Progeny testing of their offspring proved that the majority of females were originally genotypic females from the moment of fertilization and their offspring had a normal sex ratio of 1:1. Twenty four remaining females generated progeny including 69 to 79% males exceeding the normal ratio because, hypothetically, these brooders were converted maternal xy genotype and acted reproductively as phonotypical functional females. Also it is hypothetically supposed that one third of these males are super males of the "YY" genotype and two thirds should be normal males.. Further research by crossing these males after sexual maturation to normal females and testing their progeny will prove or disprove this hypothesis.

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الانقلاب الجنسى للبلطى النيلى للحصول على ذكور فائقة النمو باستخدام المحفز الانثوى ١٧- بيتا استراديول تجريبيا

محمد السيد فرج[١]، جمال عبد الناصر محمد[٢]، حسين عطية الغباشي[١]، أحمد مصطفي خاطر[٢]

١- قسم التربية والوراثة المعمل المركزي لبحوث الثروة السمكية
٢- قسم التفريخ وفسيولوجيا التكاثر المعمل المركزي لبحوث الثروة السمكية

يعتبر هذا البحث استكمالا للخطوة الأولي في برنامج شامل يتم تنفيذه في مصر لإنتاج ذكور بلطي سوبر (YY) في البلطي النيلي وذلك كحل لمشكلة التكاثر الغير مرغوب فيه لسمكة البلطي في الأحواض وزيادة الإنتاج السمكي لتميز ذكور البلطي في معدلات النمو عن الإناث. إذ أجريت تجربة كالتي تمت في الخطوة الأولي لمحاولة انقلاب جنسي لتأنيث أجنة بلطي غير مميزة جنسيا باستخدام المحفز الانثوى اثينايل استراديول عن طريق التغذية بعلف يحتوي علي نسب مختلفة من المحفزعن التي استخدمت في السابق، وقد استخدمت لذلك هابات في حوض ترابي بدلا من الأحواض الزجاجية. وكان تركيز المحفز في العلف ٥٠، ١٠٠، ١٥٠ مجم لكل كيلو جرام علف، ولفترات زمنية مختلفة ٣، ٤، ٥ أسابيع. أكدت النتائج أن زريعة البلطي النيلي التي تغذت علي علف يحتوي علي جرعات أكبر من المحفز كانت متوسطات أطوالها وأوزانها أعلي من تلك التي تغذت علي جرعات أقل بعد فترات التغذية الثلاث، مما يؤكد فاعلية اثينيل استراديول كعامل محفز لمعدلات النمو. أما بالنسبة للإنقلاب الجنسي إلي إناث فقد تم عمل اختبار أجنة للزريعة بعد انتهاء التغذية وكان تأثير المحفز الانثوى ملحوظا بدرجة كبيرة في الزريعة التي تغذت لمدة ٥ أسابيع علي علف يحتوي علي ١٥٠ مجم لكل كيلو جرام، حيث وصل متوسط نسبة الإناث إلي ٩٢,٠± ٢,٣ %. ولقد انخفضت هذه النسبة بانخفاض الجرعة في العليقة. وكان تأثير مدة التغذية بالمحفز علي الانقلاب الجنسي واضحا ، إذ انخفضت نسبة الإناث مع تقليل مدة التغذية. بعد أن وصلت الزريعة إلي النضج الجنسي في الموسم التالي تم التخلص من الذكور ووضعت الإناث المختلطة سواء المنقلبة أو الطبيعية في هابات كل أنثي في هابة مستقلة مع ذكر للتزاوج. تم عمل اختبار أجنة مرة أخري للزريعة الناتجة علي حدة لتمييز الإناث التي هي في الأصل ذكور xy وتحولت ألي إناث من الأخري التي هي في الأصل إناث طبيعية xx . وأظهر اختبار الأجنة أن ٢٤ أم من أسماك البلطي النيلي من إجمالي عدد ١٠٠ سمكة هي في الأصل ذكور وتحولت إلي إناث C-xy حيث أن معدل التجنيس في إنتاجها من الزريعة كان ١ إناث : ٣ ذكور. أذ تراوحت نسبة الذكور في الزريعة من ٦٩ إلي ٧٩% تقريبا. تم الاحتفاظ بهذه الأمهات المنقلبة وكذلك إنتاجها من الزريعة لرعايتها حتي تصل إلي النضج الجنسي لاستكمال برنامج إنتاج الذكور السوبر YY منها.