Alexandria University
Faculty of Agric. (Saba-Basha)

MANIPULATION OF BIOTECHNOLOGY FOR PRODUCTION OF GENETICALLY MODIFIED NILE TILAPIA (*Oreochromis niloticus*)

BY

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CHAPTER 1
INTRODUCTION

Success in aquaculture depends on six factors; 1) complete control of the reproductive cycle of the fish species in culture; 2) excellent genetic background of the broodstock; 3) efficient prevention and detection of disease infection; 4) thorough understanding of the optimal physiological, environmental and nutritional conditions for growth and development; 5) sufficient supply of excellent quality water; and 6) application of innovative management techniques (Thomas *et al*., 1996). By improving these factors, aquaculture industry has developed to remarkable extent during the last decade. To sustain this growth, however, newly developed technologies in molecular biology and transgenesis will have to be increasingly applied by the aquaculture industry. These technologies can be employed to enhance growth rates, control reproductive cycles, improve feed compositions, produce new vaccines and develop disease and cold resistant genetic stocks (Thomas *et al.,* 1996).

Tilapias belong to the family Cichlidae which is widely distributed in Africa, the Middle East, South and Central America, Southern India and Sri Lanka. It contains approximately 1300 species, of which approximately 150 can be called tilapia. Tilapia is perciform fish that originated in Africa and the Jordan valley. They have adapted to diverse habitats: permanent and temporary rivers, rivers with rapids, large equatorial lakes, tropical and subtropical rivers, open and closed estuaries, lagoons, swampy lakes, deep lakes and coastal brackish lakes (Trewavas, 1982).

The most commercially important tilapia genera: *Oreochromis*, *Tilapia* and *Sarotherodon.* The genus *Oreochromis* is the largest, with approximately 79 species, followed by Tilapia with approximately 41 species and the genus *Sarotherodon* with approximately 10 species.

The rapid growth of tilapia, their resistance to poor environment quality, ability to grow under sub optimal nutritional condition, and high fecundity all make them suited for aquaculture (Robert, 2003; Amal *et al.,* 2005).

The culture of tilapia is not new but its mostly conducted in freshwater and brakishwater. Fresh water are very scarce and expensive resources in many areas of the worlds, on other hand seawater is abundant and can easily be utilized for culturing fish (Cruz *et al*., 1990), and the efficient use of marine and brackish water for aquaculture becomes a vital alternative and the production of tilapia in salinity water is important in coastal areas and regions with limited supplies of freshwater.

Development of tilapia hybrids has been pursued to incorporate desirable traits into hybrids and hybrid- based varieties. The development of hybrid- based tilapia varieties is an important step toward increasing production and marketability of tilapia (Lutz, 2001). By combing the beneficial traits found in several tilapia species into hybrids, scientists and producers create fish that grow faster and are less expensive to produce.

These efforts have had varying degrees of success (Rakocy *et al*., 1993). Some hybrid–based varieties may exhibit traits beneficial to commercial – scale farming such as cold or salinity tolerance (Robert, 2003). And the progeny highly tolerant to saline condition might be produced through hybridization and other genetic manipulation techniques.

Hybrid of *O. niloticus* ♂ x *O. mossambicus* ♀ reflected better growth and feed conversion than either parenter species (Avault and Shell, 1967). Hybrid between *O. niloticus* and *O. mossambicus* were reported to grow faster in brackish water and sea water than freshwater (Liao and Chang, 1983). AL-Amoudi (1987) studied the salinity tolerance of (*O. aureus*, *O. spilurus, O. niloticus, O. mossambicus* and the hybrid between (*O. aureus* ♂ and *O. niloticus* ♀), under direct transfer and gradual acclimatization and concluded that pre- acclimatization at low salinity and gradual transfer to higher salinities, the results showed a better survival in all species.

Since 1985, a wide range of transgenic fish species have been produced by several techniques for introducing foreign DNA into fish species by Zhu *et al*. (1985 and 1986) in gold fish (*Carassius auratus*) and loach (*Misgurnus anguillicaudatus*) alternatively, Chourrout *et al*. (1986) and Maclean *et al*. (1987) in rainbow trout (*Salmo gairdneri* Rich), Brem *et al*. (1988) and Rahman and Maclean (1992 a) in Tilapia (*Oreochromis niloticus*), Inoue *et al*. (1990) in medaka (*Oryzias latipes*) and Muller *et al*. (1992) in common carp (*Cyprinus carpio*), African catfish (*Clarias gariepinus*) and Tilapia (*O. niloticus*) .

Transgenic fish is one of the attempts to increase the production of economically important fish for human consumption. The application of gene transfer in fish production including, increase in growth rate, tolerance of cold water and disease resistance.

Guillen *et al*. (1999) reported that modern marine biotechnology have permitted the generation of new strains of economically important fish species through the transfer of growth hormone genes. These transgenic fish strains show improved growth performance and therefore constitute a better alternative for aquaculture programs. Recently, they have obtained a transgenic tilapia line with accelerated growth. Experiments were performed to evaluate the behavior of transgenic tilapia in comparison with wild tilapia as a way to assess the environmental impact of introducing transgenic tilapia into Cuban aquaculture. Studies were also conducted to evaluate, according to the principle of substantial equivalence, the safety of consuming transgenic tilapia that had a lower feeding motivation and dominance status than control. Food safety assessment indicated that tilapia growth hormone has no biological activity when administered to nonhuman promates. Furthermore, no effects were detected in human healthy volunteers after the consumption of transgenic tilapia. These results showed, at least under the condition found in Cuba, no environmental implications for the introduction of this transgenic tilapia as an alternative feeding source for humans. These results support the culture and consumption of this transgenic tilapia.

Therefore, the present work aimed to evaluate the performance of Nile tilapia (*Oreochromis niloticus*), through a comparative study between interspecific cross-breeding with *Oreochromis aureus* and the injection of fragmented purified DNA [isolated from common carp, (*Cyprinus carpio*) and Blue tilapia, (*O. aureus*)] into the gonads of *O. niloticus* (males and females). In addition, the effect of two breeding methods on growth performance, body composition, feed utilization and DNA fingerprinting of *O. niloticus* were carried out.

CHAPTER 3
MATERIAL AND METHODS

The experimental work was carried out in laboratory of Breeding and Production of Fish, Animal and Fish Production Department, Fish Farm of Faculty of Agriculture (Saba-Basha), Alexandria University and Nucleic Acid Research Department, Genetic Engineering and Biotechnology Research Institute (GEBRI) Mubarak City for Scientific Research and Technological Applications, Alexandria, Egypt.

The present work aimed to study the production and genetic differentiation between genetically modified *Oreochromis niloticus* though introduction of foreign DNA isolated from *Cyprinus carpio* and *Oreochromis aureus* into *Oreochromis niloticus* gonads (males and females), and *Oreochromis niloticus* hybrid produced through interspecific cross breeding with *Oreochromis aureus*.

3.1- Fish origin

The Nile tilapia, *Oreochromis niloticus* and Blue tilapia, *Oreochromis aureus* used in this study descended from a randomly mating population at the Middle East Fish farm, Tolombat Halk El-Gamal, El-Behera Governorate, Egypt.

3.2- Experimental design:

3.2.1- Culture condition

Adult females and males of *O. niloticus* with an average weight (67.15 and 83.40. g) and *O. aureus* with an average weight (63.00 and 81.03 g), respectively were chosen and stocked into concrete ponds (3x1x1.2m) at a rate of 4 breeders/m^3 (Figure 1) .The sex ratio of the fish was 3 females: 1 male.

Readiness of females to spawn was ascertained by examining, the degree of swelling of the urogenital papille (Hussain *et al.,* 1991). Also, males were examined by the stripped out of the male sperm (Wester and Foote, 1972). Fish were fed Twice daily on pelleted diets contained 26% protein at satiation for 6 days a week (Table 1).

3.2.2- Preparation of genomic DNA:

High molecular weight DNA of *O. aureus* and *C. carpio* was extracted according to Baradakci and Skibinski (1994) method. Isolation of DNA was accomplished by reducing liver sample from *O. aureus* and *Cyprinus carpio* to small pieces. Each sample was suspended in 500 µl STE (0.1 M NaCl, 0.05 M Tris and 0.01 M EDTA, pH 8). After adding 30 µl SDS (10%) and 30 µl proteinase K (10 mg ml), the mixture was incubated at 50°C for 30 min. DNA was purified by successive extraction with phenol, phenol: chloroform: isoamyl alcohol (25:24:1) and chloroform : isoamyl alcohol (24:1), respectively. DNA was precipitated with ice-cold absolute ethanol and washed with 70% ethanol. The pellet was dried and re-suspended in 200 ul mill Q water. The concentrations of DNA were measured at 260 nm using UV-spectrophotometer.

Figure (1): Concrete ponds used for broodstocks spawning.

Table (1): Formulation and proximate analysis of the diets used in the present study.

Ingredient (%)	Diets		
	Broodstocks[1]	Fry[2]	Fingerlings and adult[3]
Wheat flour	27.0	10.0	15.0
Wheat bran	17.0	7.0	14.0
Soybean meal	26.0	48.0	37.0
Yellow corn	14.7	8.7	13.2
Fish meal	13.0	24.0	18.5
Bone meal	2.0	2.0	2.0
Vit. & min. mix[4]	0.3	0.3	0.3
Total	100	100	100
Proximate analysis (%)			
Dry matter	90.2	89.97	90.31
Crude protein	25.61	38.04	31.99
Crude fat	7.41	7.49	7.35
Crude fiber	3.75	3.40	4.06
Carbohydrates	56.01	41.58	48.42
Ash	7.00	9.51	8.19

1. Broodstocks which were used as a parents.
2. Fry fed on this diet until they reached three months of age.
3. Fingerlings and adult fish were fed on this diet from three months to end of experiment.
4. Content/kg of Vitamin & minerals mixture (P-Fizer, Cairo, Egypt). Vitamin A, 4.8 MID; Vitamin D, 0.8 MIU; Vitamin E, 4.0 g; Vitamin K, 0.8 g; Vitamin B_1, 0.4 g; Vitamin B_2, 1.6 g; Vitamin B_6, 0.6 g; Vitamin B_7, 20.0 mg; Vitamin B_{12}, 4.0 g; Folic acid, 0.4 g; Nicotinic acid, 8.0 g; Pantothenic acid, 4.0 g; Colin chloride, 200 g; Zinc, 22 g; Cupper, 4.0 g; Iodine, 0.4 g; Iron, 12.0 g; Manganese, 22,0 g; Selenium, 0.04 g

Estimation of DNA concentrations were carried out from the O.D. readings at wave length 260 nm where 1 O.D. equals to 50 µg DNA/ml (Charles, 1970). DNA purity was determined as described by Charles (1970), using O.D. ratios, 260/280 nm (2.0-2.2) and 260/230 nm (1.8-2.0). These values indicate that DNA solution is well purified. The extracted DNA was restricted by Eco Rl restriction enzyme type II. It digested DNA between guanine and adenine according to Tsai *et al.* (1993). Then, the concentrations of 10 µg/0.1 ml/fish were adjusted by extrapolating the dilutions for each type of DNA extracted using 0.1 x SSC buffer. This technique is concerned with the utilization of the whole gene, introns and exons, and not only exons through mRNA and reverse transcriptase treatments (Ali, 2001). Hereby, there is no need to initialize any kind of virus

as total DNA facilitates the introduction of foreign genes into cells with the aid of introns which act as retrotransposons (Hickey and Benkel, 1986).

3.2.3- Injection of genomic DNA into fish gonads

Adults of Nile Tilapia, *O. niloticus* with an average weight (67.15 g/female and 83.40 g/male) were chosen. Two males and four females were injected directly with the foreign DNA isolated from *O. aureus* and *Cyprinus carpio* into fish gonads using a hypodermic needle. To inoculate the adult fish, the needle was inserted into the openings of oviduct and spermduct (El-Zaeem, 2001; Lu *etl al.* 2002). Immediately, after DNA treatments were carried out, each group of injected fish was stocked separately in concrete ponds (3x1x1.2 m) at a rate of 4 breeders /m3 for natural spawning.

3.2.4- Pure line mating and interspecific hybridization

Interspecific hybridisation were carried out as follows:
*Female *O. niloticus* (N) x male *O. niloticus* (N) (N)
*Female *O. aureus* (A) x male *O. aureus* (A) (A)
*Female *O. aureus* (A) x male *O. niloticus* (N) (AN)
*Female *O. niloticus* (N) x male *O. aureus* (A) (NA)
Each hybrid and pure line was spawned in a separate pond by natural mating.

3.2.5. Fry, Fingerlings and adult fish rearing

Base generation (F_0) offspring were produced by about 2-3 weeks after being stocked to spawn. Post-hatching fry produced from each hybrid and pure line and each treatment of DNA were collected and weighed. Then, fry were transferred separately to glass aquaria (With dimensions 100x34x50 cm) at a rate of 1 fish /10 liter (Figure 2). The glass aquaria were provided with a continuous supply of de-chlorinated water and adequate aeration system, cleaned once daily by siphoning, then one-half to two-third of their water volume was replaced. All water was completely changed once every two weeks during fish sampling. Fry were fed three times daily on pelleted diet containing 38% protein to satiation for 90 days. Then, the fish fed to satiation on pelleted diet containing 32% protein to the end of experiment. Fish were weighed bi-weekly for (180) days.

3.3. Studied traits

3.3.1. Growth performance

Fry, fingerlings and adult fish were taken bi-weekly for growth measurements and feed adjustment for each treatment. Body weight (g), total gain, average daily gain (ADG**)**, specific growth rate (SGR %/day), and condition factor were calculated. Growth measurements were determined as follows:

* Total gain = Wt- Wo (Broody, 1945**)**
*ADG = Wt - Wo/n (Broody, 1945**)**
*SGR %/day = (Log_e Wt - Log_e Wo) 100/n **(**Castell and Tiews, 1980**)**

Since [n: number of days; Wo: initial weight at the beginning; Wt: final weight at the end of period].