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Sexual Reversal with 17α-Methyltestosterone in *Oreochromis* sp.: Comparison between Recirculation Aquaculture System (RAS) and Biofloc Technology (BFT)

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Abstract: Precocity in tilapia implies the use of several methods of obtaining monosex seed; the most common tends to use masculinizing hormone 17α-methyltestosterone (17αMT), with variable results. Thus the objective of this study was to compare the efficiency of the sexual reversion process using 17αMT, in a recirculation system and in biofloc. In a totally randomized design, three tanks for recirculation (T-RAS) and three tanks for biofloc (T-BIO) with a capacity of 200 L effective volume were taken and filled with 1,056 larvae of *Oreochromis* sp., without reversing and with an initial weight of 0.02 g and an initial total length of 1.4 mm. The study was carried out during 65 d, the fish were fed (10% biomass, adjusted every 15 d) by a commercial diet at 45% of crude protein that included 17αMT (60 mg/kg). Water quality, microbiology, zootechnical and gonadal analysis were monitored. Consequently the water quality results showed that just dissolved oxygen (DO), temperature (T-°C) and alkalinity did not show significant differences. Additionally, in the productive parameters there were significant differences in the final length, the gain in length and in K which were better in T-BIO. The microbiological ones did not present significant differences between the treatments. Lastly, the percentage of reversion was significantly better in T-RAS. Then, this study suggests that settleable solids concentrations above 35 cm decrease the efficiency of the sexual reversion for this species.

Key words: *Oreochromis* sp., biofloc, recirculation aquaculture system, 17α-methyltestosterone.

1. Introduction

Oreochromis sp. and *Oreochromis niloticus* (red tilapia and Nile tilapia), are the most produced species in the world after the cyprinids [1]. It is known that for the fattening of tilapia, it is better to sow males, due to several characteristics and zootechnical attributes that make this species an excellent candidate for aquaculture [2]. Several methods such as manual sexing [3], hybridization [4], transgenesis [5], androgenesis and gynogenesis [6], triploidy [7] and YY males or super-males [8] are implemented for obtaining monosex seed viable for tilapia. However, it is the method which uses the masculinizing hormone 17α-methyltestosterone (17αMT) with variable results [9-11], the most commonly used. On the other hand, there are public concerns related to the deleterious effects on the environment and human health [12] when 17αMT is being used. Therefore, it is necessary that new technologies for obtaining monosex seed are within the reach of the producer and can include a better use of water. Recirculation aquaculture system (RAS) [13] and biofloc technology (BFT) [14] could become beneficial practices to lessen these effects. Therefore, the objective of the present work was to compare the efficiency of the sexual reversion process using 17αMT in an RAS system and in BFT with a concentration of settleable solids of 35 cm.

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2. Materials and Methods

2.1 Ethical Considerations

The experiment followed the ethical procedures suggested by "The Development of Science-Based Guidelines for Laboratory Animal Care" [14] and approved by the Ethics Committee for Animal Experimentation of the Corporación Universitaria Lasallista (Act No. 12 of July 13, 2015).

2.2 Location

The experimental procedure was carried out in the RAS of the Corporación Universitaria Lasallista, in the Municipality of Caldas (Antioquia) at 1,750 m above the sea level and at an average environmental temperature of 19 °C.

2.3 Fishes and Experimental Units

In a totally randomized design, three tanks for recirculation (T-RAS) and three tanks for biofloc (T-BIO) of 200 L of effective volume were taken and filled with 1,056 red tilapia larvae (*Oreochromis* sp.) with an average initial weight and total length of 0.02 g and of 1.4 mm respectively without reversing. The study was carried out for 65 d, the fish were fed with commercial balanced 48% crude protein that included the hormone 17αMT (60 mg/kg), based on 10% of the biomass, adjusting it every 15 d by random sampling of the fish from the experimental units.

2.4 System Management

The water from the RAS was taken from the municipal aqueduct. Before the beginning of the test the water was previously dechlorinated with the addition of 5 mg/L of $Na_2S_2O_3$. Additionally, physicochemical water quality tests were carried out to maintain the comfort conditions of the species [15]. It is important to say that previously each T-BIO was inoculated with 0.7 kg of molasses, 0.7 kg of balanced feed of 19% crude protein, 1.75 g/L of NaCl and 7 mL of nitrifying bacteria. It was continually used as a source of carbon, molasses and the amount was adjusted trying to maintain a C:N ratio of 20:1 [16, 17]. Evaporation losses were replaced every 15 d with water under the same conditions mentioned. For T-RAS and T-BIO aeration was provided 24 h a day. Once both systems were stabilized, the larvae were placed in each treatment.

2.5 Water Quality and Microbiology

A total of 26 samples in the 65 experimental days were recorded for dissolved oxygen (DO, mg/L), temperature (T-°C), conductivity (μs/cm), turbidity (NTU), pH, ionized ammonia ($N\text{-}NH_4^+$, mg/L), nitrites ($N\text{-}NO_2^-$, mg/L), nitrates ($N\text{-}NO_3^-$, mg/L) which were taken with the YSI Professional Plus probe. Salinity was also recorded with refractometer (NaCl, g/L) and alkalinity (mg/L $CaCO_3$) with the Hach-FF2A kit. The sedimentation in centimeter for T-BIO was measured with the Imhoff cones which remained constant at 35 cm. For the microbiological analyses, four samplings were made in the experimental period. These samplings consisted of skin scraping to rule out the presence of *Staphylococcus*, mesophiles, fungi and yeasts, total coliforms and *Vibrio* sp. [18] for both T-RAS and T-BIO. The previously mentioned samples were taken to a dilution of 10^{-3}, inoculated by surface seeding 0.1 mL in mannitol agar. Specific culture media were applied to *Staphylococcus aureus*, mesophilic bacteria, fungi, yeast, total and fecal coliforms, *Aeromonas* sp. and *Vibrio* bacteria. The culture media were incubated in aerobic condition at 37 °C/24 h. After this time, a colony forming units (CFU) counting was performed on each one of the culture media. In all the samplings the same evaluations were carried out.

2.6 Zootechnical Parameters

The weight gain (WG) values were obtained with the following formula:

$$WG = FW - IW \quad (1)$$

where FW: final weight; IW: initial weight.

$$\text{Daily weight gain (DWG)} = WG/t \quad (2)$$

where WG stands for gain in weight and t stands for the experimental time in days.

Specific growth rate (SGR), with the formula:

$$SGR\ (\%) = \{[Ln(FW) - Ln(IW)]/t\} \times 100 \quad (3)$$

where Ln stands for the natural logarithm of the final and initial weight.

The survival rate (% S) was calculated with Eq (4):

$$\%\ S = (\text{final number of fish/initial number of fish}) \times 100 \quad (4)$$

In addition, the condition factor (K) was obtained by the equation:

$$K = FW/FL^3 \quad (5)$$

The gain in length (GL) was also calculated by Eq (6):

$$GL = FL - IL \quad (6)$$

where FL stands for the final length and IL stands for the initial length.

The daily gain in length (DGL), by the formula:

$$DGL = GL/t \quad (7)$$

2.7 Sexual Determination and Morphology of the Gonad

From the final sampling and for each treatment, 32 fish were euthanized [19]; the evisceration process of the 64 fish was performed to extract the gonads, to which light pressure was applied with coverglass to squash and subsequent staining with acetocarmine [20], light microscope was assembled for observation and evaluation of the presence of oocytes (female-H), granular tissue (male-M) or a combination of the two (intersex-I).

2.8 Statistic Analysis

The statistical design was completely randomized, the tests of normality and homoscedasticity were made for all data. For the survival value, the statistical t test was applied after transformation by arc-sine. In all cases, non-parametric statistics were applied for the comparison of independent samples. Water quality data were applied followed by the Mann-Whitney U test. For the production data, the Kolmogorov-Smirnov test was applied. A transformation was made by arc-sine for SGR data. For the data of microbiological counting, a transformation was made by Log(x). The data were analyzed in the Statgraphics Centurion XV software with a license to the Corporación Universitaria Lasallista.

3. Results and Discussion

3.1 Water Quality

RAS and BFT are increasingly implemented technologies due to environmental restrictions, eco-responsibility and the low water consumption [21, 22]. In general, the values recorded in Table 1 for T-RAS are within the appropriate ranges for tilapia [2] and for freshwater species [23]. For T-BIO, it can be said that they are within the reported ranges with some variations, due to differences in the use of the energy source that controls the C:N ratio, physiological state, species and sowing density, among others [23, 24]. As indicated in Table 1, the comparison and the significance between the sum of the ranges of the quality parameters show significant differences ($p < 0.05$) in most of the parameters evaluated, except in DO, T (°C) and alkalinity ($p > 0.05$). The DO values are the result of the auxiliary aeration by a diffuser tube, which guaranteed these levels during the whole experiment which is of obligatory use in T-BIO. This design was done in the same way for T-RAS. The values of alkalinity indicate the use of bases of sodium bicarbonate to improve the efficiency of the biofilters in the case of T-RAS and the consumption of it that occurs within the T-BIO. The non-ionized ammonium fraction ($N\text{-}NH_3$) in relation to pH and T (°C) was found within the safety margin for tilapia (0.0006 for T-RAS and 0.023 for T-BIO in mg/L $N\text{-}NH_3$), similar findings are reported in another study [24].

Fig. 1, compares T-BIO and T-RAS, the behavior of nitrogenous waste during all the samplings, it is clearly observed the differences in the concentrations.

Table 1 Values of water quality parameters and their ranges between T-BIO and T-RAS (n: 26 per treatment) (confidence level at 0.05).

Parameters	Normal data T-BIO	Normal data T-RAS	Rank sum T-BIO	Rank sum T-RAS	U	Z	*p*-level (< 0.05)
$N\text{-}NH_4^+$ (mg/L)	4.97 ± 0.97	0.15 ± 0.24	1,027.0	351.0	0.0	6.2	0.00
$N\text{-}NO_2^-$ (mg/L)	1.56 ± 0.76	0.22 ± 0.17	999.0	379.0	28.0	5.7	0.00
$N\text{-}NO_3^-$ (mg/L)	286.96 ± 57.83	0.35 ± 0.13	1,027.0	351.0	0.0	6.2	0.00
Conductivity (μs/cm)	5,060.62 ± 787.07	181.33 ± 89.54	1,027.0	351.0	0.0	6.2	0.00
DO (mg/L)	9.38 ± 1.63	9.24 ± 1.63	630.0	748.0	279.0	-1.1	0.28
T-°C	26.62 ± 1.21	26.77 ± 1.68	877.4	838.5	188.5	-2.7	0.16
Turbidity (NTU)	148.58 ± 55.26	1.10 ± 0.9	1,027.0	351.0	0.0	6.2	0.00
pH	6.89 ± 0.72	7.85 ± 0.65	442.5	935.5	91.5	-4.5	0.00
Alkalinity (mg/L $CaCO_3$)	137.21 ± 65.19	109.34 ± 8.82	732.5	645.5	294.5	0.8	0.43
Salinity (mg/L)	1.87 ± 0.88	0.18 ± 0.23	1,027.0	351.0	0.0	6.2	0.00

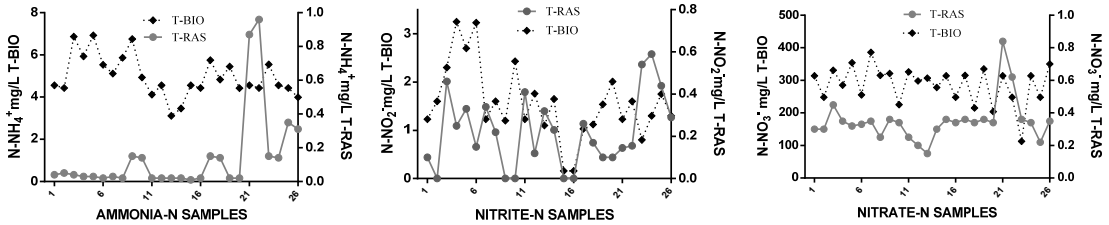

Fig. 1 Total ammonium, nitrite and nitrate values during the experiment (n: 26 per treatment).

For ammonium in T-RAS, only two samples indicated values above the trend (0.87 mg/L and 0.92 mg/L of $N\text{-}NH_4^+$, respectively) that the record had shown, but that did not imply risks for the fish. For T-BIO, a slight tendency to decrease towards the end of the experiment is observed, but maintains the highest values in comparison with T-RAS (3.1 to 6.9 and 0.01 to 0.96 as minimum and maximum in mg/L of $N\text{-}NH_4^+$, respectively). The values of nitrites in T-BIO during the first days increase substantially with a maximum value of 3.25 mg/L of $N\text{-}NO_2^-$ and from the seventh day up to the end of the trial the values tend to stabilize. For T-RAS the nitrite values were always low concentrations (0.59 mg/L of $N\text{-}NO_2^-$, maximum value) and tend to stabilize, but a clear trend is not observed. The values for nitrates are stable for the two systems and indicate in both cases that the nitrification processes are working properly, these processes have been reported as the dynamics of nitrogen compounds that is consistent with the process of autotrophic nitrification, common in biofloc [16, 25]. In addition, the assimilation of ammonia and nitrate was the process that dominated the transformation of nitrogen [26-28].

In T-BIO, the salinity was significantly higher with respect to T-RAS, the effect of chlorine on the decrease of nitrite toxicity is known, furthermore to its positive effects on growth [29]. Therefore, it is essential to maintain salinity in BFT, and increase its levels in RAS.

3.2 Productive Parameters

The anabolic effect of 17αMT on growth in tilapia is known [30, 31]. In the present study, the final values found indicate that the T-BIO fingerlings had a higher FW (3.9 ± 2.92 g), therefore WG and daily weight gain (DWG) were higher than the T-RAS fingerlings but without significant differences between treatments. In T-BIO fingerlings were significantly longer (5.79 ± 0.85 g) and affected GL, DGL and the K (4.39 ± 0.36, 0.067 ± 0.0028 and 2.43 ± 0.96 cm, respectively). The SGR was similar for the two

treatments without significant differences. For *O. niloticus* with 35 d of treatment with 17αMT (60 mg/kg), an average weight of 3.5 ± 0.26 g and an average length of 5.06 ± 0.39 cm are reported with a survival of 94% [30] similar to those found in the present study (Table 2), but achieved in a shorter experimental time. These differences can be attributed to the species, it is known that *O. niloticus* presents higher growth rates than *Oreochromis* sp. [2]. On the other hand, García-Ríos *et al.* [24] found that, when comparing BFT and a traditional system (without the addition of molasses) there were any differences in terms of productive parameters, they only reported significant differences in the percentage of survival, which was better in BFT (98%), with respect to 75% of the treatment without addition of molasses. The same researchers report a similar K and SGR higher than those reported in the present study [24]. In another trial with *O. niloticus*, survival rates of 83.05% are reported, a K of 1.7 and an SGR of 2.02 [31], lower than those reported in the present study. Little *et al.* [32], in the same species over a period of 60 d and working in hapas, found weights of 0.7 ± 0.052 g, an SGR of 7.6 ± 1.28 and a survival of 86 ± 3.2, which are lower than those found in the current study.

3.3 Efficiency in Sexual Reversal

Obtaining monosex seed through the use of 17αMT in food is the most common and most successful method in the process of sexual reversion [9, 10], although its usage every day is more restricted due to its possible impacts on health and the environment [11]. For the present study, the ANOVA in the proportion of sexes found, shows significant differences between treatments, being better in T-RAS with 92% of reversed fish. On average, 34% of the samples in T-BIO were females for the two treatments. Specimens with intersex were presented in a proportion of 1% in T-RAS, without significant differences between the treatments. Table 3 summarizes the results.

According to the reports for several species of tilapia, the usage of concentrations between 30 mg to 60 mg of 17αMT/kg of food, supplied in periods ranging from 18 d to 40 d, can result in reversion percentages that can be between 82% and 100% [33-37]. In other studies, at 75 d in the *O. niloticus* species, the reversion rate was 100% [38]. For red tilapia, using the hormone 11β-hydroxyandrostenedione (11β-OHA4), for 28 d, the percentage of males was 99.1% [39]. Other authors for the same species, using Tamoxifen, found a 100% reversion to 42 d [37]. In the present study the value was 92% of reversion for the T-RAS, value that is within the reported values. The percentage found in T-BIO is very low. Probably the lowest efficiency in the reversion process in the T-BIO is due to the concentration of settleable solids, interfering in the

Table 2 Values for the productive parameters registered in T-BIO and T-RAS (n: 495 by treatment) (confidence level at 0.05).

Zootechnical parameters	Average ± SD T-BIO	Average ± SD T-RAS	*p*-level (< 0.05)
FW (g)	3.9 ± 0.63	3.79 ± 1.81	0.95
WG (g)	3.88 ± 0.63	3.77 ± 0.15	0.95
DWG (g)	0.059 ± 0.0097	0.058 ± 0.0023	0.95
FL (cm)	5.79 ± 0.85	5.23 ± 1.24	0.0001
GL (cm)	4.39 ± 0.36	3.83 ± 0.1	0.0001
DGL (cm)	0.067 ± 0.0028	0.058 ± 0.001	0.0001
SGR (%)	8.1 ± 0.25	8.07 ± 0.062	0.99
K	2.43 ± 0.96	1.99 ± 0.05	0.0001

FW: final weight; WG: weight gain; DWG: daily weight gain; FL: final length; GL: gain in length; DGL: daily gain in length; SGR: specific growth rate; K: condition factor; S%: survival rate. $p < 0.05$ indicates significant statistical differences.

Table 3 One-way ANOVA for the means of each sex in relation to the treatment (n: 63 per treatment) (confidence level at 0.05).

Treatment	Males average	Females average	Intersex average
T-RAS	0.91a	0.08b	0.01a
T-BIO	0.61b	0.34a	0.05a

Data transformed by arc-sine; different letters indicate significant statistical differences ($p < 0.05$).

Table 4 Summation of ranges between T-BIO and T-RAS, for microbiological parameters in skin sample (n: 8) (confidence level at 0.05).

Microbiological parameters	Rank sum T-BIO	Rank sum T-RAS	U	Z	*p*-level (< 0.05)
Colimetry	73.0	63.0	27.0	0.5	0.6
Mesophiles	64.0	72.0	28.0	-0.4	0.7
Estafilococus aureus	72.0	64.0	28.0	0.4	0.7
Yeasts	73.5	62.5	26.5	0.6	0.6

$p < 0.05$ indicates significant statistical differences.

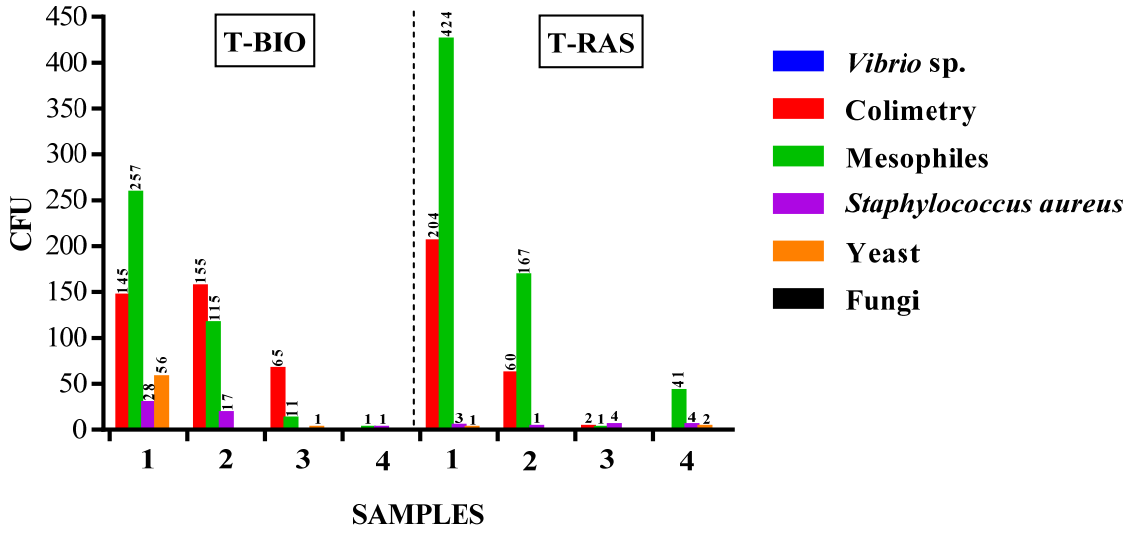

Fig. 2 Microbiological analyses carried out in four samplings for each of the treatments.
The colors indicate the organisms found by the microbiological analyze.

amount of food with hormone ingested by each larva and therefore in the efficiency of the masculinization process. There are no reports known regarding this subject.

3.4 Microbiology

The colony counting did not indicate significant differences between T-BIO and T-RAS for any microbiological parameter analyzed (Table 4).

However in Fig. 2, a decrease in the counting of microorganisms can be observed throughout the four samplings. In the first sampling, the presence of four of the six microorganisms evaluated was notable which does not exceed the microbiological values allowed for fish [18]. This presence can occur in the stabilization phase of the biofloc [40], while in the RAS there was only an increase in mesophilic counting and colimetry for the same sample. The mesophiles are microorganisms that are found in the environment and can be increased in relation to the water temperature [39] which can be notable by the fact that there was an increase in their counting for such sampling. On the contrary, colimetry countings are useful as indicators of hygiene and water quality

[41]. In this study, such countings did not exceed the allowed values with a maximum of 400 CFU for T-RAS because the quality of water was good, as it can be verified in Table 1. Regarding the presence of *Staphylococcus*, the countings did not exceed the allowed values for the microbiological requirements in fish [18] reaching values with a maximum of 28 CFU. There wasn't an incidence of *Streptococcus*, a disease that occurs mostly in poorly managed intensive cultures [42], evidencing animal welfare throughout the experiment.

In none of the samplings that were carried out, values for *Vibrio* sp. and fungi were recorded in the treatments, being a referent of innocuousness in the crop. However, it is reported that these microorganisms are common inhabitants of the aquatic environment in biofloc systems [43] and that its proliferation occurs if there is an overload of organic matter.

4. Conclusions

Sexual reversal on BFT technology is not feasible for settleable solids at a level of 35 cm. The obtained results may provide a support to test other levels of settleable solids in BFT. On the other hand, RAS and BFT have provided a suitable culture system for this physiological stage. The found values in the productive parameters show the species walfare.

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