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Research Article

Sex proportion in Nile tilapia *Oreochromis niloticus* fed estrogen mixtures: a case of paradoxical masculinization

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ABSTRACT. YY technology has been developed in response to the demand of environment-friendly techniques to produce all-male populations in Nile tilapia culture. However, the first part of this technique still requires feminization of XY fry. Use of estrogen mixtures to achieve feminization has never been tested in Nile tilapia, so the aim of this study was to evaluate the effect of different combinations of the three most important estrogens (17β-estradiol E_2, diethylstilbestrol DES, and 17α-ethinylestradiol EE_2) on sex proportion, growth, and gonadal development. Mixtures evaluated were E_2-DES, E_2-EE_2, DES-EE_2, and E_2-DES-EE_2. No significant differences in growth were observed at the end of the experiment between control fish and fish fed estrogen mixtures. However, final survival was significantly lower ($P < 0.05$) in fish fed E_2-DES and E_2-DES-EE_2. All estrogen mixture-treated groups produced progenies with a significantly higher ($P < 0.001$) proportion of males than the predicted 1:1 sex ratio. No significant differences were observed in gonadosomatic index between fish of the control group and fish fed estrogen mixtures. The proportion of deformed gonads was higher in groups fed E_2-DES and E_2-DES-EE_2. The paradoxical masculinization observed in all estrogen mixtures was probably provoked by the interaction of estrogens with androgen receptors or the inhibition of aromatase expression, which resulted in testosterone accumulation and testis development. These results address the need for further research towards understanding the role of steroids in sexual development since these findings add to a short list of studies that report paradoxical effects after using steroids.

Keywords: *Oreochromis niloticus***,** Nile tilapia, sex-reversal, paradoxical masculinization, wet weight, gonadal development, survival.

INTRODUCTION

Production of a monosex, all-male populations in Nile tilapia (*Oreochromis niloticus*) culture inhibits uncontrolled reproduction, allowing the production of marketable-sized fish in more productive systems (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, 2007; Phumyu *et al.*, 2012). Sex-reversal by feeding fry with different hormones is the most common method used to produce all-male populations. However, the use of this method is increasingly being seen as a negative procedure since there is growing number of reports of accumulation of hormones in the environment and an increasing number of consumers who are not interested in eating products that have been treated with hormones or other active substances (Piferrer, 2001; Müller & Hörstgen, 2007; Leet *et al.*, 2011).

A viable alternative on a commercial scale is the YY technology derived from males that possess a novel homogametic YY genotype, which allows for the production of genetically male tilapia based on crosses between YY males and XX females (Vera-Cruz *et al*., 1996; Mair *et al.*, 1997; Müller & Hörstgen, 2007; Alcántar-Vázquez *et al*., 2014). The initial step of YY technology requires feminization of XY fry during their sexually undifferentiated stage and the identification of these newly created "sex-reversed females" (XY females) through a progeny test (Vera-Cruz *et al*., 1996; Mair *et al.*, 1997). These XY females will be used to produce YY males at a percentage of 25% when combined with normal males (XY) (Mair *et al.*, 1997; Alcántar-Vázquez *et al*., 2014).

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Development in recent years of YY technology at the Universidad del Papaloapan has led to attempts to optimize the feminization rates, including the use in different trials of the three most important estrogens; estradiol-17β, 17α-ethynilestradiol and diethylstilbestrol with mixed results (Alcántar-Vázquez *et al*., 2015; Marín-Ramírez *et al*., 2016; Juárez-Juárez *et al*., 2017). One alternative for optimizing feminization rates in Nile tilapia could be the use of estrogen mixtures, based on the principle that similar chemicals work together in an additive manner, even in low and individually ineffective concentrations (Brian *et al*., 2007). Additionally, there is no available information on Nile tilapia about the effects of estrogen mixtures during sex differentiation, growth, or gonadal development.

The present study was undertaken to 1) determine whether high proportions of sexually undifferentiated XY *O. niloticus* fry could be sex-reversed to functional females using estrogen mixtures, and 2) describe the effect of estrogen mixtures on the gonadosomatic index, proportion of deformed gonads and growth.

MATERIALS AND METHODS

Experimental site

The present study was developed at the Aquaculture Station of the Universidad del Papaloapan, located in Loma Bonita, Oaxaca, Mexico, at the following coordinates: 18°06'N and 95°53'W, at a height of 30 m above sea level. The mean temperature and average annual rainfall are 25°C and 1845.2 mm, respectively (FAM, 2014).

Broodstock

The *O. niloticus* breeders used in this study come from the Isaluma strain, developed in the region by the Sistema Cooperativo Integral (Granja Unidad de Producción del Tesechoacan, Veracruz, Mexico) using the Egyptian and Stirling strains. This broodstock was reared for approximately 15 months in the aquaculture station of the Universidad del Papaloapan and fed twice a day with commercial pellets at 25% protein (Nutripec, Agribrands Purina, Irapuato Gto. Mexico).

Preparation of hormonal mixtures

The natural estrogen 17β-estradiol (E_2), the synthetic estrogens diethylstilbestrol (DES) and 17α-ethinylestradiol (EE_2) (Sigma Aldrich Chemical Co., St Louis, MO, USA) were mixed in equal parts in the following manner: E_2-DES, E_2-EE_2 and DES-EE_2 with 60 mg of each estrogen in each combination, and E_2-DES-EE_2 with 40 mg of each estrogen. In total, 120 mg of estrogens were set for each mixture. To allow the estrogens to mix uniformly they were dissolved in 500 mL of 95% ethanol. Once estrogen mixtures were completed, each one was added to one kilogram of commercial fish food (<0.35 mm, 53% protein, 15% lipids, fiber 2.5%, ash 12%, N-free extract 8.5%) to obtain a final concentration of 120 mg kg^{-1}. Addition of the different mixtures to fish food was done using the alcohol evaporation method described by Jiménez & Arredondo (2000). In brief, selected estrogen mixtures (previously dissolved in 500 mL of 95% ethanol) were sprayed over the food, which was distributed on a thin layer over a laboratory table, mixed several times until the food was completely moistened and maintained at room temperature for approximately six hours to allow the alcohol to evaporate. The food for the control group was treated in exactly the same manner with the exclusion of the added estrogens. Once dried, the food was stored in plastic containers at 4°C.

Fry production

O. niloticus spawners were stocked at a male: female ratio of 1:3 in two 3-m-diameter outdoor concrete tanks supplied with green water. Recently hatched fry was collected 15 d later with a fine-mesh net after 90% of water from the tanks had been siphoned. The sexually undifferentiated fry of approximately 0.02 g in wet weight were pooled, and transported to a closed recirculating system composed of 15 85-L transparent acrylic aquaria (length 45 cm, height 45.5 cm, depth 45 cm). The water in the recirculating system was filtered with a mechanical filter (Hayward, Model S310T2, Hayward Pool Products Inc., Elizabeth, NJ, USA) and a bio-filter containing only plastic bio-balls (Aquatic Eco-System, Model CBB1, Pentair Ltd., Apopka, FL, USA).

Experimental design

A completely randomized design with one factor (estrogen mixture) was used. Treatments were: Control with no estrogens; E_2-DES; E_2-EE_2; DES-EE_2; E_2-DES-EE_2. Each estrogen mixture summed up a total of 120 mg of estrogens per kilogram of food. Fifty fry were randomly assigned to each experimental unit, (initial stocking density of 0.60 fry L^{-1}). Each treatment was carried out in triplicate. Fry were fed eleven times a day at 1-h intervals (8:00 AM to 6:00 PM) at a feed rate adjusted to 20% of the total body weight per day. Water flow was closed in all aquaria for 20 min after the estrogen-enriched feeds were offered in order to encourage feeding. Hormonal treatment lasted for 15 days under a photoperiod of 12 L: 12 D and with water temperature adjusted thermostatically at 26.5 ± 1ºC. 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., Inc., Yellow Springs, OH, USA).

Fish feeding

Once the hormonal treatment was completed, fry were fed with an untreated commercial diet containing 53% protein (<0.35 mm, Nutripec Purina®) for 15 more days until the fry period was finished. All juveniles obtained from each treatment, at the end of the fry period, were counted and weighed for calculation of survival rate and mean wet weight. Juveniles were stocked at a density of approximately 19 fish per m^3 in outdoor concrete tanks and fed *ad libitum* five times a day for 20 days (50% protein, 0.1 mm, Nutripec Purina®) after which they were fed *ad libitum* four times a day (44% protein, 1.5 mm, Nutripec Purina®) for another 20 days. Fish were reared up to approximately 114 days of age when sex proportion could be safely determined by removing the gonad. Fish were fed *ad libitum* three times a day with commercial diet (40% protein, 2.4 mm, Nutripec Purina®) for approximately 30 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.

Sex identification and growth

The sex of 25-30% (n = 41 ± 4) of fish per treatment was determined by removing the gonad. Gonads were classified as ovaries and testes. Random samples of 15 fish per replicate were collected at the end of the hormonal treatment (15 d), at the end of the fry period (30 d) and during the rest of the rearing period every 21 days for calculation of mean wet weight (g) using a digital scale (± 0.01) (Ohaus Cor., Scout Pro Model Sp 202, Parsippany, NJ, USA).

Gonadal status

The extracted gonads were macroscopically examined for deformities (abnormal or reduced growth in one or the two gonads) following previous reports by Zhong *et al*. (2005), Paul-Prasanth *et al*. (2011) and Marín-Ramírez *et al*. (2016) after a sex-reversal process with synthetic estrogens. Finally, the gonads were weighed using a digital scale (± 0.01) to calculate the gonadosomatic index (Sturm, 1978) using the following formula:

GSI = [Gonad weight (g) / Fish weight (g)] × 100.

Statistical analysis

The proportion of males identified in each treatment was tested against the 1:1 expectation using a chi-square test at a probability of 0.1% ($P < 0.001$). Percentages of males were arcsine transformed and analyzed using a one-way analysis of variance (ANOVA), with a Tukey test performed on treatment means *a posteriori*. Average wet weight obtained at different times through the experiment was analyzed using the same statistical methods. Survival was analyzed using a chi-square test. Gonadosomatic index values and proportion of deformed gonads were analyzed using a Kruskal-Wallis nonparametric analysis. When significant differences occurred ($P < 0.05$) a Conover test was applied.

RESULTS

All estrogen mixture-treated groups produced progenies with a significantly higher (Chi-square; $P < 0.001$) proportion of males than the predicted 1:1 sex ratio (Table 1).

The ANOVA indicated significant differences between treatments ($P < 0.05$), with a significantly higher percentage of males observed in the fish, fed E_2-DES-EE_2 in comparison to the fish fed E_2-EE_2 and DES-EE_2 (Tukey; $P < 0.05$). No significant differences were observed between the fish fed E_2-DES-EE_2 and E_2-DES (Tukey; $P > 0.05$). Final survival was significantly lower (Chi-square; $P < 0.05$) in the fish fed the estrogen mixtures of E_2-DES and E_2-DES-EE_2 in comparison to that registered for the control fish (Table 1).

No significant differences (Conover; $P > 0.05$) were observed in the GSI between treatments (Table 1). However, the proportion of deformed gonads was higher (Conover; $P < 0.05$) in the groups fed estrogen mixtures E_2-DES and E_2-DES-EE_2. No deformed gonads were observed in the control fish or the fish fed E_2-EE_2 (Table 1).

No significant differences in weight (Tukey; $P > 0.05$) were observed at 15 days of age (end of the hormonal treatment) between the fry of the control group and the fry fed estrogen mixtures. At the end of the fry period (30 days of age) a significantly lower (Tukey; $P < 0.05$) mean weight was registered for the fry fed E_2-DES in comparison to the control fry and the fry fed the estrogen mixtures of DES-EE_2 and E_2-EE_2. In the post-treatment part of the experiment, a significantly higher (Tukey; $P < 0.05$) mean weight was observed at 51 days of age for the control fish and the E_2-EE_2-treated fish than that observed in the fish fed DES-EE_2 and E_2-DES-EE_2. At 72 and 93 days of age, fish fed E_2-DES showed a significantly higher (Tukey; $P < 0.05$) mean weight than the fish fed DES-EE_2 and E_2-DES-EE_2. No significant differences (Tukey; $P >$

Table 1. Percentage of survival (S), percentage of males, gonadosomatic index (GSI) (± SE) and percentage of deformed gonads (PDG) of Nile tilapia (*Oreochromis niloticus*) fed different estrogen mixtures. Data collected at the end of the experiment (114 days of age). *Percentage of survival significantly different from the control group (Chi-square; $P < 0.05$). [1]Significantly different from the expected 1:1 distribution (Chi-square; $P < 0.001$). Values in each column superscripted with different letters indicate significant differences between treatments (Tukey; $P < 0.05$ Male percentage; Conover; $P < 0.05$ PDG).

Estrogen mixture	S	Male (%)	GSI	PDG
Control	82	56.0 ± 1.5^{d}	0.16 ± 0.04	0.0 ± 0.0^{c}
E_2-DES	72*	85.3 ± 1.7^{1ab}	0.20 ± 0.04	27.0 ± 1.2^{a}
E_2-EE_2	80	74.3 ± 1.7^{1c}	0.25 ± 0.11	0.0 ± 0.0^{c}
DES-EE_2	79	80.0 ± 1.5^{1bc}	0.15 ± 0.03	3.0 ± 1.0^{b}
E_2-DES-EE_2	70*	88.7 ± 0.7^{1a}	0.10 ± 0.01	30.0 ± 1.9^{a}

0.05) were observed at the end of the experiment (114 days of age) for the control fish and the fish fed the estrogen mixtures (Table 2).

DISCUSSION

One of the most important alternative techniques being researched today for decreasing the use of hormones during sex-reversal treatments in Nile tilapia culture is the production of YY males (Mair *et al.*, 1997; Alcántar-Vázquez *et al*., 2014). Feminization of XY fry to obtain XY females is one of the critical stages of YY-male technology, requiring the use of exogenous estrogens to achieve this (Mair *et al.*, 1997; Alcántar-Vázquez *et al*., 2014, 2015; Marín-Ramírez *et al*., 2016).

The use of exogenous estrogens to reverse sex in fish is based upon its potential to disrupt the natural differentiation process, even after its initiation, by overriding the normal developmental pattern of gene expressions and physiological regulations leading to sex reversion (Devlin & Nagahama, 2002).

The idea behind the present work was to investigate if applying estrogen mixtures at low concentrations for 15 days could optimize the feminization process developed in our laboratory (Alcántar-Vázquez *et al*., 2015; Marín-Ramírez *et al*., 2016; Juárez-Juárez *et al*., 2017). The duration of the hormonal treatment was strongly based on two aspects: the current trend in commercial cultures and Nile tilapia market demand of a reduction in the use of exogenous steroids to achieve sex reversal, and the previous works carried out, both in Nile tilapia and Mozambique tilapia (Pandian & Varadaraj, 1988; Varadaraj, 1989; Hiott & Phelps, 1993; Rosenstein & Hulata, 1994; Phelps & Popma, 2000; Bertolla-Afonso *et al*., 2001), that reported high sex-reversal rates, when applying treatments for 11, 13, 14 or 15 days. Phelps & Popma (2000) suggest that, as a rule, fish should receive at least 14 days of hormone treatment to achieve a successful sex-reversal rate. Additionally, Piferrer (2001) reports that duration of estrogen treatment can be reduced as much as possible if estrogen concentration (or in this case potency) is increased.

Typically, the application of exogenous estrogens feminizes reproductive tissues; however, in our case masculinization was observed instead of feminization in all groups fed estrogen mixtures. Although some estrogens are known to have masculinizing effects on several aspects of normal male development in vertebrates (Warner *et al*., 2014), this is the first report in fish of estrogenic compounds applied during early stages (single or in a mixture) to have caused masculinization of the gonads in a high percentage of the progeny. Warner *et al.* (2014) have reported similar results in *Chrysemys picta* and *Chelydra serpentina*, two species of turtles, after applying E_2 to the eggs at different temperatures and at different periods of the incubation process.

Studies in vertebrates indicate that E_2 can upregulate androgen receptor expression and bind, albeit with a lower affinity, to androgen receptors (Heinlein & Chang, 2002; Richter *et al*., 2007). It is possible that the presence in blood plasma of high levels of two or three estrogens, including E_2, provoked an increase in the androgen receptor expression and/or the binding with androgen receptors, resulting in the masculinization of the reproductive tissue. Another possibility is that the presence of these estrogens at high levels could have inhibited the aromatase expression, blocking the conversion of androgens to estrogens, resulting in the accumulation of testosterone in blood plasma and in testis development (Warner *et al*., 2014). Finally, masculinizing effects of high concentration of estrogens (E_2) have been reported in other vertebrates (Hayes, 1998). Therefore, it is possible that the naturally synthesized estradiol coupled with the applied exogenous estrogens increased the total concentration

Table 2. Mean wet weight (WW) (g ± SE) (n = 3) of Nile tilapia (*Oreochromis niloticus*) fed different estrogen mixtures. Values in each row superscripted with different letters indicate significant differences between treatments (Tukey; $P < 0.05$).

Days	WW - estrogen mixture				
	Control	E_2-DES	E_2-EE_2	DES-EE_2	E_2-DES-EE_2
0	0.02 ± 0.01^a	0.02 ± 0.01^a	0.02 ± 0.01^a	0.02 ± 0.01^a	0.02 ± 0.01^a
15	0.20 ± 0.02^a	0.20 ± 0.03^a	0.20 ± 0.02^a	0.19 ± 0.03^a	0.19 ± 0.02^a
30	2.76 ± 0.18^a	2.02 ± 0.20^b	2.76 ± 0.19^a	2.97 ± 0.21^a	2.33 ± 0.25^{ab}
51	16.34 ± 0.41^a	14.77 ± 0.59^{ab}	17.05 ± 0.44^a	12.79 ± 0.51^b	9.59 ± 1.10^b
72	24.03 ± 1.37^{ab}	29.14 ± 1.71^a	24.94 ± 1.17^{ab}	22.39 ± 0.97^b	23.24 ± 1.49^b
93	43.91 ± 2.55^{ab}	54.20 ± 3.36^a	44.67 ± 2.92^{ab}	40.93 ± 2.57^b	50.75 ± 2.92^{ab}
114	68.10 ± 3.93^a	79.56 ± 3.37^a	69.96 ± 5.15^a	73.23 ± 3.11^a	80.76 ± 4.02^a

above physiological levels, producing a paradoxical masculinization, by altering the expression of aromatase or estrogen receptor genes in the gonad (Katsu *et al.*, 2004; Warner *et al*., 2014). A paradoxical feminization has been reported in several species of fish after applying high concentrations of androgens or rearing at high water temperatures (Nakamura, 1975; Piferrer & Donaldson, 1991; Varadaraj *et al.*, 1994; Rinchard *et al*., 1999).

Water temperature during hormonal treatments is considered a factor responsible for deviations from expected sex ratios (Wang & Tsai, 2000). However, in this experiment, water temperature during fry stage was maintained at approximately 27 ± 0.5ºC, while the minimum water temperature reported resulting in a masculinization of the gonads and therefore a higher proportion of males is 28ºC (Wessels & Hörstgen-Schwark, 2011; Alcántar-Vázquez *et al*., 2015). Additionally, experiments made in our laboratory at a mean water temperature of 28ºC only resulted in a maximum male percentage of 59.6%. This makes it unlikely that the higher percentage of males observed in the groups fed estrogen mixtures could be a consequence of water temperature. Additionally, if water temperature were responsible for such deviation in male percentages, it should have also been observed in the control group; however, the control group showed a percentage of males within normal ranges for Nile tilapia.

Although survival was lower in the groups fed estrogen mixtures, it was high enough in comparison to the control group to rule out the possibility that differential mortality between male and female fry was responsible for the high percentage of males observed. Additionally, our ability to sex juveniles based on a protocol developed in our laboratory reduces the possibility that our manipulations generate the sex proportions observed. However, we need to consider that the sex percentages observed may have been due to an expired stock. Estrogens used were obtained two and a half years earlier, and worked effectively in several trials carried out in our laboratory until 2014. This experiment was performed during the summer of 2015, so we cannot rule out the possibility that the estrogens expired (although they were stored below 4ºC the entire time) and their effect at physiological level was altered, resulting in a masculinization instead of a feminization of the reproductive tissue.

In recent years, some steroids have been found to act as promoters of growth; however, in most cases they have been associated with a decrease in the growth rate observed after a sex-reversal treatment (Varadaraj, 1989; Blázquez *et al.*, 2001; Piferrer, 2001; Marín-Ramírez *et al*., 2016). In the case of estrogens, Rhida & Lone (1995) report that they show no anabolic effect in most teleost. This agrees with that observed in our work since exposure to estrogen mixtures did not cause a significant reduction or increase in growth rate in the treated groups in comparison to the control group. Results obtained in previous work carried out in our laboratory using the three estrogens separately on Nile tilapia have shown similar results (Alcántar-Vázquez *et al*., 2015; Juárez-Juárez *et al*., 2017), with the exception of DES (Marín-Ramírez *et al*., 2016), which has shown a negative effect on growth rate in some trials. It is possible that applying DES in combination with other estrogens cancels its negative effect on growth rate; however, we cannot rule out the paradoxical masculinizing effect of the estrogen mixtures used on the growth rate observed.

Although no significant differences in final weight were registered, it was possible to observe that at a higher percentage of males a greater final weight was obtained in the groups treated with estrogen mixtures. Toguyeni *et al.* (1996) report that this is the result of the sexual dimorphism present in Nile tilapia, since males of Nile tilapia show higher growth rates than females as a direct consequence of sex hormones and indirectly to

sex-related behavior and physiological factors (Phumyu *et al*., 2012).

Several authors (Zhong *et al.*, 2005; Hamdoon *et al.*, 2013; Marín-Ramírez *et al.*, 2016; Juárez-Juárez *et al.*, 2017) have reported a decrease in final survival after an estrogen treatment; however, in some cases no significant increase in mortality has been detected (Varadaraj, 1989; Van Aerle *et al*., 2002; Andersen *et al*., 2003; Alcántar-Vázquez *et al*., 2015). According to Piferrer (2001), this depends on a number of factors including the type of estrogen (natural or synthetic), the concentration used, the timing (based on the timing of gonadal differentiation, Lin *et al*., 2012) and the duration of the hormonal treatment. In our work, only the estrogen mixtures that combined E_2 with DES were the ones that showed a significant reduction in final survival. It is probable that the combination of these two estrogens provoked an increase in the susceptibility to infections during growth as proposed by Shved *et al*. (2009), therefore reducing survival. Similar observations using DES (Marín-Ramírez *et al*., 2016) and E_2 (*unpubl. data*) in our laboratory support this.

Continuous exposure to synthetic compounds, including estrogens in single or binary mixtures, has shown to provoke, in several species, a decrease in gonadal development. This is characterized by a reduction in GSI, as well as morphological and histological alterations undergone by the gonads (Linderoth *et al.*, 2006; Marchand *et al.*, 2008; Louiz *et al.*, 2009; Paul-Prasanth *et al.*, 2011; Zhengyan *et al*., 2012; Song *et al*., 2014; Marín-Ramírez *et al*., 2016). In our work, although the gonadal evaluation of fish fed estrogen mixtures did not show a significant reduction in the GSI values in comparison to the control group, it was possible to observe an increase in the proportion of deformed gonads in the estrogen mixtures that include DES, especially in mixtures that including both DES and E_2. These deformations as previously reported consisted mainly of a reduction of size or abnormal growth. Piferrer (2001) reports that these deformations or abnormalities could be caused by the administration of the estrogens in the diet. Milnes *et al.* (2006), reported that exposure of males to estrogens can result in the reduction of testicular growth or testicular atrophy due to testicular lesions such as fibrosis and histological alterations. This could explain the high presence of deformities in the gonads of males, especially in those fed DES. Similar findings have been reported by Marín-Ramírez *et al*. (2016) for Nile tilapia by using DES to feminize the strain of Nile tilapia developed in our laboratory. Finally, Song *et al*. (2014) report in the goldfish (*Carassius auratus*) an increase in gonadal atrophy in several treatments using individual or binary mixtures of estrogens. This increase in gonadal deformities could probably be related to the significant reduction of final survival also observed in the estrogen mixtures that combined E_2 and DES; however, more research is needed to clarify this.

CONCLUSIONS

Although the sex proportion obtained was not what we expected, it is important to report because it draws attention to the issue of publication of negative or unusual results in the scientific literature. Warner *et al*. (2014) mention that if paradoxical results like these are left unreported, then such findings might not be as unusual as one would expect and our overall understanding of the impacts of sex steroids will not be skewed. In light of the sex proportions observed here, it would be necessary to do further research using a new batch of estrogens and repeat the experiment at least two times with different genetic lines of Nile tilapia to ensure that this result is not limited to the genetic line breed in our laboratory.

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