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Research Article

Effect of the combination of a cold-water temperature and exogenous estrogens on feminization, growth, gonadosomatic index and fat muscle content of Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758)

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ABSTRACT. The use of water temperature to modify sex proportion in Nile tilapia has been suggested as an alternative to eliminating the use of steroids since it was discovered that it has an important role in the sex differentiation process. However, the use of cold-water temperatures to achieve feminization has rarely been explored and never has it been combined with exogenous estrogens. This work aimed to determine if the proportion of females could be increased by combining low concentrations of estrogens with a cold-water temperature, and describing its effect on growth parameters, gonadosomatic index, and fat muscle content. Two experiments were carried out, each one set at a specific water temperature (27.5 and 21.5ºC). In each experiment four treatments (per triplicate) were evaluated (control -no estrogens-, estradiol-17β E_2, 17α-ethinylestradiol EE_2 and diethylstilbestrol DES). Higher feminization rates were observed in all groups, including the control group, reared at 21.5ºC. Growth parameters showed significantly higher values ($P < 0.05$) for the groups reared at 27.5ºC. The control group and the group fed E_2 reared at 21.5ºC showed significantly higher ($P < 0.05$) values of gonadosomatic index than that observed in the groups reared at 27.5ºC. Fat muscle content was significantly higher ($P < 0.05$) in both experiments for the groups fed E_2 and EE_2. The fry used showed a high tendency towards feminization by exposure to a cold-water temperature. Our results allow us to consider E_2 (21.5ºC) the best option for the development of YY technology.

Keywords: *Oreochromis niloticus*; feminization; thermosensitivity; growth depression; gonads; fat in muscle

INTRODUCTION

Worldwide production of the fish group known as tilapia has increased steadily, turning its culture into an agro-industrial activity of high economic and social impact through which it is possible to generate income, improve the quality of life in rural areas and offer food of high nutritional value to the world population (FAO, 2009). Currently, Nile tilapia (*Oreochromis niloticus* Linnaeus, 1758) is responsible for more than 99.2% of the world tilapia production, while the remaining 0.8% corresponds to other species including Mozambique tilapia (*Oreochromis mossambicus* Peters, 1852), blue tilapia (*Oreochromis aureus* Steindachner, 1864), redbreast tilapia (*Coptodon rendalli* Boulenger, 1897) and redbelly tilapia (*Coptodon zillii* Gervais, 1848) (FishStatJ, 2016).

In Mexico, tilapia (mainly Nile tilapia) represents approximately 75% of the production of farmed fish, with an annual production of 117,806 t per year (SAGARPA, 2017). Nile tilapia offers many advantages over other fish species; however, and despite its relatively low fecundity (Phelps & Popma, 2000), in mixed-sex cultures the control of its early maturity is a recurring problem. Nile tilapia fish reach sexual maturity (30-50 g) long before commercial size (between

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200 to 800 g, depending on the market and country) (Jiménez & Arredondo, 2000; Arboleda-Obregón, 2005; DeLong *et al*., 2009; Negroni, 2013; Romana-Eguia *et al*., 2013; Arredondo-Figueroa *et al*., 2015). Therefore, in commercial farming of Nile tilapia, reproduction during grow-out is a major problem leading to the presence of fry and juveniles that overpopulate ponds and ultimately resulting in a wide range of fish sizes at harvest instead of the larger and more uniform fish expected from the original stocking (Mair *et al*., 1997; Tariq-Ezaz *et al*., 2004).

To avoid this, a series of techniques have been developed, the production of a monosex, all-male population being the most commonly used. All-male population culture eliminates sex behavior and therefore uncontrolled reproduction, allowing the production of marketable-sized fish (Varadaraj, 1989; Ponzoni *et al*., 2005). Therefore, it has been recognized for many years as the most effective technique to increase Nile tilapia production under commercial culture conditions (Mair *et al*., 1997; Müller & Hörstgen-Scharwk, 2007; Phumyu *et al*., 2012).

Sex-reversal by feeding fry with different steroids is the most common method used to produce all-male populations. However, in recent years this method has been increasingly criticized. The accumulation of hormones in the environment, as well as the increasing number of consumers that are not interested in eating products that have been treated with hormones or other active substances (Piferrer, 2001; Müller & Hörstgen-Scharwk, 2007; Leet *et al*., 2011) have led to the search for alternative techniques for the production of all-male Nile tilapia populations.

One of the most promising techniques involves the production of YY males, which when combined with normal females (XX) produce progenies composed of, in theory, 100% genetic males. The initial development of the YY technology requires using hormones to feminize sexually undifferentiated male fry (XY) and to identify these newly created XY females through progeny test (Vera-Cruz *et al*., 1996; Mair *et al*., 1997; Alcántar-Vázquez *et al*., 2014). These females will be used to produce YY males in a percentage of 25% when combined with normal males (XY).

Production in the last three years of YY males at the Universidad del Papaloapan has led to attempts to optimize the feminization process (Alcántar-Vázquez *et al*., 2015; Marín-Ramírez *et al*., 2016). One viable alternative for optimizing the production of XY females is feminization through the combination of a low concentration of estrogens (to avoid the observed reduction in survival and growth) and a cold-water temperature during early days of the fry period. The exposure to cold-water temperatures has been associated with an increase in the proportion of females (gonadal feminization) in several species including the Mozambique tilapia (Wang & Tsai, 2000. Only a few works have addressed the effect of cold-water temperatures on sex proportions in Nile tilapia (Baroiller *et al*., 1995a; Desprez & mélard, 1998; Abucay *et al*., 1999; Azaza *et al*., 2008); however, currently, there are no reports of the use of a cold-water temperature in combination with estrogens to achieve a higher feminization rate of Nile tilapia XY fry.

Previous work carried out in our laboratory using the three most important estrogens (estradiol-17β, diethylstilbestrol, and 17α-ethinylestradiol) per separate in different trials (Alcántar-Vázquez *et al*., 2015; Marín-Ramírez *et al*., 2016; Juárez-Juárez *et al*., 2017) has shown that lower concentrations (100-200 mg kg^{-1}) did not achieve a 100% rate of feminization in Nile tilapia. Taking this into account, the present study was developed to (1) determine whether a higher proportion of sexually undifferentiated XY-Nile tilapia fry could be sex-reversed to functional XY females by combining a low concentration of estrogens with a cold-water temperature during the early days of fry period, and (2) describe the effect of this combination on growth, gonadosomatic index and fat muscle content after sex differentiation in cultivated Nile tilapia.

MATERIALS AND METHODS

Broodstock

Nile tilapia males (450-600 g) used in this research were produced in the Aquaculture Station of the Universidad del Papaloapan, from locally available strains (Centro Acuícola de Temazcal, Oaxaca, México and Granja Unidad de Producción del Tesechoacan, Veracruz). Nile tilapia females were donated (300-350 g) by the Granja Unidad de Producción del Tesechoacan and acclimated for 20 days in 3 m outdoor concrete tanks, before starting the experiments. During this time, males and females were fed twice a day with commercial pellets with 32% protein (optimum protein-energy ratio of 110-120 mg per kcal digestible energy (Shiau, 2002) (Nutripec, Agribrands Purina, Irapuato Gto. Mexico).

Preparation of estrogen treatment

Three estrogen treatments were prepared for the present experiments, one including the natural estrogen estradiol-17β (E_2) and the other two including the synthetic estrogens 17α-ethinylestradiol (EE_2) and diethylstilbestrol (DES) (Sigma Aldrich Chemical Co., St Louis, MO, USA). Each estrogen was added to one kilogram of the commercial fish meal (<0.35 mm, 53% protein, 15% lipids) using the method described by

Guerrero (1975). In short, 120 mg of each estrogen was dissolved in 500 ml of 95% ethanol, sprayed over a kilogram of fish food and maintained overnight at room temperature to allow the alcohol to evaporate. The food for the control group was handled in exactly the same manner with exception of the added estrogen. In total, four treatments (in triplicate) were evaluated in each experiment; control group (no estrogens), E_2 (120 mg kg^{-1}), EE_2 (120 mg kg^{-1}) and DES (120 mg kg^{-1}).

Fry production

Nile tilapia breeders (male:female) were stocked at a ratio of 1:3 in two 3 m diameter outdoor concrete tanks (28-30ºC) supplied with fertilized water. Fry was collected 14 days later with a fine-mesh net after 90% of the water in the tanks had been siphoned. The recently hatched and sexually undifferentiated fry (~0.02 g wet weight and 8 mm length) were pooled, transported to a closed recirculating system and randomly divided into 12 acrylic aquaria of 85 L (three aquaria per treatment) at an initial stocking density of 0.6 fry L^{-1}. The water in the recirculating system was filtered using a mechanical filter (Hayward, Model S310T2, Hayward Pool Products Inc., Elizabeth, NJ, USA), a biofilter containing only plastic bio-balls (Aquatic Eco-System, Model CBB1, Pentair Ltd. Apopka, FL, USA) and UV sterilizer (Lumiaction, Model BE1X20, Lumiaction Co. Ltd, Taipei, Taiwan).

Experimental conditions

In total, two experiments developed at different water temperatures (during the estrogen treatment) were carried out. For the first experiment, water temperature was thermostatically adjusted and controlled at 27.5 ± 1ºC, while for the second experiment, once fry was stocked in the acrylic aquaria, water temperature (~27.0ºC) was lowered thermostatically over a period of 24 h until reaching a final temperature of 21.5 ± 0.5ºC. Once the estrogen treatment was completed, water temperature was gradually raised to 27ºC. In both experiments estrogen treatment lasted for 20 d under a photoperiod of 12 light: 12 dark. Fry were fed *ad libitum* with the food supplemented with estrogens 11 times a day 1 h intervals. Water flow was closed in all aquaria for 20 min after the estrogen-enriched feeds were offered in order to encourage feeding.

Once the estrogen treatment was completed, fry were fed with an untreated commercial diet containing 53% protein (<0.35 mm, Nutripec Purina®) for 10 more days for the first experiment and for 20 more days for the second experiment (due to the smaller size of the fry). Aquaria were siphoned daily to remove feces and dead fry. Water temperature was monitored daily using a multiparameter display system (YSI model 655, Yellow Springs Instrument Co., OH, USA). Random samples of 25% of fish per replicate were collected every 10 d during the fry period. Mean weight was obtained using a digital scale (±0.01 g) (Ohaus Cor., Scout Pro Model Sp 202, Parsippany, NJ, USA), and total length was recorded from a digitized image using imaging software (ImageJ version 1.3).

All the fingerlings obtained from each treatment at the end of the fry period were counted; weighed and measured for the calculation of survival rate, mean wet weight and total length. The juveniles were nursed in outdoor 1.2 m diameter floating net cages and fed *ad libitum* six times a day (50% protein, 0.1 mm, Nutripec Purina®) for 20 days, followed by a commercial diet at 44% protein (1.5 mm, Nutripec Purina®) for another 20 days. Finally, fish were marked with visible implant elastomer tags (one color for each treatment replica) (Northwest Marine Technology, Inc.) then transferred to 3 m diameter concrete tanks and reared up to approximately 114 days of age until sex proportion could be safely determined by removing the gonad. During this time, fish were fed *ad libitum* three times a day with commercial diet (40% protein, 2.4 mm, Nutripec Purina®) for approximately 15 days, followed by a commercial diet at 35% protein (3.5 mm, Nutripec Purina®) three times a day for another 20 days and finally a commercial diet with 25% protein (3.5 mm, Nutripec Purina®) until the end of the experiment. Total length and wet weight were registered every 21 days using a digital scale (±0.01 g) and an ichthyometer (Aquatic Eco System, Inc., Apopka, FL, USA). Water temperature ranged from 28 to 31ºC during all the outdoor (culture in net cages and concrete tanks) part of the experiment.

Growth

Growth performance was evaluated in terms of final body weight (FBW), final total length (FTL), biomass gained (BG), body weight gain per day (BWG), specific growth rate (SGR) and condition factor (CF). This evaluation was performed at the end of the fry period (30 days) and at the end of the experiment (114 days) for each experiment (27.5ºC and 21.5ºC). The following formulae were used:

$$BG = FBW - IBW$$

$$BWG\ (g\ d^{-1}) = (FBW - IBW) / DT$$

$$SGR = \ln (FBW/IBW) \times 100\ DT^{-1}$$

where dt is the duration of the experiment in days; FBW and IBW are the final body weight and initial body weight, respectively.

$$CF = 10^3\ (\text{mean weight} / \text{total length}^3)$$

Evaluation of sex proportion

The sex of the fish in each treatment was determined by external examination using dye (methylene blue at 1%) to highlight the differences in the papilla structure. Each fish was classified as male, female or undifferentiated. Additionally, 30% of the fish in each treatment were weighed, measured and sexed again by removing the gonads. Gonads were classified as ovaries, testes or undifferentiated.

Gonasomatic index (GSI)

The extracted gonads were weighed using a digital scale (±0.01 g) to calculate the GSI using the following formula:

$$GSI = [\text{gonad weight (g)} / \text{fish weight (g)}] \times 100$$

Fat muscle content

Analyses were made on muscle (including skin) samples obtained from the lateral part of the fish. Muscle samples were cut into slices (5-10 g), oven dried at 120ºC for 24 h and homogenized with the aid of an industrial blender. Fat content was measured using the method 920.39 (AOAC, 1997) and expressed as a percentage of fat in muscle.

Statistical analysis

Results of growth performance were subjected to a one-way ANOVA, and the significance of the difference between means was tested using Duncan´s multiple range test. Differences were deemed to be significant at $P < 0.05$. Final survival was analyzed using a chi-square test. The proportion of females identified in each treatment was tested against the 1:1 expectation using a chi-square test at a probability of 0.1% ($P < 0.001$). Differences in the gonadosomatic index and fat muscle content between treatments were analyzed using a Kruskal-Wallis nonparametric analysis. Finally, a binomial test (two-tailed) was used to analyze the proportion of females obtained between experiments (water temperatures).

RESULTS

Growth performance

Final body weight (FBW), final total length (FTL), biomass gained (BG), body weight gained per day (BWG), condition factor (CF) and specific growth rate (SGR) for all groups at both water temperatures are given in Table 1. At the end of the fry period, FBW and BG were significantly higher ($P < 0.05$) in the control group reared at 27.5ºC in comparison to the estrogen-treated groups reared at the same water temperature, and all the groups reared at 21.5ºC. Between the estrogen-treated groups, the ones reared at 27.5ºC showed significantly higher ($P < 0.05$) values of FBW and BG than that registered at 21.5ºC (Table 1).

The same tendency was observed for the values of FTL, BWG, and SGR; however, in this case, the control group and the group fed E_2 reared at 27.5ºC were the ones showing the significantly higher ($P < 0.05$) values in comparison to that observed for the synthetic estrogens (EE_2 and DES) reared at the same water temperature, and all the groups reared at 21.5ºC. On the contrary, the control group reared at 21.5ºC showed the significantly higher ($P < 0.05$) value of CF in comparison to the estrogen-treated groups reared at the same water temperature, and all the groups reared at 27.5ºC. Between the estrogen-treated groups, the groups reared at 21.5ºC registered significantly higher ($P < 0.05$) values than that observed in the groups reared at 27.5ºC (Table 1).

At the end of the experiment (114 days of age) significant differences ($P < 0.05$) were observed in the values of FBW and BG, with the groups fed E_2 and EE_2 reared at 27.5ºC showing the higher values in comparison to the group fed DES and all the groups reared at 21.5ºC (Table 1). Between the groups reared at 21.5ºC, the group fed E_2 showed the significantly higher values ($P < 0.05$) of FBW and BG. A significantly lower ($P < 0.05$) value of FTL was observed in the groups fed DES (27.5ºC), and EE_2 (21.5ºC) in comparison to the control group and the group fed E_2 reared at 27.5ºC (Table 1).

The significantly higher ($P < 0.05$) CF value was registered in the group fed EE_2 followed by the group fed E_2, both reared at 27.5ºC. No significant differences were observed in the values of CF between all the groups reared at 21.5ºC, and the rest of the groups reared at 27.5ºC. In terms of SGR, significant differences ($P < 0.05$) were registered, with the group fed E_2 (21.5ºC) showing a higher value than that observed in the control group and the groups fed EE_2 and DES reared at the same temperature and all the groups reared at 27.5ºC. Between the groups reared at 27.5ºC, the group fed EE_2 showed a significantly higher ($P < 0.05$) value of SGR compared to the control group and the groups fed E_2 and DES (Table 1).

Final survival was significantly lower ($P < 0.05$) at the two water temperatures analyzed in the groups fed the synthetic estrogens EE_2 and DES in comparison to the control group and the group fed E_2 (Table 2).

Gonadosomatic index (GSI)

No significant differences in GSI were observed between the control group and the estrogen-treated groups at each water temperature. However, between water temperatures, the control group and the group fed E_2 reared at 21.5ºC showed significantly higher ($P < 0.05$) values of GSI in comparison to that observed in

Table 1. Effect of two water temperatures and feeding estrogens for 20 days on growth performance (mean ± standard error; n = 3) of Nile tilapia fry (*Oreochromis niloticus*). E_2: Estradiol-17, EE_2: 17-ethinylestradiol, DES: diethylstilbestrol. FBW: Final body weight, FTL: final total length, BG: biomass gained, BWG: body weight gain per day, CF: condition factor, SGR: specific growth rate. Values in each row superscripted with different letters indicate significant differences between groups ($P < 0.05$).

Parameter	Water temperature (ºC)							
	27.5 ± 1.0				21.5 ± 0.5			
	Control	E_2	EE_2	DES	Control	E_2	EE_2	DES
30 days								
FBW	1.39 ± 0.06^{a}	1.25 ± 0.05^{b}	0.83 ± 0.08^{c}	0.78 ± 0.09^{c}	0.49 ± 0.03^{d}	0.42 ± 0.04^{e}	0.41 ± 0.03^{e}	0.44 ± 0.03^{e}
FTL	4.38 ± 0.14^{a}	4.14 ± 0.16^{a}	3.58 ± 0.11^{b}	3.45 ± 0.12^{b}	2.46 ± 0.15^{c}	2.43 ± 0.12^{c}	2.41 ± 0.13^{c}	2.51 ± 0.10^{c}
BG	1.37 ± 0.06^{a}	1.23 ± 0.05^{b}	0.81 ± 0.08^{c}	0.76 ± 0.09^{c}	0.47 ± 0.03^{d}	0.40 ± 0.04^{d}	0.39 ± 0.03^{d}	0.42 ± 0.03^{d}
BWG	0.045 ± 0.005^{a}	0.041 ± 0.005^{a}	0.027 ± 0.004^{b}	0.025 ± 0.002^{b}	0.015 ± 0.002^{c}	0.013 ± 0.003^{c}	0.013 ± 0.003^{c}	0.014 ± 0.004^{c}
CF	1.65 ± 0.05^{d}	1.76 ± 0.09^{cd}	1.80 ± 0.08^{cd}	1.89 ± 0.06^{c}	3.29 ± 0.11^{a}	2.92 ± 0.08^{b}	2.92 ± 0.08^{b}	2.78 ± 0.07^{b}
SGR	14.13 ± 0.07^{a}	13.78 ± 0.07^{a}	12.41 ± 0.06^{b}	12.21 ± 0.05^{b}	10.66 ± 0.07^{c}	10.14 ± 0.07^{d}	10.06 ± 0.04^{d}	10.30 ± 0.04^{cd}
114 days								
FBW	82.50 ± 5.49^{ab}	99.91 ± 4.18^{a}	99.84 ± 4.28^{a}	60.29 ± 4.54^{c}	69.6 ± 3.30^{c}	80.19 ± 3.64^{b}	62.15 ± 3.76^{c}	65.53 ± 3.03^{c}
FTL	15.94 ± 0.23^{a}	16.20 ± 0.27^{a}	14.82 ± 0.22^{ab}	14.21 ± 0.35^{b}	15.08 ± 0.55^{ab}	15.55 ± 0.45^{ab}	14.54 ± 0.20^{b}	14.92 ± 0.32^{ab}
BG	81.11 ± 4.94^{ab}	98.66 ± 5.81^{a}	99.01 ± 3.22^{a}	59.51 ± 4.62^{c}	69.11 ± 3.32^{bc}	79.77 ± 3.10^{b}	61.74 ± 3.53^{c}	65.11 ± 3.32^{c}
BWG	0.96 ± 0.05^{b}	1.17 ± 0.06^{a}	1.18 ± 0.06^{a}	0.70 ± 0.04^{c}	0.82 ± 0.04^{bc}	0.94 ± 0.03^{b}	0.73 ± 0.05^{c}	0.77 ± 0.04^{c}
CF	2.03 ± 0.11^{c}	2.34 ± 0.07^{b}	3.06 ± 0.12^{a}	2.10 ± 0.08^{c}	2.02 ± 0.09^{c}	2.13 ± 0.09^{c}	2.02 ± 0.10^{c}	1.97 ± 0.08^{c}
SGR	4.86 ± 0.06^{e}	5.21 ± 0.06^{d}	5.70 ± 0.04^{c}	5.17 ± 0.05^{d}	5.90 ± 0.05^{b}	6.25 ± 0.04^{a}	5.97 ± 0.06^{b}	5.95 ± 0.02^{b}

the control group and the estrogen-treated groups reared at 27.5ºC (Table 2).

Percentage of females

Sex-reversed females were produced at both water temperatures; however, at 27.5ºC only the groups fed EE_2 and DES produced a progeny significantly skewed toward female with a sex ratio that deviated significantly ($P < 0.001$) from the 1:1 sex ratio expected in Nile tilapia crosses between normal males (XY) and normal females (XX) (Table 2). At 21.5ºC a significant increase ($P < 0.001$) in the percentage of females was observed in all estrogen-treated groups and the control group. A 100% feminization rate was registered in the groups fed E_2 and DES (Table 2).

Percentage of females between water temperatures

The results of the binomial test (two-tailed) showed significant differences between experiments for the control group (Z = 6.3, d.f. = 2, $P < 0.01$) and the group fed E_2 (Z = 3.9, d.f. = 2, $P < 0.01$). No significant differences were observed for the groups fed EE_2 (Z = 1.1, d.f. = 2, $P = 0.1335$) and DES (Z = 1.0, d.f. = 2, $P = 0.1562$) (Table 2).

Fat muscle content (FMC)

Significant differences ($P < 0.05$) were observed in FMC, with the groups fed E_2 and EE_2 showing the higher values at both water temperatures in comparison to the control group and the group fed DES (Table 2). No significant differences were observed between the control group, and the group fed DES at both water temperatures.

DISCUSSION

In a commercial culture of Nile tilapia, production of monosex all-male populations through exogenous steroids reduces the time needed to produce fish of commercial size thereby eliminating the problems associated with gonadal maturation (Varadaraj & Pandian, 1989). However, current trends in both the market and the research being conducted show an interest in reducing the use of hormones during sex-reversal treatments to produce all-male populations. One of the most important alternative techniques at the commercial level is the production of YY males (Mair *et al.*, 1997). Although the initial stage of the YY technology requires the feminization of XY fry to produce XY females, hormones are not administered to the final product, which significantly reduces the concentration of hormones in the farm effluents (Marín-Ramírez *et al.*, 2016).

Feminization, therefore, is one of the critical stages of YY-male technology. Production and identification of females with an XY genotype will ensure an adequate number of YY males. As for masculinization, feminization is achieved mainly through the addition of hormones (in this case estrogens) to the food provided

Table 2. Performance at the end of the experiment (114 days of age) of Nile tilapia fry (*Oreochromis niloticus*) reared at two water temperatures and fed different estrogens for 20 days (mean ± standard error; n = 3). *Survival percentage significantly different from the control group ($P < 0.05$). [1]Significantly different from the expected 1:1 distribution ($P < 0.001$). [2]Significantly different between water temperatures, binomial test ($P < 0.001$). Values in each column superscripted with different letter indicate significant differences between groups ($P < 0.05$). S: survival, M: males, F: females, U: undifferentiated, GSI: gonadosomatic index, FMC: fat muscle content.

Treatment (ºC)	S	Sex percentage			GSI	FMC
27.5 ± 1.0		M	F	U		
Control	98	54	45[2]	1	0.9 ± 0.2[bc]	10.7 ± 0.34[b]
E_2	91	33	64[2]	3	0.5 ± 0.2[c]	12.6 ± 0.05[a]
EE_2	71*	14	83[1]	3	0.6 ± 0.1[c]	14.5 ± 0.79[a]
DES	70*	5	91[1]	4	0.6 ± 0.1[c]	10.6 ± 0.06[b]
21.5 ± 1.0		M	F	U		
Control	99	-	98[1,2]	2	1.5 ± 0.2[a]	9.7 ± 0.07[b]
E_2	98	-	100[1,2]	-	1.7 ± 0.3[a]	12.8 ± 0.09[a]
EE_2	63*	-	93[1]	7	1.3 ± 0.2[ab]	12.6 ± 0.54[a]
DES	54*	-	100[1]	-	1.4 ± 0.2[ab]	9.9 ± 0.51[b]

provided to the fish during the fry period (Piferrer, 2001); however, the control of water temperature to achieve sex reversal is an alternative which will further contribute to the reduction of hormones in the commercial culture of Nile tilapia.

The application of specific water temperatures during fry period has been considered an option to reverse the sex in Nile tilapia since it was discovered that sex determination was controlled by the interaction of the following three components; a complex genetic sex determination system with a major determinant locus (sex chromosomes XX/XY), some minor genetic factors (parental factors), and the influence of environmental factors (Baroiller *et al.*, 2009), especially the water temperature during the fry period (Wang & Tsai, 2000; Baroiller *et al.*, 2009). However, only a few works have actually used water temperature to alter the sex proportion, specifically to try to masculinize Nile tilapia (Baroiller *et al.*, 1995a,b, 1996; Desprez & Melard, 1998; Baras *et al.*, 2001; Azaza *et al.*, 2008). Wang & Tsai (2000) reported that water temperatures above 32°C would increase the production of reductase and/or an androgen receptor that in turn would produce a masculinization of the gonads, while cold-water temperatures (close to 21°C), such as the one used in this work, would increase the production of aromatase and/or an estrogen receptor which in turn would produce a feminization of the gonads. The former supports our results, as we obtain a higher rate of feminization using a cold-water temperature compared to the water temperature of 27.5°C in all four treatments. However, this increase was only significant for the control group, and the group fed E_2. The high percentage of females observed in the control group suggests that the cold-water temperature used was potent enough to induce a high gonadal feminization without the need of exogenous estrogens. The 100% feminization rate obtained in the groups fed E_2 and DES was probably produced by an increase in the production of aromatase stimulated by the cold-water temperature, which resulted in a higher quantity of androgen substrate transformed into functional estrogen, which in turn when added to the orally administered estrogen resulted in a more potent feminizing effect of the gonadal tissue.

The fact that the control group showed almost complete feminization (98%) of its progeny at 21.5°C indicates that the progeny produced by our breeders have a high sensitivity to water temperature. This thermosensitivity has been previously reported by Wang & Tsai (2000) in the Mozambique tilapia using a water temperature of 20.0ºC. However, in Nile tilapia several authors report no feminizing effect at cold-water temperatures (Baroiller *et al.*, 1995a; Desprez & Melard, 1998; Abucay *et al.*, 1999; Tessema *et al.*, 2006), only a masculinizing effect at high-water temperatures (Baroiller *et al.*, 1995a,b, 1996; Desprez & Mélard, 1998; Abucay *et al.*, 1999; Baras *et al.*, 2001; Azaza *et al.*, 2008; D´Cotta *et al.*, 2008; Rougeot *et al.*, 2008; Wessel & Hörstgen-Schwark, 2011). Azaza *et al.* (2008) reported that these variations can be explained by the variability of strains and populations used between experiments. This indicates that the strains from which we obtain our progeny have this thermosensitivity. In relation to the above, Baroiller *et al.* (2009) report that a clear parental effect with respect to thermosensitivity with an influence of both parents has been demonstrated at the individual level. That is, both males and females used may be responsible for the sensitivity observed in the percentages of feminization.

Wang & Tsai (2000) suggest that the period of action to achieve feminization of the gonad by cold-water temperatures is before the fry reach 10 days of age, while high-water temperatures induce masculinization of the gonad after the 10 days of age. Thus, water temperature can induce the feminization or masculinization of the gonads during a short period during the development of the fry. In our experiment, feminization was initiated once the fry was transferred to the recirculating system (less than five days of age), ensuring a high percentage of sexual reversion.

Despite being synthetic hormones, EE_2 and DES do not frequently induce 100% feminization according to previous studies reported for Nile tilapia (Hopkins *et al.*, 1979; Potts & Phelps, 1995; Hamdoon *et al.*, 2013; Marín-Ramírez *et al.*, 2016; Juárez-Juárez *et al.*, 2017) explaining why the percentages of feminization obtained in the groups reared at 27.5ºC did not achieve a 100% feminization rate. However, they are higher when compared to the percentages reported in previous studies for Nile tilapia, 64% for E_2 (compared to 60-66%), 83% for EE_2 (compared to 80-90% reported) and finally 91% for DES (compared to 80-87%) (Potts & Phelps, 1995; Hamdoon *et al.*, 2013; Alcántar-Vázquez *et al.*, 2015). These percentages, as previously discussed, increased considerably for the groups reared at 21.5ºC as a consequence of a physiological interaction between the cold-water temperature and the exogenous estrogen. This highlights the feminizing effect of cold-water temperatures and underlines its importance as a tool to reduce or eliminate the use of hormones during the sex reversal of Nile tilapia with the objective of producing YY males.

In both experiments, it was possible to observe a decrease in final survival in the groups fed exogenous estrogens, especially the two synthetic estrogens (EE_2 and DES) in comparison to the natural one (E_2). Piferrer (2001) reports that the application of estrogens can adversely affect survival in a large number of species of the teleost, especially if a certain limit (depending on each particular species) is exceeded. Shved *et al.* (2009) reported that exposure to EE_2 during early development negatively affects the IGF (insulin-like growth factor) system in Nile tilapia immune organs and could potentially interfere with the antigen presentation capacity of the immune system thereby altering the susceptibility to infections during growth and reducing survival. This could explain the reduction in survival observed in the two synthetic estrogens used in the present work. A similar reduction in survival using DES at different concentrations has been reported in previous works (Zhong *et al.*, 2005; Hamdoon *et al.*, 2013). Previous works conducted in our laboratory using DES (Marín-Ramírez *et al.*, 2016) and EE_2 (Juárez-Juárez *et al.*, 2017) support this.

Final survival in the control group and the group fed E_2 reared at 21.5°C did not show a significant reduction in comparison to that observed for the same groups reared at 27.5ºC. This indicates that mortality observed in the groups fed synthetic estrogens was probably the result of the previously reported negative effect produced at a physiological level for these estrogens. However, we cannot rule out the possibility that the feminizing effect of cold-water temperature acted synergistically at the physiological level with the synthetic estrogens supplied producing the same effect as a much higher estrogen concentration. Taking into account that the synthetic estrogens used in our experiments are known to be the most potent for feminizing Nile tilapia, this could explain the high mortality observed in those groups at 21.5ºC.

Although some androgens have been shown to be growth-promoting agents when administered at low concentrations (Piferrer, 2001), in general, estrogens either natural or synthetic do not have a positive effect on the growth of teleost (Ridha & Lone, 1995). However, a decrease in growth has been reported for several species, especially when the estrogen concentration is increased (Piferrer, 2001; Hendry *et al.*, 2003; Arslan *et al.*, 2009; Hamdoon *et al.*, 2013, Marín-Ramírez *et al.*, 2016; Juárez-Juárez *et al.*, 2017). In our experiments, higher growth, biomass, and specific growth rate were observed using the natural estrogen E_2 in comparison to the synthetic estrogens, especially DES. This agrees with previous experiments carried out in our lab using E_2 and DES in independent trials. In this case, groups fed E_2 showed a similar growth compared to the control group (Alcántar-Vázquez *et al.*, 2015) while groups fed DES showed a decrease in growth rate at all concentrations used (Marín-Ramírez *et al.*, 2016). Similar findings were reported by Król *et al.* (2014) for the European catfish (*Silurus glanis* Linnaeus, 1758), in which case a decrease in growth rate was observed using the synthetic estrogen DES, while the natural estrogen E_2 did not cause such decrease. In other studies evaluating only E_2, groups fed this estrogen have shown in comparison to the control group either a similar growth rate (Wang *et al.*, 2008; Balali *et al.*, 2012), a significant lower one (Komen *et al.*, 1989; Gorycczko *et al.*, 1991) or a significant increase (Shreck & Fowler, 1982; Degani, 1986; Chiba *et al.*, 1993; Güzel *et al.*, 2006). Herman & Kinkaid (1988) report that different metabolic pathways between species may be responsible for this increase/decrease in growth. Piferrer (2001) and Alcántar-Vázquez *et al.* (2015) indicate that the presence of negative, neutral or positive effects on growth after a treatment with estrogens seems to be highly dependent on the biology of the particular species, type of hormone and its concentration. Several authors have reported that the negative effects of estrogens on growth are related to the disruption or alteration of the thyroid and/or the GH/IGF-I axis (growth hormone/insulin-like-growth factor) (Leatherland, 1985; Ikuta *et al.*, 1987; Cyr & Eales, 1996; Arsenault *et al*., 2004).

Wang *et al.* (2008) report that this increase or decrease in growth parameters can change between experimental stages; during the estrogen treatment stage of the experiment, growth rate of groups treated

with estrogens decreases in comparison to the control group, while in the post-feminization stage this growth rate increases until reaching a similar value compared to the control group or surpasses it significantly. This growth pattern was observed in our work, especially in the groups fed synthetic estrogens. During the feminization stage (fry period) FBW, SGR, BG, and BWG showed lower values in the estrogen-treated groups, independently of water temperature, while in the post-feminization stage FBW, SGR, BG, and BWG were similar (especially for EE_2) or higher (E_2) in comparison to the control group. This growth recovery following hormonal treatment may be regarded as compensatory growth (Avelar *et al.*, 2014). This growth is described as an accelerated growth commonly seen when favorable conditions are restored after a period of growth depression (Ali *et al.*, 2003). Several authors have reported this compensatory growth phase after a steroid treatment (Vera-Cruz & Mair, 1994; Wang *et al.*, 2008; Avelar *et al.*, 2014). In our experiment, compensatory growth is probably what is responsible for the higher SGR observed in the fish reared at 21.5ºC in comparison to the ones reared at 27.5ºC. This could be explained if we take into account that in this case the growth depression observed during the fry period was caused not only by the estrogen treatment but also by the cold-water temperature at which the fish were reared, leading to a compensatory growth enforced by an increase in metabolic rate and in food intake. Azaza *et al*. (2008) reported that at cold-water temperatures the observed growth depression is due mainly to the loss of appetite and to the higher energy cost for maintenance metabolism. In those cases, a substantial amount of the energy obtained though the food is used to cover maintenance metabolism at the expense of somatic growth (Brett, 1979). This explains the lower FBW, SGR, BWG, and BG observed in all the groups reared at 21.5ºC in comparison to that reared at 27.5ºC.

The CF observed an opposite pattern in comparison to the rest of the growth parameters during the fry period, with higher values in all groups reared at 21.5ºC. This could be the result of the combination of several factors, including the gastric rate of evacuation (which increases with temperature), the metabolic balance and the state of fullness of the alimentary canal (Weatherly, 1972; Azaza *et al*., 2008). Slow rates of metabolic activity (at a cold-water temperature) combined with a slow motion of the food through the alimentary canal could lead to the temporary accumulation of fat (which later will be used mainly for maintenance metabolism during the growth depression phase), which results in a higher CF and gives the impression of a healthy normally growing fish. Final values for CF remained above 2.0 for almost all groups, indicating a normal and healthy growth based on previous reports for Nile tilapia (Fish Breeding Association, 2003; El-Saidy & Gaber, 2005; Crab *et al.*, 2009; Gupta *et al.*, 2012). These results show the plasticity of the fish in adapting to growth depression stages and making a full recovery once this stage is over.

The value of GSI is important during the development of the culture since it is an indicator of the sexual maturity of the fish and consequently of their health and nutritional status (Dadzie & Wangila, 1980; Zeyl *et al.*, 2014; Marín-Ramírez *et al*., 2016). In recent studies it has been observed that continuous exposure to steroids, especially synthetic ones, can induce a deterioration in gonadal development; this is based on an observed reduction of GSI as well as on the morphological and histological changes suffered by the gonads of fish exposed to the steroids (Linderoth *et al.*, 2006; Marchand *et al.*, 2008; Louiz *et al.*, 2009). This is important since the objective of the present work is for the XY females produced by feminization to serve as key players in the successful obtaining of YY males. Therefore, an XY female with impaired gonadal growth will have a reduced probability of being selected as a breeder.

In our experiments, it was possible to observe a reduction of GSI with respect to the control group only in the estrogen-treated-groups reared at 27.5ºC. This agrees with the decrease in survival observed after using estrogens at normal temperatures reported by Zhong *et al*. (2005) for Chinese minnow *Gobiocypris rarus* and Marín-Ramírez *et al.* (2016) for Nile tilapia. In both cases they observed a reduction of GSI in groups exposed to the estrogen, as well as an overall reduction in growth, resulting in a smaller body weight and body length compared to the control group. In our case, only the group fed DES, reared at 27.5ºC, showed a similar reduction in FBW and FTL.

The fact that significantly higher values of GSI were observed in all groups reared at 21.5ºC is probably the result of the change from males to females. In Nile tilapia, the females have heavier gonads than the males (Gómez-Marquez *et al*., 2003; Peña-Mendoza *et al.*, 2005) as a consequence of the production of oocytes, especially large ones like those produced by the female of this species, in comparison to the GSI obtained in sperm-producing males (Juárez-Juárez *et al*., 2017). Taking this into account, it is possible that the higher values of GSI observed could be explained by the fact that in the control group and the estrogen-treated groups no males (with smaller GSI values) were registered, in comparison to the groups reared at 27.5ºC, where the percentage of males ranged from 5 to

54%. The presence of only females could also explain why no significant differences were observed between the groups reared at 21.5ºC. However, this argument explains only partially why no reduction in GSI was observed at 21.5ºC for the estrogen-treated groups in comparison to the control group. This could be explained by either an alteration in the way the estrogens act at physiological level provoked by a lower metabolic rate of the fish reared at 21.5ºC, or a differential response to the estrogens from the fry used in each experiment (although the same breeders were used), or small changes in the development of the experiments (although the same protocol was followed); however further research is needed to clarify this.

One aspect that has gained importance in recent years is the impact of sex-reversal treatments on the biochemical composition of the reversed fish. Degani (1986) and Chiba *et al*. (1993) report an increase in the amount of FMC after a treatment with E_2 for the European eel (*Anguilla anguilla* Linnaeus, 1758) and the Japanese eel (*Anguilla japonica* Temminck & Schlegel, 1846), respectively. In our work, the increase of FMC was particularly high in the groups fed E_2 and EE_2 (at both water temperatures). Alcántar-Vázquez *et al*. (2015) have reported similar findings for Nile tilapia after having observed an increase of FMC following a treatment of 30 days with different concentrations of E_2. Chiba *et al*. (1993) suggest that E_2 plays a key role in the regulation of lipid metabolism in fish muscle. Based on this information, on the chemical structure of EE_2 and on the results obtained, it is probable that EE_2 can also perform the functions of E_2, which could explain the increase of FMC in the groups fed E_2 and EE_2 with respect to the control group and the group fed DES. In other freshwater species, Panter *et al.* (1998) report for the fathead minnow (*Pimephales promelas* Rafinesque, 1820) an increase in vitellogenin synthesis after exposure to E_2 while Olivereau & Olivereau (1979) and Haux & Norberg (1985) report a depletion of liver glycogen after repeated intramuscular injections of E_2 for females of freshwater eel and juveniles of rainbow trout (*Salmo gairdnerii* Walbaum, 1792), respectively. The increase of FMC observed is probably caused by the accumulation of glycogen and other lipid products in the muscle after an excessive stimulation of E_2 or similar estrogens on the liver. This effect is produced independently of the water temperature and will last until the adult stage in the sex-reversed fish, as was observed by Alcántar-Vázquez *et al.* (2015) for sex-reversed Nile tilapia females obtained by orally supplied E_2 during the fry stage.

Concluding, in the present study a 100% feminization rate was achieved in several treatments combining the use of a cold-water temperature and exogenous estrogens, which will ensure an adequate production of XY females to be used as breeders in the production of YY males. These results suggest that the strain of breeders used has a tendency towards feminization of the gonads at low water temperatures, making it possible for future experiments to obtain a feminization rate of 100% without the use of exogenous estrogens. Finally, the observed proportion of females in the group fed E_2 at 21.5ºC in addition to the obtained values of growth, survival, and GSI allow us to consider the natural estrogen E_2 the best option in comparison to synthetic estrogens for the development of the YY technology.

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