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Production of a high percentage of male offspring with a natural androgen, 11β-hydroxyandrostenedione (11βOHA4), in Florida red tilapia

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Abstract

This paper reports the effects of a natural androgen, 11β-hydroxyandrostenedione (11βOHA4), on sex-reversed fry of the Florida red tilapia. In a first approach, the optimal dosage and duration were determined in the laboratory. The sex-reversal treatment was applied on post-yolksac stage fry (10 days after fertilisation at 27 °C). Dosages of 20, 30, 40 and 50 mg of 11βOHA4 kg^{-1} of food were used during periods ranging from 10 to 35 days. Dosages of 40 or 50 mg of 11βOHA4 kg^{-1} of food during 21–35 days significantly increased the male percentage in sex-reversed groups in comparison to control groups. A lower but still significant deviation of the male percentage was observed when a dosage of 40 mg kg^{-1} was given during at least 28 days. Lower dosages did not significantly affect the sex ratio of treated groups. Based on the results of these observations, a follow-up study was conducted to evaluate the efficiency of this natural androgen for an intensive production of sex-reversed fry. Optimal dosage (50 mg kg^{-1}) and treatment duration (28 days) were applied to increasing stocking density of fry (8000–11,000 fish m^{-2}), producing a mean male percentage of 99.1% on the 510,000 treated fry. This study demonstrates the masculinizing efficiency of 11βOHA4 in Florida red tilapia. Interest of a natural

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androgen, used in sex-reversal treatment, is discussed, particularly for the Florida red tilapia strain.

Keywords: Sex reversal; Natural androgen; 11β-hydroxyandrostenedione; Florida red tilapia

1. Introduction

Oreochromis species are characterised by a precocious sexual maturity and a high reproductive efficacy, resulting in overpopulation in ponds. A monosex male population is a solution to control reproductive activity and also to increase production because the male grows faster than the female in these species (Ruwet et al., 1976; Hanson et al., 1983). Sex reversal with synthetic androgens is nowadays one of the most frequently applied techniques to produce monosex male populations in tilapia. This technique gives good results both at experimental and commercial stages (Guerrero, 1975; Berger and Rothbard, 1987; Mc Geachin et al., 1987; Guerrero and Guerrero, 1988; Vera Cruz and Mair, 1994; Mélard et al., 1995). However, little is known about the actual impact of the residuals on the environment. The use of synthetic androgens is also currently prohibited in European countries. The use of a natural hormone such as 11β-hydroxyandrostene-dione (noted down, 11βOHA4) may also alleviate the problems of marketing fish, which have been exposed to synthetic hormone. In gonochoric teleosts, few studies have focused on the endocrine environment and the steroidogenic potential of the gonads during sex differentiation (Van den Hurk et al., 1982; Rothbard et al., 1987; Baroiller, 1988; Baroiller et al., 1988; Feist et al., 1990). In salmonids (Van den Hurk et al., 1982), tilapia (Baroiller, 1988) and African catfish *Clarias gariepinus* (Van den Hurk et al., 1989), the androgen 11βOHA4 has been specially identified as a potential metabolite of androstenedione during testicular differentiation. It has further been shown to have a masculinizing effect in both the rainbow trout *Oncorhynchus mykiss* (Van den Hurk and Van Oordt, 1985; Feist et al., 1995) and the African catfish *C. gariepinus* (Van den Hurk et al., 1989). In salmonids, however, transient masculinization (Van den Hurk and Van Oordt, 1985) and malformations of the genital ducts and/or gonads (Feist et al., 1995) have been reported and immersing the fish in 11βOHA4 or feeding them with it did not result in 100% male population. In tilapia *O. niloticus*, functional male monosex population have been produced with this natural androgen (Baroiller and Toguyeni, 1996).

The aim of this study was to test the masculinizing efficiency of a natural androgen, 11β-hydroxyandrostenedione (11βOHA4), in Florida red tilapia. First, the effect of dosage and treatment duration was evaluated on undifferentiated fry of the Florida red strain female tilapia, reared at low density in the laboratory. Next, a second study was conducted to test the efficiency of this natural androgen for intensive production of sex-reversed fry. Optimal treatment (50 mg kg^{-1} over a period of 28 days) was applied to increasing stocking density of fry (8000–11,000 fish m^{-2}), to test the influence of density on survival and sex reversal. The Florida red tilapia is one of the most commercially important tilapia strains, notably in Latin America, Asia and Caribbean. In Reunion

Island, commercial interest in red tilapia is due to its resemblance to seawater species such as sea bream, *Chrysophis major*.

2. Materials and methods

This study was carried out at the Freshwater Aquaculture Centre in Reunion Island (Indian Ocean). The 11β hydroxy-4-androstene-3, 17 dione (11βOHA4) used in this study came from Sigma and is identified under number A 3009/34H4100.

2.1. Origin of fish

The Florida red tilapia originated from a cross between a red mutant male of *O. mossambicus* and a female *O. urolepis hornorum*. In order to improve its growth rate and its resistance to low temperature, this hybrid has been crossed with *O. niloticus* and *O. aureus* (Behrends and Smitherman, 1984; Galman and Avtalion, 1983). The strain, used in Reunion Island, originally came from Martinique (1990) and Jamaica (1991).

2.2. Definition of optimal sex-reversal treatment (Study 1)

The experimental design for this follow-up experiment is indicated in the first part of Table 1.

2.2.1. Fry production

Florida red tilapia (one male and three or four females) breeders were maintained in a 400-l aquarium thermoregulated at 27 ± 1 °C with recirculating culture system. The reproductive status was checked every day by looking for dilation of the bucco-pharyngeal cavity of females due to mouthbrooding behaviour. Mouthbrooding females were isolated in their respective aquaria. Post-yolksac stage (i.e. free-swimming) fry were collected from the mouth of the female over a period of 10 days after fertilisation.

2.2.2. Hormonal treatment

Progeny was divided into groups of 100–200 fry and each group was reared in a tank (200 l/0.4 m^2) at a temperature of 27 ± 1 °C (thermoregulated recirculating culture system). The sex-reversal treatment was applied on post-yolksac stage fry (10 days after

Table 1
Experimental design for 11βOHA4 sex-reversal treatment of Florida red tilapia fry

Study	Dosage (mg of 11βOHA4 kg^{-1} of food)	Duration (days)	Environment	Density (fry m^{-2})
1	20, 30, 40 or 50	10, 14, 21, 25, 28, 35	Tank (200 l/0.4 m^2) recirculating culture system	250–500
2	50	28	Tank (200 l/1.0 m^2) open-culture system	8000–11,000

fertilisation at 27 °C). To determine the optimal dosage and duration of sex-reversal treatment with 11βOHA4, concentrations of 20, 30, 40 and 50 mg of 11βOHA4 kg^{-1} of food were tested during periods ranging from 10 to 35 days (Table 1). Fry were fed at 30% of their biomass per day for the first week, 15% for the second week, 12% for the third week and 10% for the last week to the sexing. The food was given by an automatic feeder, except for the first week where fry were fed six times daily. A fry sample from each experimental group was weighed weekly for the adjustment of the food ration. Hormonally treated food (commercial salmonids food with 54% protein) was prepared by the alcohol evaporation method (0.6 l of 95% ethanol kg^{-1} of food) at the different concentrations tested. Control feed was prepared in the same way without hormone.

2.2.3. Sexing

Between 60 and 90 days of growth and/or an average weight of > 3 g, the sex ratio in treated and control groups was determined by gonadal microscopic examination (Guerrero and Shelton, 1974). Sexing was carried out on a sample of 52–100 individuals. At this stage of development, oocytes are easily identifiable in their auxocytosis or previtellogenis stages and a typical lobular configuration is observed in the testes (Baroiller, 1988).

2.3. Intensive production of sex-reversed fry (Study 2)

The experimental design for this follow-up experiment is indicated in the second part of Table 1.

2.3.1. Fry production

Males and females of Florida red tilapia were maintained in five spawning tanks (12 m^2/5 m^3) at a density of six individuals per square meter and a sex ratio of one male for three females. Eggs, sacfry and post-yolksac stage (i.e. free-swimming) fry were collected over a period of 10 days by removing clutches from mouthbrooding females. Eggs and nonswimming sacfry were placed in up welling incubators thermoregulated at 27 ± 1 °C until post-yolksac stage.

2.3.2. Hormonal treatment

At post-yolksac stage (10 days post-fertilisation at 27 °C), fry were placed in tank (1 m^2/0.2 m^3) where the water flow was sufficient to ensure 500% of turnover per hour. Optimal dosage of 11βOHA4 (50 mg of 11βOHA4 kg^{-1} of food during 28 days) was applied to fry stocked in tank (1 m^2/0.2 m^3) at increasing densities (8000, 9000, 10,000 and 11,000 fry m^{-2}). Each density consisted of at least five replicate tanks.

Hormonally treated and control feeds were prepared as described in Study 1. The fry were fed by an automatic feeder at 20% of the biomass for the first week, 18% for the second week, 16% for the third week and 15% for the fourth week. Afterwards, food ration was calculated according to Mélard et al.'s (1995) formula, which includes the rearing temperature and the body weight. A fry sample was weighed weekly for the adjustment of the food ration. Following the sex-reversal treatment (28 days), survival rate (%) was determined in each tank. Next, treated fry, originating from the same control day, were rounded up in a bigger tank (12 m^2/10 m^3) to form a new group (as noted in results Table 3). Then fry were

Table 2
Survival and sex ratio of Florida red tilapia fry 11βOHA4-treated

Treatment (dosage/duration, mg kg $^{-1}$/day)	Initial fry number	GSR (%)	Females	Males	Sex ratio (% male)	χ^2 test
50/35	100	86	0	86	100	51.6*
50/28	100	86	1	88	98	45.1*
Control	100	86	27	39	59	
50/28	192	52	2	98	98	115.1*
50/28	175	57	2	98	98	115.1*
50/25	126	79	0	100	100	122.6*
50/25	172	58	1	99	99	118.8*
50/21	178	56	10	90	90	88.9*
50/21	200	50	2	98	98	115.1*
Control	196	51	76	24	24	
50/35	100	74	1	73	98	63.4*
50/21	100	42	8	34	80	22.2*
Control	100	84	43	41	48	
50/28	100	67	0	67	100	35.3*
50/21	100	52	0	52	100	35.3 *
50/14	100	98	20	78	79	2.1
50/10	100	82	30	52	63	1.1
Control	100	72	21	51	70	
40/35	100	83	17	66	79	42.9*
40/28	100	97	37	60	61	15.7*
40/21	100	73	39	34	46	3.5
40/14	100	91	58	33	36	0.2
40/10	100	97	75	22	22	3.0
Control	100	96	64	32	33	
40/35	100	100	1	99	99	74.2*
40/28	100	100	28	72	72	16.1*
40/21	100	76	34	42	55	2.4
Control	100	100	56	44	44	
40/21	100	100	34	66	66	10.7**
40/14	100	100	53	47	47	0.3
40/10	100	100	59	41	41	0.1
Control	100	100	57	43	43	
30/35	100	100	49	51	51	1.0
30/28	100	100	43	57	57	0.02
30/21	100	100	31	69	69	2.6
30/14	100	89	41	48	53	0.5
Control	100	92	38	54	58	
20/35	100	100	52	48	48	0.2
20/28	100	100	41	59	49	0.1
20/21	100	88	35	53	60	1.6
Control	100	76	37	39	51	

Tank 200 l/0.4 m^2. Mean temperature: 27.0 ± 1.0 °C; GSR: survival rate at the end of the growing period. χ^2 test: compared with the respective control group; $df = 1$.

* $P < 0.001$.

** $P < 0.01$.

reared to an average weight of >3 g, prior to sexing gonad squash (Guerrero and Shelton, 1974). Efficiency of sex-reversal treatment was tested on a sample of 500 fish per group.

3. Results

In the first study, sex-reversal treatment was applied to nine Florida red tilapia offspring. The survival rate in the treated groups ranged from 42.0% to 100.0%. The

Table 3
Results of intensive sex-reversed fry production (50 mg of 11βOHA4 kg^{-1} during 28 days) in Florida red tilapia

Group	Density (fry m^{-2})	SR (%)	Sex ratio (% male)	Group	Density (fry m^{-2})	SR (%)	Sex ratio (% male)
1	8000	88.2	98.0*	8	9000	96.5	99.2*
	8000	93.8			9000	95.2	
	10,000	97.1			10,000	53.6	
	10,000	78.9			10,000	81.8	
	10,000	72.4					
2	8000	99.1	99.4*	9	10,000	93.3	99.6*
	10,000	96.3			10,000	84.6	
	10,000	67.1			11,000	84.5	
	10,000	76.6					
3	8000	84.9	99.6*	10	10,000	92.9	98.6*
	10,000	91.2					
	10,000	82.8					
	10,000	92.0					
	10,000	63.7					
	10,000	74.5					
	11,000	88.6					
4	10,000	85.8	99.4*	11	9000	79.3	99.6*
	10,000	81.6			10,000	85.7	
	10,000	95.2			10,000	97.9	
	10,000	95.2					
	11,000	76.2					
5	9000	98.9	98.6*	12	8000	99.3	97.4*
	9000	89.4			9000	96.0	
	9000	96.1					
	10,000	49.9					
6	9000	85.5	99.6*	13	10,000	90.9	98.4*
	9000	96.0			10,000	96.9	
	9000	78.9			10,000	85.1	
					9000	84.4	
7	10,000	84.4	99.6*	14	8000	90.6	98.8*
	10,000	99.1			9000	84.9	
	10,000	90.1			9000	50.1	
	11,000	84.4					
	11,000	82.9					
Control	10,000	80.8	31.0				
	10,000	91.3	53.0				

Tank 200 l/1 m^2. Temperature: 27.0 ± 1.0 °C. SR: survival rate (%) after sex-reversal treatment.

* Significant difference when compared with the mean of the two control groups (χ^2 test; $df=1$; $P<0.001$).

mean survival rate in treated groups (82.3 ± 18.8%) was not significantly different from survival rate of control groups (81.9 ± 17.0%). (Mann–Whitney test; $U = 134$; $n = 9$; $m = 32$; $P > 0.05$) (Table 2). Dosages of 40 or 50 mg of 11βOHA4 kg^{-1} during 21–35 days significantly increased the male percentage in sex-reversed groups in comparison to control groups (Table 2, $P < 0.001$). On the other hand, lower dosage (20 or 30 mg kg^{-1}) or shorter treatment duration (40 mg kg^{-1} during 10–21 days) had no significant effect on the sex of treated hormone fish (Table 2, $P > 0.01$). In groups treated for 25–35 days, the mean male percentage was significantly higher at 50 mg kg^{-1} (98.9 ± 0.9%) than at 40 mg kg^{-1} (77.8 ± 13.8%) ($\chi^2 = 21.62$; $df = 1$; $P < 0.001$). Monosex male population were produced in groups treated at 50 mg kg^{-1} for 21–35 days. At 40 mg kg^{-1} for 35 days of treatment, 99% of fish were male in one treated group and only 79% in a second one ($\chi^2 = 20.43$; $df = 1$; $P < 0.001$). Similar results were observed at 50 mg kg^{-1} for 21 days (80.0% and 100.0%; $\chi^2 = 22.22$; $df = 1$; $P < 0.001$). The mean male percentage in control groups was 47.8 ± 13.1%, ranging from 24% to 70%.

In the second study, survival rate in sex-reversed fry at 50 mg kg^{-1} for 28 days ranged from 49.9% (10,000 fry m^{-2}) to 99.3% (8000 fry m^{-2}) and the 510,000 fry treated had a mean survival rate of 85.3%. Similar results were observed in the two control groups (80.8% and 91.3%). The mean percentage survival rate of treated fry reared at 8000 m^{-2} ($n = 6$; 92.7 ± 5.3%) was not significantly different than in those stocked at 9000 ($n = 13$; 87.0 ± 12.5%) (t-test; $t = -1.04$; $df = 17$; $P > 0.05$) and 10,000 ($n = 31$; 84.2 ± 12.3%) (t-test; $t = -1.64$; $df = 36$; $P > 0.05$). However, it was significantly higher than in 11,000 fry m^{-2} ($n = 5$; 83.3 ± 4.1%) (t-test; $t = -3.207$; $df = 9$; $P < 0.05$) (Table 3). Some of the batches ($n = 4$) reared at 9000 and 10,000 fry m^{-2} have a survival lesser than 70% (49.9–67.1), whereas a minimum of 84.9% was obtained at 8000 fry m^{-2}.

The treatment at 50 mg kg^{-1} for 28 days administered to 510,000 fry produced a mean male percentage of 99.1%, and results from the 14 treated group ranged from 97.4 (group 12) to 99.6 (groups 3, 6, 7, 9, 11) (Fig. 1). The male percentage in treated groups was significantly higher (χ^2-test; χ^2-values > 72.7, $df = 1$; $P < 0.001$) than the mean of the two control groups (42.0%) (Table 3).

4. Discussion

In the first study, a treatment for 21–35 days at 50 mg of 11βOHA4 kg^{-1} of food produced nearly 100% male population of Florida red tilapia. Lower doses (20, 30 or 40 mg of 11βOHA4 kg^{-1} of food) or less duration (10, 14 days) had variable or no effect on treated groups. Results showed that high male percentages (≥ 98.0%) were systematically obtained with 50 mg of 11βOHA4 kg^{-1} of food applied for 25 days minimum. To yield a monosex male population, Florida red tilapia requires a higher dose of 11βOHA4 (50 mg kg^{-1}) than *O. niloticus* (10 mg kg^{-1}) (Baroiller and Toguyeni, 1996). The proportion of males significantly increased when the dosage of either steroid exceeded 5 mg kg^{-1} in *O. niloticus* (Baroiller and Toguyeni, 1996) and 40 mg kg^{-1} in Florida red tilapia (present study). This natural androgen has previously been used for sex-reversal treatment in only three species. In rainbow trout (*Onchorynchus mykiss*), sex-reversal results with 11βOHA4 ranged from 69% to 72% of male when fish were submitted to a combination of immersion (0.4 mg l^{-1}) and feeding (3 mg kg^{-1}) (Feist et al., 1995), from 76% to 78% when fish were fed at 6–60 mg kg^{-1} (Van den Hurck and Van Oordt, 1985). A sex-reversal treatment at 300 mg kg^{-1} for 4 weeks in African catfish (*C. gariepinus*) produced 77% males (Van den Hurk et al., 1989) with 11βOHA4. Generally, synthetic androgens are more potent masculinizing agents than those of natural origin (Hunter and Donaldson, 1983). In rainbow trout, a combination of immersion and feeding (400 mg m^{-3} for 2 h + 3 mg kg^{-1}) resulted in a monosex male population after treatment with 17α-methyltestosterone and a 69–72 male population in fish treated with 11βOHA4 (Feist at al., 1995). In tilapia *O. niloticus*, 98.1–100.0% of male were obtained with sex-reversal treatment at 10–50 mg of 11βOHA4 kg^{-1}. The 17α-methyltestosterone still induced monosex male population at 5 mg kg^{-1}.

However, in intensive culture systems, the natural androgen 11βOHA4 is as efficient as artificial ones. In Florida red tilapia (present study), 99.1% of males have been obtained after a sex-reversal treatment of 510,000 fry with 11βOHA4 (50 mg kg^{-1} of food for 28 days). Indeed, high and similar male percentage were obtained with 17α-methyltestosterone or 17α-ethynyltestosterone at 60 mg kg^{-1} of food for 25–28 days in intensive production system: 91.2–99.4% in *O. niloticus* (Vera-Cruz and Mair, 1994), 96.0–100.0% in *O. niloticus* x *O. aureus* (Rothbard et al., 1983; Buddle, 1984), 97.3–100.0% in Taiwan red tilapia (Rothbard et al., 1983; Berger and Rothbard, 1987) and 83.0–97.0% in *O. aureus* (Mélard and Ducarme, 1993; Mélard et al., 1995). A lower dose of 17α-methyltestosterone (30 mg kg^{-1} for 21 days) produced 99.0% males in *O. niloticus* (Guerrero and Guerrero, 1988). This natural androgen (11βOHA4) had a similar masculinizing effect as a synthetic androgen (17α-methyltestosterone or 17α-ethynyltestosterone) in intensive production of sex-reversed fry.

Increasing density does not induce a reduction of survival rate in Florida red tilapia, except for 11,000 fry m^{-2}. The variability of survival rate in replicates was more important at 9000 or 10,000 fry m^{-2} than at 8000 fry m^{-2}. However, high stocking density in tank (8000–11,000 fry m^{-2}) combined with 500% turn over of water per hour have given excellent survival rate (85.3%). In comparison to *O. niloticus*, Vera-Cruz and Mair (1994) obtained 68.9% of survival at 5000 fry m^{-2} in hapas, but with poor water quality and exchange.

Tilapia species present a great potential for aquaculture in tropical and subtropical countries. To control the reproductive efficiency and due to the better growth of males, monosex male population of tilapia are usually used to optimise the production system. To control the reproductive efficiency and utilise the better growth of males, monosex male progeny of tilapia are usually referred to optimise the production system (decreasing of reproduction, reduction of sexual/territorial behaviour, achievement of higher average growth rate...). Several techniques are used to produce progeny with a high percentage of males: hand sexing according to the urogenital papilla (Chervinsky and Rothbard, 1982), hormonal sex reversal with androgen (Vera-Cruz and Mair, 1994; Mélard and Ducarme, 1993), interspecific hybridisation (Majumdar and Mc Andrew, 1983; Mair et al., 1991b), genetic approach in *O. aureus* (ZZ pseudofemale line: Jensen and Shelton, 1979; Mair et al., 1991b; Lahav, 1993; Desprez et al., 1995) and in *O. niloticus* (YY super male line: Scott et al., 1989; Mair et al., 1991a; Beardmore et al., 2001). Hand sexing has pretty much vanished due to high labour costs and the risk of misclassification errors. For instance, the sex-reversal process with synthetic androgen (17α-methyltestosterone) was used to produce high male percentage population in tilapia species (Guerrero and Guerrero, 1988; Mélard and Ducarme, 1993; Vera-Cruz and Mair, 1994). Using synthetic androgens set down the problem of androgens residues in marker-sized fish and the possibility of contamination of the environment. Recent studies showed that genetic technology for the production of monosex male populations in tilapia could be developed in aquaculture industry (Beardmore et al., 2001). For example, high male percentage populations were produced with ZZ pseudofemales in *O. aureus* (Lahav, 1993; Desprez et al., 1995) and with YY super-males in *O. niloticus* (Mair et al., 1997; Beardmore et al., 2001). However in a complex hybrid such as Florida red tilapia, involving four species and both male homogamety (ZZ/ZW in *O. hornorum* and *O. aureus*) and female homogamety (XX/XY in *O. mossambicus* and *O. niloticus*), a similar genetic approach can be excluded at present. Therefore, the use of a natural androgen as 11βOHA4 can, thus, constitute the one and only alternative to use synthetic hormone, without altering the percentage of males in Florida red tilapia. This process can also be applied to other tilapia species and may alleviate problems of marketable fish which have been exposed to synthetic steroids (Feist et al., 1995; present study). The recommended hormone dosage and stocking density for 28 days treatment to achieve nearly 100% sex-reversal in Florida red tilapia fry are 50 mg of 11βOHA4 kg^{-1} of food and 8000–10,000 fry m^{-2} in tank with high water flow turn over.

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