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Production of a high percentage of male offspring in growth-enhanced transgenic tilapia using *Oreochromis aureus ZZ* selected pseudofemales

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Abstract

Gene transfer has offered a new tool for the development of improved fish strains for aquaculture. Monosex fish populations could minimize the impact of genetically modified organisms in the environment. In *Oreochromis aureus*, the use of pseudofemale spawners (sex-reversed male with a female phenotype) is an alternative technique for producing genetically male tilapia offspring. *O. aureus* fry were treated with 17β-estradiol at 100 mg/kg of food for 45 days. We obtained 77.1% females and 45.9% in the control group. Females randomly taken from the treated group were crossed with normal males. Fry from pseudofemales producing more than 90% male progenies were submitted to 17 β-estradiol treatment to obtain F_2 pseudofemales. The results of the sex-reversal were low and variable ranging between 66.0 and 84.3% females. F_2 pseudofemales were crossed with transgenic males from the F70 line (*O. aureus* × *O. urolepis hornorum*) and non-transgenic (*O. aureus*) males. The sex ratio of progeny of F_2 pseudofemale deviated significantly ($P<0.01$) in favor of males in the crosses with transgenic (90.2%) and non-transgenic (89.3%) males compared to the results observed with normal females (51.0 and 52.3%, respectively). The mean fry production with pseudofemales (per m^2/day) was similar to the normal females in the crosses with transgenic and non-transgenic males. To our knowledge this is the first report on the production of a near monosex population in genetically modified fish.

Keywords: *Oreochromis aureus*; Growth hormone; Monosex; Tilapia; Sex reversal; Transgenic

1. Introduction

Although exogenous application of growth hormone (GH) results in a significant growth enhancement in fish (Agellon et al., 1988) it may not be cost effective. If new strains of fish producing optimal levels of ectopic GH can be produced, it would bypass many of the problems associated with exogenous GH treatments. Moreover, once these improved fish strains have been generated, their culture would be more cost effective than the culture of their ordinary counterparts (Martínez et al., 1999).

We have reported a new growth-enhanced line of tilapia (*Oreochromis urolepis hornorum*), which was

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generated from a construct containing human CMV 5' regulatory sequence linked to complementary tilapia growth hormone gene in a single copy (Martínez et al., 1996). Martínez et al. (1999) reported the generation of homozygotic transgenic broodstock and the Mendelian transmission of the transgene when crossing the transgenic animals with *O. aureus*, the new line was named F70.

The first genetically engineered animal commercially marketed for the recreational, hobby aquarium market in the USA and several other countries, was the GloFish, a transgenic fish that 'glows' due to the skeletal muscle expression of a fluorescent protein genetic construct (FDA, 2003). Confinement of genetically engineered fish and shellfish can be accomplished physically (e.g. by screens and other mechanical barriers to prevent escape from rearing tanks and ponds), physico-chemically (e.g. by lethal water temperatures or chemicals applied to water in existing fish tanks), or biologically (e.g. by rendering the organism incapable of reproducing or of surviving outside of the aquaculture system). It is unlikely that 100% confinement would be achieved by a single method (Kapuscinski, 2005).

The production of male monosex tilapia (*Oreochromis* species) populations with 17 α-methyltestosterone treatment is well established (Rothbard et al., 1983; Guerrero and Guerrero, 1988). This technique gives good results both at experimental and commercial stages but requires the utilization of steroids (Melard, 1995). In some countries, local legislation prohibits the use of synthetic androgens for fish production purposes (Desprez et al., 1995). Melard (1995) succeed in producing a stock of F_2 pseudofemale *Oreochromis aureus* (female phenotype, male genotype ZZ), using sex-reversed (17 α-ethynylestradiol treatment) offspring of selected F_1 pseudofemales that gave 100% male progeny.

The aim of this study was to obtain an *O. aureus* pseudofemale stock to achieve an all-male offspring of F70 transgenic tilapia as a result of the breeding between F_2 pseudofemales and males from homozygotic F70.

2. Materials and methods

2.1. Origin of fish

O. aureus (200–400 g) strain was supplied by the fish farm Alevi–Cuba, in Camagüey, Cuba. Homozygous (two transgene copies/cell) males from the growth-enhanced transgenic tilapia line F70 (*O. aureus* × *O. u. hornorum*) were supplied by the Center for Genetic Engineering and Biotechnology in Havana (Martínez et al., 1999).

2.2. Fry production

The *O. aureus* spawners (three females and one male) were maintained in spawning tanks of 2 m^3 under a photoperiod of 12 h light: 12 h dark. Eggs were collected every week from the mouths of females and incubated at 28 ± 1 °C in 17 mmol/l NaCl, 0.27 mmol/l $CaCl_2.2H_2O$, 0.41 mmol/l KCl, 1.33 mmol/l $MgSO_4$, 0.0001% [weight/volume] methylene blue, pH 8, until the larvae had reabsorbed their yolk sac.

2.3. Hormonal treatments

One hundred milligrams of 17β-estradiol (1, 3, 5 [10]-estratriene-3, 17 β-estradiol; Sigma E-8875, USA) was dissolved in 0.8-l of 95% ethanol. The solution was sprayed over 1 kg of food (Economac-2, Aquafauna Bio-Marine, Inc., USA), while stirring thoroughly. The food for the control group was prepared in the same manner but without hormone. After alcohol evaporation, the food was stored at 4 °C.

A batch of 100 fry resulting from the cross between normal *O. aureus* was submitted to a treatment with 17β-estradiol incorporated in the food during 45 days. The fry were reared in small 60-l glass aquaria at 28 ± 1 °C using the steroid-enriched feeds after the total yolk sac re-absorption.

After 90 days, the animals were sexed by genital examination and the sex ratio was compared using a 2 × 2 contingency χ^2 test. The females were reared up to sexual maturity (200–250 g; age 6 months) in 10 m^3 fiberglass tanks at 28 ± 1 °C. The sex was re-checked every month and males were eliminated.

2.4. Offspring testing

Twenty females were randomly taken from the treated group and placed (10 females to three males) in a spawning tank of 10 m^3 at 28 ± 1°C. The females were individually tagged with an Avid microchip (I.D. Systems, USA). The eggs were collected every week from the mouths of females and incubated in the same conditions as explained in Section 2.2. After the fry had re-absorbed their yolk sac, they were reared for two months in a small labelled 60-l glass aquarium at 28 ± 1 °C. A minimum of 140 fish from each progeny were sexed using the aceto-carmine squash method (Guerrero and Shelton, 1974). The pseudofemales (female with a male ZZ genotype) were identified when the sex ratio of their offspring was significantly different (2 × 2 contingency χ^2 test) from an expected sex ratio of 1:1. Only pseudofemales giving over 90% male offspring were selected to produce F_2 pseudofemales.