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Research Article

Effect of the combination of fluoxymesterone and a commercial blend of additives on the sex proportion, growth, and survival of Nile tilapia (*Oreochromis niloticus*) fry

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ABSTRACT. In the commercial culture of Nile tilapia (*Oreochromis niloticus*), monosex, all-male populations obtained through steroid sex reversal is the most commercially used method to eliminate unwanted reproduction and increase final productive and economic yield. However, the rapid growth of Nile tilapia culture has led, in some regions, to an increase in infectious diseases and mortality in all stages of commercial culture. To reduce this, without the abuse of antibiotics or similar substances, feed additives are currently used in all stages, including the fry period, during the sex reversal process. The objective of this work was to evaluate the effect of synthetic steroid Fluoxymesterone (FM) at various concentrations in combination with a commercial Blend of additives (BA) on the percentage of males, growth, and survival of Nile tilapia fry. Five treatments (FM20, FM5+BA, FM10+BA, FM20+BA, and BA+FM20) and a control group were evaluated. Treatments differ in the order in which the commercial blend (2 g) was added and the FM concentration (5, 10, and 20 mg). The feeding trial lasted for 30 days using a sexually undifferentiated population of Nile tilapia. The results showed a decrease in the percentage of males in all treatments supplemented with the BA, compared to the FM20 treatment (containing 20 mg without BA). Growth and survival were positively affected by the inclusion of the BA in all treatments, showing higher values than those observed in the control group. Further studies are necessary to determine the FM-BA interaction.

Keywords: *Oreochromis niloticus*; sex reversal; fluoxymesterone; commercial blend; growth; survival

INTRODUCTION

It is well known that monosex, all-male Nile tilapia (*Oreochromis niloticus*) populations show better growth than mixed populations, eliminating unwanted reproduction and allowing the production of homogeneous size fish (Varadaraj & Pandian 1989, Mair et al. 1997, Müller-Belecke & Hörstgen-Schwark 2007, Alcántar-Vázquez et al. 2014) at harvest. In recent years, the production of all-male populations has been recognized as the most effective technique to increase Nile tilapia yield under commercial farming conditions (Müller-Belecke & Hörstgen-Schwark 2007, Phumyu et al. 2012, Trejo-Quezada et al. 2021). Currently, the most used method to achieve monosex populations is sex reversal, through the inclusion of steroids in the

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balanced food supplied during the fry period (Piferrer 2001, Alcántar-Vázquez 2018) before sexual differentiation, which allows obtaining a population composed of 100% male fish (Jiménez & Arredondo 2000, Daza et al. 2005, Trejo-Quezada et al. 2021).

In recent years, a lot of research effort has been invested in the development of the Nile tilapia's monosex population technology to exploit their benefits for commercial aquaculture (Beardmore et al. 2001, Abucay & Mair 2004, Trejo-Quezada et al. 2021, Ramírez-Ochoa et al. 2023). In parallel, the incorporation of food additives to the commercial culture of Nile tilapia to improve growth, health, and survival is becoming a widespread protocol for producers seeking to increase culture yield (Toyama et al. 2000, Cavichiolo et al. 2002, Bombardelli et al. 2005, de Araújo et al. 2018, Rahman et al. 2019, Jo-Rivero & Espinoza 2020, Zamora-Vera 2020). However, its application during the fry period has not been extensively evaluated and could interfere with the sex reversal (masculinization) process.

One of the commercial food additives that are currently being used in the southern region of Mexico is a blend composed of a prebiotic (inulin), vitamin C, garlic (*Allium sativum*), cinnamon (*Cinnamomum verum*), and sodium chloride and is mainly used in the juvenile and adult stages of commercial culture. However, producers of masculinized fry are conducting efforts to incorporate it into the fry stage during the sex-reversal process without knowing the potential effects on the sex-reversal percentages. Therefore, the present work was undertaken to determine the effect of commercial androgen (Fluoxymesterone, FM) in combination with a commercial blend of additives on the proportion of males in the growth and survival of Nile tilapia.

MATERIALS AND METHODS

Nile tilapia fry

Undifferentiated Nile tilapia fry with yolk sac (four days of age) was acquired from the Tilapia del Papaloapan farm, a commercial production unit in San Juan Bautista Valle Nacional, Oaxaca. Following the commercial sex-reversal protocols (before the beginning of exogenous feeding to ensure a successful masculinization process), 1000 fry were transported to Papaloapan University, Agricultural Science School, Laboratory of Aquaculture. Upon arrival at the laboratory, they underwent thermal acclimation to adapt the experimental fish to the new laboratory conditions (>1°C).

Experimental diets preparation

In the present study, the steroid used was the synthetic hormone FM (Pharma, Hamshire, UK), while the commercial feed additive evaluated was a mixture commonly named "Blend" (BA). The basal diet used was Purina Agribrands 53% protein (PN53).

Treatment containing PN53 with FM was prepared using the method described by Guerrero (1975). In brief, 5, 10, or 20 mg of FM were dissolved in 500 mL of 95% ethanol, sprayed over 1 kg of PN53, distributed on a thin layer over a laboratory table, and mixed several times until the feed was completely moistened. Sprayed food was maintained at room temperature for 4 h to allow the alcohol to evaporate. The supplementation of the commercial blend was conducted as follows: 2 g of BA was grounded using a mortar to homogenize its particle size and manually added to 1 kg of PN53 before or after being sprayed with one of the selected concentrations of FM. Finally, it was mixed until achieving a homogeneous mix. Once dried, all experimental diets were stored in plastic containers and refrigerated at 4°C (DFR-9010, Daewoo) until use. The control group was PN53 (without supplementation of FM or BA).

Experimental design

Five treatments and a Control group (C) were used in a mixed population of Nile tilapia. The treatments used were: FM20 (PN53 + 20 mg of FM), FM5+BA (PN53, 5 mg of FM + 2 g of BA), FM10+BA (PN53, 10 mg of FM + 2 g of BA), FM20+BA (PN53, 20 mg of FM + 2 g of BA) and BA+FM20 (PN53, 2 g of BA + 20 mg of FM). The difference in the last two treatments consisted in the order in which the steroid and blend were added. For the experiment, the sexually undifferentiated fry were transported to a closed recirculating system and randomly divided into 18 acrylic aquaria of 85 L at an initial stocking density of 0.6 fry L^{-1} (51 fry per aquaria). Each treatment was carried out in triplicate.

Experimental diets were administered for 30 days under a photoperiod of 12:12 h light:dark. Water temperature was thermostatically controlled and adjusted at 27 ± 1°C. The water in the recirculating system was filtered using a mechanical filter (Hayward, Model S310T2) and a biofilter containing only plastic bio-balls (Aquatic Eco-System, Model CBB1). Fry were fed six times a day at 2 h intervals (08:00 to 18:00 h) at a feed rate adjusted to 10% of the total body weight per day. Water flow was closed in all aquaria 10 min before and 20 min after the administration of experimental diets to encourage feeding. Aquariums were siphoned daily to remove feces and dead fry.

Once the feeding test was completed, juveniles were fed an untreated commercial diet (50% protein, 16% lipids, Silvercup, El Pedregal®) until the end of the experiment (60 days). During this period, juveniles were fed four times daily at a feeding ratio of 5% of their total body weight. To maximize growth, juveniles were stocked in outdoor plastic tanks at a density of 0.2 fish L^{-1}.

Growth

Random samples of 20% fry per aquaria were collected every 15 days to assess wet weight (WW) and total length (TL). WW was obtained using a digital scale (±0.01 g) (Ohaus Cor., Scout Pro Model Sp 202), and TL was recorded from a digitized image using imaging software (ImageJ version 1.36). Growth performance was evaluated in terms of gained biomass (GB), average daily weight gain (ADW), average daily length gain rate (ADL), and condition factor (CF) using the following formulas:

GB = final biomass (g) - initial biomass (g)

ADW = [final wet weight (g) - initial wet weight (g)] / culture days

ADL = [final length (g) - initial length (g)] / culture days

CF = [fish wet weight (g)] / [fish length^3 (cm)] × 100

Final survival

Final survival (FS%) was obtained using the following formula:

$$FS\% = [NFE / NFS] \times 100$$

where NFE is the number of fishes at the end of the experiment, and NFS is the number of fishes stocked.

Sex proportion

The sex of 50% fish per replicate treatment was determined by the squash technique proposed by Guerrero & Shelton (1974). In brief, fish were anesthetized (0.3 mL L^{-1}, 2-phenoxyethanol, Sigma-Aldrich) and sacrificed to extract the gonads. The collected gonads were mounted individually on a glass slide, and a few drops of acetocarmine stain were added and lightly squashed with a cover slip. The criteria to identify the sex of the fish under the microscope (40x) was the following: if uniform tissue with fine-like granular structure (spermatogonia) was observed, it was classified as male, while if large and circular structures (oogonia) were observed in the tissue, it was classified as a female (Fig. 1).

Statistical analysis

Assumptions of normality (Shapiro-Wilk's test) and homoscedasticity (Bartlett's test) were performed on the data obtained. Differences in WW, TL, and growth indices were analyzed using a one-way analysis of variance, with a Tukey test performed on treatment means *a posteriori*. Differences were deemed to be significant at $P < 0.05$. Final survival was analyzed using a chi-square test. The proportion of males identified in each treatment was tested against the 1:1 (male: female) expectation using a chi-square test at a probability of 0.1% ($P < 0.001$).

RESULTS

Growth

WW and TL of the C and fish-fed experimental diets groups are shown in Table 1. Significant differences ($P < 0.05$) were observed in WW at 30 days, with groups FM20+BA, BA+FM20, and FM10+BA showing higher values compared to the C group. No significant differences were registered between the FM20 and FM5+BA groups compared to the rest of the treatments or the C group. At 45 days, a significantly higher ($P < 0.05$) value of WW was shown for the FM10+BA group compared to the BA+FM20 group. No significant differences were observed between the C group and experimental groups. At day 60, all experimental groups showed significantly ($P < 0.05$) higher WW values than those observed in the C group. There were no significant differences between the experimental groups.

At 15 days, TL showed a significantly ($P < 0.05$) higher value in the BA+FM20 group than in C and the rest of the experimental groups. On day 30, no significant differences ($P > 0.05$) were detected among the experimental groups. In contrast, at day 45, the FM5+BA and FM10+BA groups showed significantly ($P < 0.05$) higher TL values than those observed in C and the FM20, FM20+BA, and BA+FM20 experimental groups. At 60 days of the experiment, a significant ($P < 0.05$) higher TL value was observed in the FM20, FM5+BA, and FM10+BA groups compared to the C group. No significant differences were recorded in TL values obtained in the C, FM20+BA, and BA+FM20 groups.

The results obtained for GB, ADW, ADL, and CF are shown in Table 2. The experimental groups observed no significant differences between 15 and 30 days. At 45 days, CF of the FM5+BA and FM10+BA groups showed significantly ($P < 0.05$) lower values

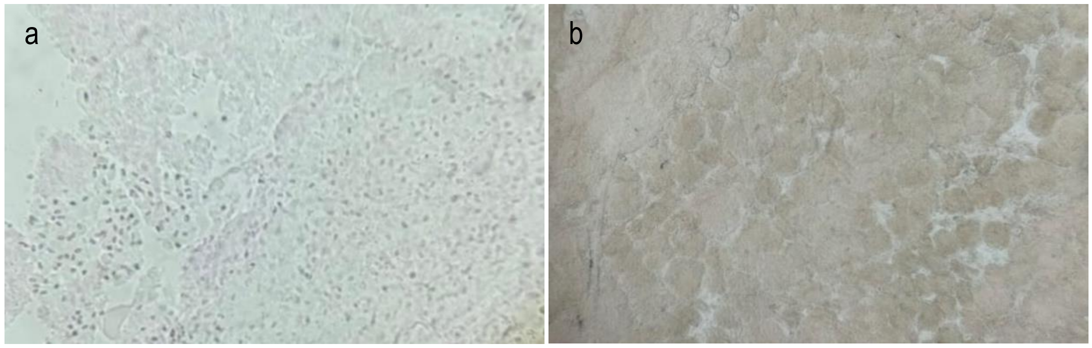

Figure 1. Aceto-carmine stain technique (squash) of a) male and b) female of Nile tilapia (*Oreochromis niloticus*) fed with Fluoxymesterone (FM) and a commercial Blend of additives (BA) at different concentrations and forms of preparation (40x).

Table 1. Wet weight (WW, g) and total length (TL, cm) of Nile tilapia fry (*Oreochromis niloticus*) fed with Fluoxymesterone (FM) and a commercial Blend of additives (BA) at different concentrations and forms of preparation. Mean ± standard deviation. Values in each row superscript with different letters indicate significant differences between groups ($P < 0.05$). C: control treatment, FM20: commercial food supplemented with 20 mg of FM, FM20+BA: commercial food supplemented with 20 mg FM and subsequently 2 g of BA, BA+FM20: commercial food supplemented with 2 g of BA and subsequently 20 mg of FM, FM5+BA: food supplemented with 5 mg of FM and subsequently 2 g of BA, FM10+BA: food supplemented 10 mg of FM and subsequently 2 g of BA.

	Group					
	C	FM20	FM20+BA	BA+FM20	FM5+BA	FM10+BA
Day 1						
WW	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.001^{a}	0.01 ± 0.00^{a}
TL	0.78 ± 0.08^{a}	0.78 ± 0.08^{a}	0.78 ± 0.08^{a}	0.78 ± 0.08^{a}	0.78 ± 0.08^{a}	0.78 ± 0.08^{a}
Day 15						
WW	0.21 ± 0.02^{a}	0.23 ± 0.01^{a}	0.24 ± 0.03^{a}	0.25 ± 0.01^{a}	0.24 ± 0.02^{a}	0.20 ± 0.02^{a}
TL	1.72 ± 0.05^{a}	1.76 ± 0.06^{a}	1.82 ± 0.06^{a}	2.07 ± 0.06^{b}	1.82 ± 0.05^{a}	1.79 ± 0.05^{a}
Day 30						
WW	0.93 ± 0.08^{a}	1.26 ± 0.13^{ab}	1.37 ± 0.14^{b}	1.41 ± 0.19^{b}	1.22 ± 0.12^{ab}	1.39 ± 0.14^{b}
TL	2.95 ± 0.08^{a}	3.34 ± 0.12^{a}	3.00 ± 0.08^{a}	3.12 ± 0.09^{a}	3.10 ± 0.09^{a}	3.38 ± 0.08^{a}
Day 45						
WW	4.53 ± 0.28^{ab}	4.85 ± 0.27^{ab}	3.97 ± 0.29^{ab}	3.81 ± 0.23^{b}	4.17 ± 0.19^{ab}	4.86 ± 0.27^{a}
TL	5.18 ± 0.11^{a}	4.92 ± 0.10^{a}	4.90 ± 0.16^{a}	4.87 ± 0.11^{a}	5.72 ± 0.15^{b}	5.95 ± 0.13^{b}
Day 60						
WW	6.54 ± 0.50^{a}	9.96 ± 0.86^{b}	9.58 ± 0.55^{b}	9.77 ± 0.53^{b}	9.97 ± 0.55^{b}	10.89 ± 0.83^{b}
TL	6.03 ± 0.19^{a}	7.20 ± 0.22^{bc}	6.61 ± 0.12^{ab}	6.71 ± 0.11^{abc}	7.40 ± 0.14^{c}	6.94 ± 0.23^{bc}

than those recorded in the C, FM20, and BA+FM20 groups. No significant differences were observed between the FM20+BA group and C or the rest of the experimental groups. ADW observed in the FM20+BA group registered a significantly ($P < 0.05$) lower value than the FM20 and the FM10+BA groups. There were no significant differences in ADW between C and the rest of the experimental groups. The values obtained for ADL showed significant differences ($P < 0.05$), with the C, FM5+BA, and FM10+BA groups recording higher ADL values than those shown in the FM20 group.

At day 60, the GB and ADW observed in the C group showed significantly ($P < 0.05$) lower values than the rest of the experimental groups. No significant differences between FM20 and the experimental groups supplemented FM and BA were observed in GB and ADW. CF values obtained in the FM20+BA, BA+ FM20, and FM10+BA groups were significantly ($P <$ 0.05) higher than those observed in the FM20 and

Table 2. Gained biomass (GB, g), condition factor (CF), average daily weight gain (ADW, g), and average daily length gain (ADL, cm) of Nile tilapia fry (*Oreochromis niloticus*) fed with Fluoxymesterone (FM) and a commercial Blend of additives (BA) at different concentrations and forms of preparation. Mean ± standard deviation. Values in each row superscript with different letters indicate significant differences between groups ($P < 0.05$). C: control treatment, FM20: commercial food supplemented with 20 mg of FM, FM20+BA: commercial food supplemented with 20 mg FM and subsequently 2 g of BA, BA+FM20: commercial food supplemented with 2 g of BA and subsequently 20 mg of FM, FM5+BA: food supplemented with 5 mg of FM and subsequently 2 g of BA, FM10+BA: food supplemented 10 mg of FM and subsequently 2 g of BA.

	Group					
	C	FM20	FM20+BA	BA+FM20	FM5+BA	FM10+BA
Day 15						
GB	0.20 ± 0.03^{a}	0.22 ± 0.01^{a}	0.23 ± 0.05^{a}	0.24 ± 0.02^{a}	0.23 ± 0.03^{a}	0.19 ± 0.03^{a}
CF	4.62 ± 0.09^{a}	4.78 ± 2.31^{a}	3.65 ± 1.00^{a}	2.76 ± 0.70^{a}	4.02 ± 0.92^{a}	3.31 ± 0.98^{a}
ADW	0.0114 ± 0.002^{a}	0.015 ± 0.00^{a}	0.015 ± 0.004^{a}	0.016 ± 0.002^{a}	0.016 ± 0.003^{a}	0.013 ± 0.003^{a}
ADL	0.059 ± 0.006^{a}	0.065 ± 0.02^{a}	0.074 ± 0.016^{a}	0.089 ± 0.013^{a}	0.070 ± 0.003^{a}	0.070 ± 0.004^{a}
Day 30						
GB	0.76 ± 0.23^{a}	1.02 ± 0.05^{a}	1.08 ± 0.23^{a}	1.07 ± 0.04^{a}	1.04 ± 0.09^{a}	1.14 ± 0.40^{a}
CF	3.70 ± 1.11^{a}	3.48 ± 1.27^{a}	5.05 ± 1.51^{a}	4.93 ± 2.15^{a}	3.97 ± 0.53^{a}	3.62 ± 1.41^{a}
ADW	0.051 ± 0.017^{a}	0.068 ± 0.003^{a}	0.072 ± 0.015^{a}	0.071 ± 0.003^{a}	0.069 ± 0.006^{a}	0.076 ± 0.027^{a}
ADL	0.088 ± 0.007^{a}	0.108 ± 0.036^{a}	0.075 ± 0.039^{a}	0.064 ± 0.044^{a}	0.090 ± 0.011^{a}	0.102 ± 0.011^{a}
Day 45						
GB	3.30 ± 0.78^{a}	3.07 ± 0.43^{a}	2.37 ± 0.19^{a}	2.32 ± 0.43^{a}	2.76 ± 0.48^{a}	3.27 ± 0.34^{a}
CF	3.27 ± 0.26^{a}	3.86 ± 0.06^{a}	3.41 ± 1.40^{ab}	3.17 ± 0.29^{a}	2.28 ± 0.014^{b}	2.09 ± 0.17^{b}
ADW	0.193 ± 0.009^{ab}	0.224 ± 0.028^{a}	0.158 ± 0.012^{b}	0.163 ± 0.016^{ab}	0.196 ± 0.014^{ab}	0.217 ± 0.022^{a}
ADL	0.138 ± 0.012^{ab}	0.090 ± 0.009^{c}	0.107 ± 0.015^{bc}	0.137 ± 0.015^{ab}	0.165 ± 0.039^{a}	0.179 ± 0.009^{a}
Day 60						
GB	2.02 ± 0.70^{a}	5.10 ± 1.12^{b}	6.02 ± 0.54^{b}	5.87 ± 0.62^{b}	5.63 ± 0.98^{b}	5.97 ± 0.88^{b}
CF	2.66 ± 0.73^{ab}	2.19 ± 0.33^{a}	3.05 ± 0.27^{b}	3.01 ± 0.08^{b}	2.27 ± 0.58^{a}	2.96 ± 0.42^{b}
ADW	0.163 ± 0.033^{a}	0.306 ± 0.022^{b}	0.378 ± 0.023^{b}	0.392 ± 0.041^{b}	0.379 ± 0.037^{b}	0.389 ± 0.052^{b}
ADL	0.042 ± 0.006^{a}	0.165 ± 0.038^{bc}	0.150 ± 0.005^{b}	0.129 ± 0.009^{c}	0.124 ± 0.038^{bc}	0.054 ± 0.007^{a}

FM5+BA groups. There were no significant differences in CF between the C and the rest of the experimental groups. Finally, ADL recorded significantly ($P < 0.05$) lower values in the C and FM10+BA groups than those observed for the FM20, FM20+BA, BA+FM20, and FM5+BA groups.

Sex proportion and final survival

The results obtained for sex proportion and final survival are shown in Table 3. A percentage of males that deviates significantly ($P < 0.001$) from the proportion of males expected (50%) was observed only in the FM20, BA+FM20 and FM10+BA groups. Final survival with respect to the C group showed a significantly ($P < 0.05$) lower value for the FM20 group and a significantly ($P < 0.05$) higher value for the BA+FM20 group.

DISCUSSION

FM is a steroid that is increasingly used commercially due to its availability and capacity to achieve high percentages of males at very low concentrations. Moreno-Enríquez et al. (2003) and Ramírez-Ochoa et al. (2023) reported 95 and 92% of males using only 5 mg during 35 and 30 days, respectively. Twenty milligrams of FM were used by Villafuerte-Rincón (2008) and Calzada-Ruiz et al. (2020), who reported 100% masculinization. These results illustrate the importance of FM as an alternative to 17α-methyltestosterone. Although it is the most used steroid for sex reversal in Nile tilapia, over the years, it has faced numerous accusations as a result of its environmental and consumer risks (Megbowon & Mojekwu 2013, Straus et al. 2013).

In the present study, to ensure that the steroid supplied reached an optimal level in the bloodstream before sexual differentiation was initiated, the fry used still had remnants of their yolk sac at the beginning of the experiment, as suggested by Ramírez-Ochoa et al. (2023) to guarantee an optimal start of the sex-reversal process. However, in the present work, the percentage of males obtained was low (under 75%) in all the treatments fed the combination of FM and BA.

Table 3. Sex proportion and Final survival (FS) (mean ± standard deviation) of Nile tilapia fry (*Oreochromis niloticus*) fed with Fluoxymesterone (FM) and a commercial Blend of additives (BA) at different concentrations and forms of preparation. •Significantly different from the control group ($P < 0.05$). *Significantly different from the expected 1:1 distribution ($P < 0.001$). C: control treatment, FM20: commercial food supplemented with 20 mg of FM, FM20+BA: commercial food supplemented with 20 mg FM and subsequently 2 g of BA, BA+FM20: commercial food supplemented with 2 g of BA and subsequently 20 mg of FM, FM5+BA: food supplemented with 5 mg of FM and subsequently 2 g of BA, FM10+BA: food supplemented 10 mg of FM and subsequently 2 g of BA.

Group	Sex proportion		FS
	Males (%)	Females (%)	(%)
C	53.4 ± 2.4	47.3 ± 3.2	88.1 ± 1.8
FM20	89.9 ± 2.7*	11.2 ± 2.1	82.5 ± 2.6•
FM20+BA	66.5 ± 3.3	34.6 ± 3.1	86.3 ± 2.8
BA+FM20	68.9 ± 2.8*	33.1 ± 2.3	94.2 ± 2.4•
FM5+BA	65.1 ± 2.6	35.7 ± 2.2	88.6 ± 2.9
FM10+BA	71.3 ± 3.4*	29.6 ± 4.1	90.0 ± 3.1

Regarding masculinization, the most successful treatment was the FM20 group, which obtained a percentage of males close to 90%. Our results showed that low percentages of masculinization were obtained in all treatments supplemented with the steroid and the commercial blend, regardless of FM concentration or the order in which the commercial blend was added. Our results indicated that the differences in the percentage of males obtained could have been caused by the differential ingestion of FM and the commercial blend. The decrease in the intake of FM by ingesting the commercial blend during the critical stage (labile period) of Nile tilapia could have caused the decrease observed in the percentages of males, regardless of the concentration of FM used. During the labile period of Nile tilapia, the fry must increase the steroid levels (in this case, FM) in the bloodstream and keep them elevated during the days that sex reversal is achievable; the reason that for successful masculinization or feminization of Nile tilapia, it is recommended to feed every few hours to maintain adequate levels of the steroid in the bloodstream (El-Greisy & El-Gamal 2012, Alcántar-Vázquez et al. 2015, Marín-Ramírez et al. 2016, Juárez-Juárez et al. 2017, Trejo-Quezada et al. 2021, Ramírez-Ochoa et al. 2023). Another explanation for the low percentage of males observed in several groups could be the mixing and homogenization of the steroid when adding it to commercial food. Although FM used in the present study showed certain difficulties for dissolving in alcohol during the inclusion in the feed (as part of the evaporation method used for adding the steroid to the commercial food), as in previous studies in our laboratory, low percentage of males had not been previously observed as in the present experiment. Therefore, the low male percentages observed may result from the commercial blend in the experimental diets rather than the manufacturing method.

Although the commercial blend used during our experiment is recommended for all stages of development of Nile tilapia, its supplementation in the commercial feed during the fry period may have caused interference in the masculinization process. Studies reporting the supplementation of additives, alone or in blends, during the fry stage only cover aspects related to growth, body composition, immune response, and survival, with generally positive results in one or several of such aspects (Bombardelli et al. 2005, Kalko-Schwarz et al. 2011, Abdelhamid et al. 2012, Ibrahem et al. 2012, Ortiz et al. 2013, Sakaguti-Graciano et al. 2014, Tiengtam et al. 2015, Boonanuntanasarn et al. 2018, de Araújo et al. 2018, Zamora-Vera 2020, González-Cobián 2021). However, steroid-additive interaction and its effects on sex percentages have generally not been reported. Only a few authors have included this aspect, reporting no negative effects on the male percentage by the supplementation of an additive (Toyama et al. 2000, Mörschbächer et al. 2014, Rahman et al. 2019, Faust et al. 2023). In this respect, our work represents the first negative report of the androgen-additive interaction during the sex reversal process in Nile tilapia.

Currently, companies in Mexico that commercialize additives in blends do not have background studies contemplating the effect of manual supplementation at this critical stage. In consequence, farms that produce masculinized Nile tilapia are not certain about the use of these commercial blends without negative effects on sex reversal percentages. Therefore, the information obtained in our work could be of great value for short-term decision-making at the commercial level. However, first, it is necessary to determine the percentage of males obtained after the supplementation of a commercial additive to assess if these results depend on four factors: 1) type of additive, 2) concentration level, 3) type of mixture used (ingredients in the blend) or 4) supplementation method (namely: if the additive is added during or after the elaboration of the experimental diet).

At the end of the experiment, a higher growth was observed in the treatments that received the commercial blend or FM compared to that recorded in the control group. Previous reports, in terms of reproduction, sex reversal, and growth effects of FM in Nile tilapia (Moreno-Enríquez et al. 2003, Torres-Hernández et al. 2010, Ornelas-Luna et al. 2017, Trejo-Quezada et al. 2021), have concluded that, although it does not have a clear and powerful anabolic effect as 17α-methyltestosterone does (El-Saidy et al. 2005, Abdelghany 2010, Hafeez-ur-Rehman et al. 2014, Ajiboye et al. 2015, Trejo-Quezada et al. 2021), FM has shown, in some instances, a positive effect on growth at the end of the sex-reversal process (Manosroi et al. 2004, Gutiérrez-Sigueros et al. 2018, Ramírez-Ochoa et al. 2023). Ramírez-Ochoa et al. (2023) reported that FM withdrawal caused a growth depression in Nile tilapia juveniles.

In our work, all groups supplemented with the commercial blend showed a final growth performance higher than that observed in the C group. Although the supplementation of the commercial blend ended at 30 days, its effect remained for several weeks after its withdrawal. This result is more relevant considering the low percentage of males in this group compared to the FM20 group. Phelps & Popma (2000) suggested that improved growth of steroid-treated Nile tilapia is more related to the superior growth of males than to the anabolic response related to increased protein synthesis and muscle gain. It could explain the results obtained in the FM20 group, which presented the highest percentage of males and whose final growth is comparable to those that received the combination of FM and the commercial blend. The growth performance observed in all groups that received the commercial blend is comparable to that recorded in a predominantly male group (FM20), even though it was composed of a higher female population, which could indicate that the commercial blend improved the growth of females and considering that the sex reversal process was altered by the addition of the commercial blend, of the males as well; probably the reason behind the growth performance observed, especially in the last 15 days of the experiment (as seen in the values of GB, ADW and ADL).

Commercial additives containing one or several of the ingredients included in the blend used in this work (cinnamon, garlic, vitamin C, inulin) have been shown to improve growth performance (Toyama et al. 2000, Naftal-Gabriel et al. 2019, Rahman et al. 2019, Fattahi et al. 2021, Habiba et al. 2021, Pérez-Jiménez et al. 2022, Faust et al. 2023). However, in other studies, although evidence of improved health, chemical, digestive, or hematological parameters has been observed, no positive effect on growth was recorded (Kalko-Schwarz et al. 2011, Abdelhamid et al. 2012, Mörschbächer et al. 2014, Sakaguti-Graciano et al. 2014, de Araújo et al. 2018, de la Cruz-Marín et al. 2023). These variations in growth performance could result from several aspects, including species, age, duration of additive supplementation, and, more importantly, concentration and type of additive (Dawood et al. 2018).

Sodium chloride is an important component of the commercial blend used in the present study. This component may be responsible for the improved growth in experimental fish fed this additive blend. This ingredient increases appetite (Fontaínhas-Fernandes et al. 2000), contributing to increased food intake and improved growth rate in all groups supplemented with the commercial blend. The effect of sodium chloride on appetite and other ingredients (such as cinnamon, garlic, vitamin C, and inulin) could have contributed to the results observed in the growth parameters, especially GB and ADW. The beneficial effect of feeding sodium chloride in the diet could be related to a metabolic need for sodium and chloride ions in hypotonic environments (such as freshwater) where loss and environment uptake of these ions are major problems (Gatlin 2001, Lim et al. 2006).

In the present study, the addition of the commercial blend showed no apparent effect on fish survival compared to the control group, except for the BA+FM20 group. However, considering that the lower value of final survival was observed in the group supplemented with only FM at 20 mg per kg, adding the commercial blend might have helped reduce the steroid's negative effects on survival. Although FM is not considered a steroid with strong negative effects (Ramírez-Ochoa et al. 2023) on survival, our work showed that at a concentration of 20 mg kg^{-1}, it caused a decrease in the percentage of survival at the end of the experiment. Several authors have reported in Nile tilapia and its hybrids survival rates lower than those obtained in the present study, ranging from 24 to 83% for fish exposed to 17α-methyltestosterone and similar steroids through feeding (21 to 30 days) (Carranza 1990, Pérez 2002, Pinza-Pinza 2014) or immersions (López et al. 2007, Miranda-Cerritos et al. 2008, Botero et al. 2011).

The final survival observed in the group BA+FM20 supports the idea that the order in which the commercial additive and the steroid are included in the diet could affect the outcome of some parameters. However, more work is necessary to elucidate this. Previous studies evaluating the survival of fry supplemented with commercial additives (Bio-mos® and similar products made from *Saccharomyces cerevisiae*, T-Protphyt 2000, mannan-oligosaccharides, AminoGut®, fructooligosaccharides, inulin, probiotics, and prebiotics) during a sex reversal process showed similar results compared to those shown in our work, observing no differences in survival percentages (Abdelhamid et al. 2012, Mörschbächer et al. 2014, de Araújo et al. 2018, Rahman et al. 2019) or higher survival in the additive-supplemented fish (Samrongpan et al. 2008, Sakaguti-Graciano et al. 2014, García-Curbelo et al. 2017, Opiyo et al. 2019, Pérez-Jiménez et al. 2022, de la Cruz-Marín et al. 2023, Faust et al. 2023). These results depend on several variables (Dawood et al. 2018), including species, stage of development, type of commercial additive (prebiotic, probiotic, synbiotics, blends, or immunostimulants), and concentration. Additionally, it is important to consider the additive supplementation method (manually added to the commercial feed or during the formulation and elaboration of the feed) and when the additive is manually added, the supplementation order (in which the commercial additive and the steroid are added to the food).

In previous studies, individual components (inulin, vitamin C, cinnamon, and garlic) of the commercial blend used in the present study have been demonstrated to improve survival in several species. Cota-Gastélum (2011) reported a 100% survival rate of Nile tilapia when fed a diet supplemented with inulin. Furthermore, Ibrahem et al. (2012) reported that the survival percentage increased significantly in the experimental group that received a combination of inulin and ascorbic acid (vitamin C). Several authors have reported a positive effect of vitamin C on survival in various species after its single or combined supplementation for 14 days in the Siberian sturgeon *Acipenser baerii* (Xie et al. 2006), 45 days in the Colombian shark catfish *Hexanematichthys seemanni* (Dallos-Rodríguez 2007), and 60 days in the rohu *Labeo rohita* (Tewary & Patra 2008) and the totoaba *Totoaba macdonaldi* (Enciso-Contreras 2016). Furthermore, cinnamon and garlic have been shown to improve health parameters and, in consequence, positively affect final survival in Nile tilapia fed experimental diets during 90 days (Shalaby et al. 2006), swordtail *Xiphophorus helleri* in a 42 days feeding period (Kalyankar et al. 2013), grass carp *Ctenopharyngodon idella* during 60 days (Ghafoor et al. 2020), European sea bass *Dicentrarchus labrax* fed experimental diets during 90 days (Habiba et al. 2021), and the rainbow trout *Oncorhynchus mykiss* in 56 days (Fattahi et al. 2021).

CONCLUSION

The use of the commercial blend of additives during the sex reversal period negatively affected the percentage of males obtained, regardless of the concentration of FM used or the order in which the commercial blend was added, which could be the result of the reduction in the intake of FM and the increase in the intake of the commercial blend. On the other hand, growth and survival were positively affected by the inclusion of the additives, even 30 days after its withdrawal, showing that the commercial blend's ingredients have a long-lasting effect on the fish physiology. Finally, the commercial blend of additives has started being incorporated into the commercial fry feed during its elaboration, so further studies are suggested to evaluate the percentage of males obtained after using this new supplementation method.

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