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Production of Male *Oreochromis niloticus* GET Excel Tilapia by Egg Immersion in Methyl Testosterone Hormone

Dan M. Arriesgado, Helen J. Vicente and Danilo A. Vicente
Mindanao State University at Naawan, 9023 Naawan, Misamis Oriental
Email: danarriesgado@yahoo.com

ABSTRACT

The masculinization of Nile tilapia, *Oreochromis niloticus,* GET Excel strain was conducted through egg immersion in two concentrations of 17 α-methyltestosterone (500 and 1000µg L^{-1}) at different immersion times (24, 48, 72 and 96 h). The effects of hormone concentration (HC) and immersion time (IT) on egg hatching rates, fry growth, survival and sex ratio were evaluated. Immersion time significantly influenced hatching with decreasing hatching rates at increasing IT. Highest hatching rate of 85% occurred after 24h IT regardless of HC. Low hatching rate of 63 to 65% was obtained at 96h IT. Survival rate of post-treated fry in the net enclosure was not significantly different among treatments. Higher growth occurred in 1000µg L^{-1} HC although not significantly different from the other treatment. Higher proportions of male juveniles were obtained with increasing HC especially at 1000µg L^{-1} (97-100%) across all IT. The interaction effect of HC x IT demonstrated that HC at 1000µg L^{-1} induced production of higher percentages of tilapia males. This implies that HC is a primary factor contributing to the high production of male population of *O. niloticus*. Cost and return analysis showed an inverse proportion of the return on investment (ROI) with immersion time. Highest average ROI was obtained in 500µg L^{-1} (55.32%) and 1000µg L^{-1} (55.25%) HC at 24h IT and lowest in 1000µg L^{-1} (28.50%) and 500µg L^{-1} (32.42%) HC at 96h IT. This study has identified the HC and IT of eggs that contributed to highest percentage of male production, a vital information in improving efficiency of all-male tilapia juvenile production to support the growing aquaculture of this important fish species.

Keywords: Aquaculture, masculinization, 17-α methyltestosterone, hormones

INTRODUCTION

The decline in the catch of tilapia in freshwater ecosystems and its early sexual maturity in culture systems are well-recognized problems in tilapia farming (Guerrero, 1975). The Nile tilapia (*Oreochromis niloticus.*), one of the most popular aquaculture species, is a prolific breeder capable of attaining sexual maturity as early as 60 days especially when reared in a natural ratio of around 50% male and 50% female. This species will breed frequently, often every 30 days, resulting in stiffer competition for food and space between the stocked fish and the recruits and hence, resulting in smaller sizes of the cultured fish. At reduced nutrition the best attainable market size after four months of culture is 150g (Guerrero, 1975).

In comparison with mixed populations, all-male tilapia populations provide several advantages for aquaculture, including superior growth where males grow faster than females, as well as prevention of unwanted reproduction which diverts energy away from somatic growth. Some techniques, including the manual separation of males and selective hybridization, are technically feasible but are cumbersome and time-consuming which are management constraints in commercial operations. This method is also only 80% accurate due to human error.

The masculinization technique by oral administration of a hormone (Carrasco *et al*., 1999; Tayamen and Shelton, 1978; Phelps, 2006) is the most common method used in the Philippines for the production of all-male tilapia. Sex reversal of newly hatched tilapia is accomplished via oral administration of 17α-methyltestosterone (MT), which has been incorporated into starter fish feeds. The technique, however, has some limitations, such as the uniform age of fish that should be used at the first feeding stage to ensure high masculinization rate. Another constraint is the lack of control over reversal efficiency particularly when the fish are in the natural environment where natural food is present. Dietary treatment with MT is an effective means of producing all-male tilapia populations, however, the treatment requires several weeks of exposure or a longer treatment period. Moreover, the use of large quantities of sex reversal hormone in the hatchery may pose a health risk to workers during prolonged exposure (Mair, 1997).

Only a few studies are available on masculinization of tilapia by egg immersion in hormone solution of MT although many studies exist that are focused on fry immersion (Varadaraj and Pandian, 1989; Abd El-Aziz, 2000; Gale *et al*., 1999). Experiments conducted on sex reversal of *O. niloticus* by egg immersion technique showed that eggs immersed at 800µg L^{-1} for 96h obtained 91% male population (Cagauan *et al.,* 2004). The use of exogenous estradiol valerate applied by both immersion method and oral administration is found to be effective for altering sex ratios, producing monosex females of rainbow trout (Gullu *et al*., 2005).

Testosterone produced during fetal/neonatal life plays a role in programming the development of a "male" hypothalamus and brain. The administration of testosterone to a female during this critical programming period will result in masculinization of hypothalamic function and consequent acyclicity and anovulation in adulthood (Dohler, 1991; Gorski, 1996). The relative role of testosterone, dihydrotestosterone and estradiol varies according to behavior in a species-specific manner for both organizational and activational influences. In some species, all three hormones play a role in masculinization (Cooke *et al*., 1998). The genotypic sex can be modified by exogenous sex steroids at early developmental stages long before gonadal differentiation (Iwamatsu *et al.,* 2006). The estradiol-17 beta content of fertilized eggs increased when eggs were incubated in medium containing exogenous testosterone at the concentrations of 100 and 500ng ml^{-1}, but not in low concentrations of 10ng ml^{-1} or less.

The central role of testosterone in facilitating masculinization has two important downsides. First, if a genotypic male fails to produce testosterone it will not masculinize and will develop as a phenotypic female but with testes. Conversely, if a genotypic female is exposed to sufficient testosterone (or other androgens) she will be masculinized but will have ovaries (Simpson and Rebar, 1995). In other words, partial masculinization of the female or partial failure of masculinization of the male can occur. Complete or partial sex reversals of the gonads can be caused by early exposure of eggs, larvae, or juvenile animals to estrogens or androgens (Hayes, 1998a, 1998b). In addition, androgens usually inhibit female duct (Müllerian) development while enhancing male duct (Wolffian) development. The androgenic nature of methytestosterone was clearly demonstrated by masculinization of exposed females (Hayes, 1998a; 1998b). Vitellogenin induction was observed in both sexes, probably a result of aromatization of the administered androgen. The 5 alpha-reductase is the enzyme responsible for the conversion of testosterone to 5 alpha-dihydrotestosterone (DHT), a more potent androgen receptor agonist that acts specifically to masculinize the external genitalia of the male.

Sex reversal by egg immersion may lessen the duration of treatment and lowers the cost of hormone used. This alternative technique of administering the sex- manipulating hormone provides more efficient management of tilapia hatcheries employing artificial incubation because of higher male production and lower risk to health of workers from constant exposure to the chemical. This study is aimed to determine the effect of varied hormone concentrations using MT at different immersion times on the egg hatchability rate, fry survival rate, growth, and sex ratio of *O. niloticus* by egg immersion technique. The study also aims to evaluate the economic viability of this protocol for the refinement of the GET Excel tilapia hatchery operations.

MATERIALS AND METHODS

Experimental Organism

The Genetically Improved Farmed Tilapia (GIFT) strain of the Nile Tilapia, *Oreochromis niloticus*, was developed in the Philippines under the coordination of ICLARM from 1987 to 1997. Through further selective breeding, this strain was developed into GET 2000 (Genetically Enhance Tilapia) and later GET EXCEL 2002 or Genetically Enhanced Tilapia with Excellent qualities. The GET Excel tilapia grows 10 percent faster and 38 percent larger than the conventional tilapia (dela Cruz, 2003).

GET Excel fingerlings of *O. niloticus* were procured from BFAR and were domesticated into breeders at the MSU-Naawan tilapia hatchery complex in Naawan, Misamis Oriental. The breeders were domesticated in broodstock tanks and paired at a sex ratio of 1 male: 3 females m^{-3}, fed with high protein diet four times a day at 3-5% average body weight (ABW) until the eggs were spawned.

Experimental Design

A 2 x 4 factorial experiment was used to determine the efficacy of egg immersion at different hormone concentrations (HC) and immersion times (IT) for male production of tilapia within five months of rearing period. The eight treatment combinations had three replicates each arranged in a completely randomized design (Table 1). Twenty four units of 1.5L plastic container filled with filtered fresh water were used for the treatment combinations during the immersion period.

Table 1. The experimental treatment combinations of two variables (HC & IT) in a 2x4 factorial experiment.

Treatment	Dose of methyltestosterone ($\mu g\ L^{-1}$)	Immersion Time (hours)
1	500	24
2	500	48
3	500	72
4	500	96
5	1000	24
6	1000	48
7	1000	72
8	1000	96

A stock solution of 1.0g L^{-1} ethanol was prepared and used to dissolve the methyltestosterone before application. One milliliter of the stock solution was applied per liter of water of the incubating units for 24, 48, 72 and 96h IT.

Egg Collection, Immersion and Incubation

Eggs were collected from the mouth of the brooding female tilapia early in the morning (06.00-07.00h) following the established procedure for egg collection. Eggs were pooled and stocked in experimental 1.5L capacity plastic containers using a calibrated scoop at an average stocking density of 200 eggs per container. Pooled eggs were immersed in 17α-methyltestosterone (MT) inside different plastic containers with hormone concentrations of 500 and 1000µg L^{-1} for different immersion times of 24, 48, 72 and 96h. After the designated immersion time, treated eggs were transferred to 27 units of 10L capacity plastic jars for a continuous incubation until hatching (after about 3-5 days). All eggs were incubated in clean fresh water provided with sufficient aeration to facilitate continuous movement of eggs in the water column. Favorable temperatures of 28 to 30ºC were maintained for optimum hatching of eggs.

Rearing, Water Management and Feeding Protocols

Water change started after the designated immersion periods prior to oral administration of commercial diets. Daily water management was done with 100% water change using pre-filtered fresh water during incubation period to maintain good water quality. Once the yolk sac was absorbed the fry were adapted to exogenous feeding and were given commercial feeds four times a day at a feeding rate of 25-30% ABW. The fry were reared for at least 20 days, after which they were transferred to a 1x1x1 m net enclosure. The fry were reared for four months with regular water management and fed at 10% BW until they were large enough for manual sex determination.

Data Management and Analysis

Data on egg hatching rate, fry growth, survival rate and sex ratio were evaluated. The time of development of the fry from the period of immersion up to oral administration of commercial diet was closely monitored. Growth, survival and health condition of the fish were determined every month throughout the experimental period. Manual sex determination of tilapia was made after four months of culture when sexual differentiation was already possible.

Statistical analysis of data was conducted using the linear general model in the Statistical Package for Social Sciences (SPSS) software. Analysis of variance in 2 x 4 factorial with three replicates was used to compare the hatchability, growth, survival and male production of *O. niloticus* exposed to different HC and IT. Comparison of means was done using Duncan's Multiple Range Test. Percentage data (i.e. hatching rate, survival rate, growth rate and masculinization rate) were arc-sine-transformed before testing for significant differences.

Economic Evaluation

Cost and return analysis on the egg immersion technique in all-male production of *O. niloticus* fry (from egg production to introduction of commercial diet) was conducted for the different treatments. Production cost, net profits and return on investment (ROI) were determined. Production cost includes breeders, feeds, hormones, chemicals, electricity and manpower.

RESULTS

Hatching Rate

The interaction between hormone concentration (HC) and immersion time (IT) had no significant effects on the hatching rate of eggs (p>0.05 ANOVA). Hatching rate of the eggs was equally high in the 1000µg L^{-1} (85.33%) and 500µg L^{-1} (85.17 %) at 24h IT (p > 0.05). Hatching rate, on the other hand, was lower at 96h IT in 500µg L^{-1} and 1000µg L^{-1} HC at 65% and 63%, respectively (Table 2).

Table 2. Hatching percentage of *O. niloticus* eggs at different hormone concentrations and immersion times.

HC	Hatching (%) at different IT (hrs)			
(µg L^{-1})	24	48	72	96
500	67.50[a]	61.61[a]	54.35[b]	53.56[b]
	(3.00)	(0.72)	(1.44)	(1.65)
1000	67.66[a]	60.67[a]	52.24[b]	55.55[b]
	(2.33)	(3.37)	(2.25)	(3.81)

note: Mean with similar superscript are not significantly different at p>0.05. Values in parentheses are standard error of the mean.

Immersion time has significant effect on the hatchability of eggs (p<0.05) where the highest hatching rate generally occurred at 24h IT at 85.33% and 85.17% in 1000µg L^{-1} HC and 500µg L^{-1} HC, respectively. Low hatching rates were observed at 96h IT and in 500µg L^{-1} (64.67%) and 1000µg L^{-1} HC, (62.50%). Results showed a decreasing trend in hatching percentage as IT increased at higher HC.

Growth Performance

Statistical test showed no significant difference on the interaction effect of HC and IT on the growth performance of *O. niloticus* in the net enclosure. Relatively higher average growth rates (30-39%) were obtained on tilapia exposed to 1000µg L^{-1} HC across all IT, while lower growth rates (28-32%) were obtained from 500µg L^{-1} HC,

although these values did not differ significantly (p>0.0, Fig. 1). Results showed that immersion time had no significant influence on the growth of *O. niloticus* exposed to varying hormone concentrations. Mean growth rates of tilapia as a function of immersion time ranged between 29.86- 35.21%.

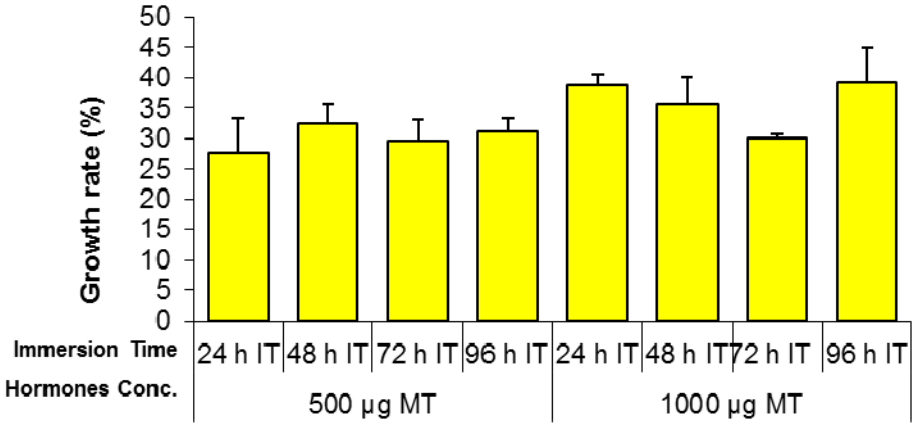

Figure 1. Mean growth rate of *O. niloticus* as a function of hormone concentration across all immersion times.

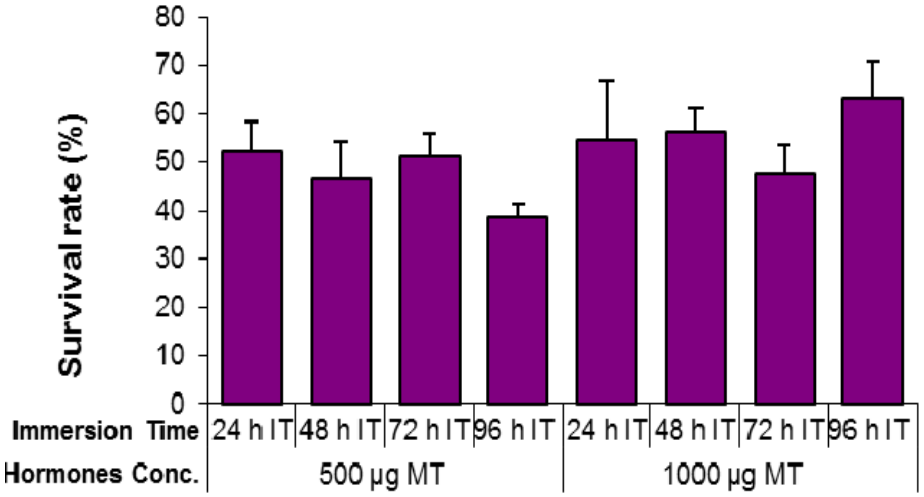

Figure 2. Mean survival of *Oreochromis niloticus* as a function of hormone concentration across different immersion times.

Survival

Average survival of post-treatment tilapia juveniles exposed to 500µg L^{-1} HC at various IT range from 39-52% while juveniles exposed to 1000µg L^{-1} HC had survival rates between 47-63% across various IT. Differences in survival rate (Fig. 2), however, are not significant ($P>0.05$), suggesting that the different HC have no significant effect on the mean survival rate of tilapia fry and juveniles.

The mean survival rates as a function of IT ranged from 29.86-35.21%, and are not significantly different from each other ($P>0.05$). This result suggests that there is no significant interaction between HC and IT in the survival rates of O. *niloticus*.

Male Tilapia Production

A significant increase in the production of male population of tilapia was observed with increasing HC (Table 3, Fig. 3). Mean reversal rate as a function of HC was significantly higher at 1000µg L^{-1} HC (98-99%) across all IT ($P< 0.05$). Production of male *O. niloticus* at 500µg L^{-1} HC obtained a lower average percent male production (89-90%). The main interaction effect of HC and IT in monosex production of *O. niloticus* by egg immersion technique was not significantly different from each other.

Table 3. Percent male production of *O. niloticus* by egg immersion across HC and IT. Mean with similar superscript are not significantly different at $P>0.05$. Values in parenthesis are standard error of the mean.

Hormone Concentration	Immersion Time (hrs)			
(µg l^{-1})	24	48	72	96
500	90.36^{b}	89.01^{b}	89.57^{b}	89.93^{b}
	(1.69)	(1.17)	(2.06)	(3.65)
1000	98.44^{a}	98.57^{a}	97.99^{a}	97.87^{a}
	(0.88)	(1.43)	(1.03)	(0.45)

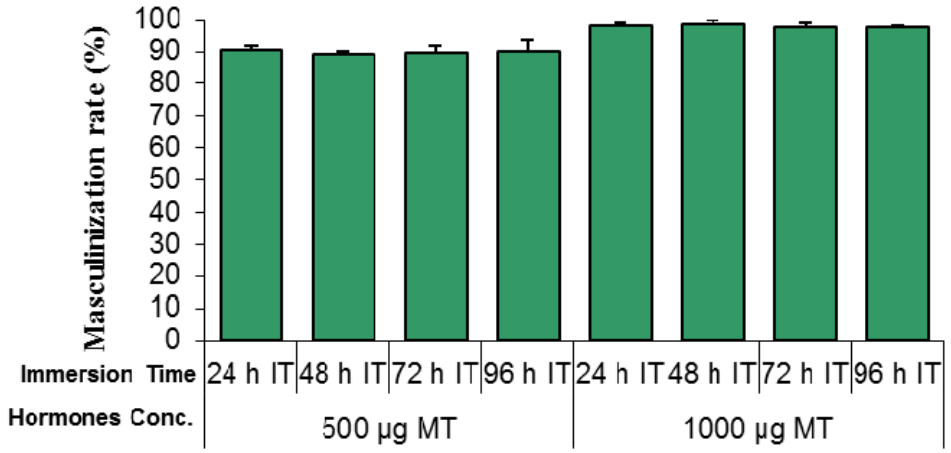

Figure 3. Male production of *Oreochromis niloticus* as influenced by Hormone concentrations across all Immersion Treatments (IT).

The main effect of IT on the production of male populations across all hormone concentrations was not significantly different among treatments. A 100% male production rate obtained from eggs immersed in 1000 µg L^{-1} HC at 48h IT was also observed at 96h IT. Monosex production rates of 90% were observed in 24, 72 and 96h IT and the lowest rate of male production was observed in 48h IT at 500µg L^{-1} HC.

Cost and Return Analysis

The use of 17α-methyltestosterone in egg immersion technique resulted in high production of male tilapia fry ranging from 275-379 pieces with a monetary value of PhP165-227.00. Highest returns on investment (ROI) were obtained in 500µg L^{-1} (55.32%) and 1000µg L^{-1} HC (55.25%) at 24h IT. Low ROI of 32.42% and 28.50% obtained in 500µg L^{-1} HC and 1000µg L^{-1} HC at 96h immersion time can be attributed to low hatching rate observed in these treatments.

DISCUSSION

Results of this study showed the efficacy of egg immersion protocol on the hatchability, growth, survival and sex development of GET Excel strain of *O. niloticus*. Although the main effect of IT on the hatching percentages had significant variations in the different treatments, no trend is apparent on the effect of HC on hatching rates. As IT increased the hatching rate decreased, indicating that the tolerance level of the eggs may have been affected by the longer IT. The work of Cagauan *et al*. (2004) demonstrated that during embryonic development, the eggs can be affected by the administration of methyltestosterone in the holding water especially when they are exposed at longer periods of time without water change. It was observed that during embryonic development the water quality deteriorated, becoming turbid, if not changed within one day. This is because during embryonic development, large quantities of ammonia and carbon dioxide may be produced (Coche and Edwards, 1989). These metabolites need to be flushed out by maintaining a continuous flow of water or frequent water change because this may adversely affect the hatchability of tilapia eggs.

The hatchability of the eggs in this study was lower compared to that obtained in the study of Cagauan *et al.* (2004) who had administered HC of 200 to 800µg L^{-1}, lower than the concentration used in this study. This low hatchability may be attributed to some factors such as dosage of hormone, age of the breeders and water quality. Typically breeders should be replaced every 12 months during continuous breeding. In the hatchery, the ideal age of the breeders undergoing continuous breeding and spawning must not exceed 24 months. Breeders in MSU Naawan used in this study were already 4 years old.

The 17α-methyltestosterone is a potent inducer of male tilapia production. Based on the results, sex development of *O. niloticus* GET Excel strain leading to the production of monosex or nearly monosex tilapia was possible through egg immersion protocol. The rate of sex reversal tended to increase as HC increased. The production of monosex tilapia during prolonged exposure to 17α-methyltestosterone treatment may have been triggered by the specific effects of this exogenous androgen. According to Rang and Dale (1994) this anabolic hormone is rapidly absorbed and metabolized resulting in increased protein synthesis, enhancement of muscle development, and leads to higher growth promoting properties.

Higher rates of male tilapia production, on the other hand, were obtained from higher HC used in this study than were reported by Cagauan *et al.* (2004) and comparable to the average masculinization rate obtained from employing the traditional sex reversal technique by oral administration of hormone-treated feed. High monosex production of male tilapia fry may be attributed to the use of higher concentration of methyltestosterone during immersion and incubation protocol. According to Iwamatsu *et al*. (2006), the estradiol (E2) content of fertilized eggs increased when eggs were incubated in medium containing exogenous testosterone at

the concentrations of 100 and 500ng ml^{-1}, but not in low concentrations of 10 ng ml^{-1} or less. The presence of 500ng ml^{-1} MT in the incubation medium also induced an increase in the E2 content of embryos. Masculinization of *O. niloticus* by egg immersion lessens the duration of the treatment and lowers the cost of hormone used relative to the traditional technique of oral administration, and reduces the health risks on the aquaculture workers from prolonged exposure to the hormones. This technique will reduce the treatment period while increasing the frequency of producing fry to marketable size.

This study has demonstrated that HC and IT had little effect on the general health condition of the fish. Although small erosions of the caudal fin were commonly observed in some treatments, the fish exhibited normal ocular, tail, fin and defense reflexes as well as feeding and escape reflex. The economic analysis had shown that shorter duration of immersion resulted in a high return on investment. Moreover, egg immersion in hormone rather than regular oral administration through feeding poses less exposure to health risks on aquaculture workers. Results of this study have significant implications on aquaculture development in producing high quality and quantity of male tilapia fry through a sustainable operation.

CONCLUSION AND RECOMMENDATIONS

The production of a high quality tilapia in grow-out culture systems relies on the sustained high production of good quality, fast-growing male tilapia fry produced under proper husbandry practices in the hatchery operation. Production of monosex tilapia by egg immersion technique using 17α-methyltestosterone can result in mass production of functional male populations. This would improve and refine the hatchery production of fry by shortening the treatment period for sex reversal and the days of culture to marketable size.

This technique is believed to increase transport of hormones from water into fish to result in a more consistent and higher rate of male production of tilapia fry, thus, refining and improving the current hatchery techniques of tilapia using oral administration. The results clearly indicate that this technique has the potential to replace the currently practiced technique of manually feeding methyltestosterone for producing male fry of tilapia with higher return on investment.

Since the use of egg immersion technique has been proven to be viable in the production of male tilapia fry, a further study is recommended on the efficacy of immersion protocol on the sac fry of *O. niloticus* to explore the possibility of further increasing production of male tilapia fry. Improving management practices during immersion and incubation, including the optimum stocking density of eggs, and efficient water quality management, should be considered in the refinement of this technique to achieve higher rates of sex reversal.

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