

Action of two endocrine disruptors on the sexual differentiation of Nile tilapia¹

Ação de dois desreguladores endócrinos na diferenciação sexual da tilápia do Nilo

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ABSTRACT - Endocrine disruptors are exogenous substances that can impair one or more functions of the endocrine system and cause adverse effects to the health of an intact organism. The main goal of this study was to evaluate the effect of the endocrine disruptors 17 β -estradiol (E2) and 17 α -ethinyl estradiol (EE2) on the sexual differentiation of Nile tilapia during spawning. Six treatments and one control with three replications, totaling 21 experimental units, were assessed in a completely randomized design. Hormones were dosed at concentrations of 250, 500, and 1,000 ng L⁻¹ (treatments E2-250, E2-500, E2-1,000, EE2-250, EE2-500, and EE2-1,000). Exposure to hormones occurred during the first 28 days of life. After this period, water was completely renewed and individuals kept in their respective experimental units for another 60 days, totaling 88 days. At the end of this period, animals were euthanized by ice desensitization for gonad extraction, slide preparation, and observation under an optical microscope. At the end of the experiment, the results indicated that the type of hormone and hormone concentration differentially affected the parameters of zootechnical performance. A significant difference ($p < 0.05$) was observed in the condition factor between control and treatments. Intersex individuals were observed in treatments of both hormones, except for the control treatment. In addition, malformations were observed in individuals submitted to treatments, mainly in the head region. Thus, the presence of the chemical compounds 17 β -estradiol and 17 α -ethinylestradiol severely affected the development and sexual differentiation of Nile tilapia.

Key words: Emerging pollutants. *Oreochromis niloticus*. 17 α -ethinylestradiol. 17 β -estradiol.

RESUMO - Desreguladores endócrinos são substâncias exógenas que possuem a capacidade de alterar uma ou várias funções do sistema endócrino com efeitos adversos a saúde um organismo intacto. O objetivo principal do presente trabalho foi avaliar a ação dos desreguladores endócrinos 17 β -estradiol (E2) e 17 α -etinilestradiol (EE2) na diferenciação sexual da tilápia do Nilo durante o período de alevinagem. Foram avaliados seis tratamentos e um controle, com três repetições, totalizando 21 unidades experimentais em um delineamento inteiramente casualizado. Os hormônios foram dosados nas concentrações de 250; 500 e 1.000 ng L⁻¹ (E2-250; E2-500; E2-1.000; EE2-250; EE2-500; EE2-1.000). A exposição aos hormônios ocorreu durante os primeiros 28 dias de vida. Após este período a água foi totalmente renovada e os indivíduos mantidos em suas respectivas unidades experimentais por mais 60 dias, totalizando 88 dias. Ao final deste período os animais foram eutanasiados por insensibilização em gelo para extração das gônadas, preparação das lâminas e observação sob microscópio óptico. Ao final do experimento foi possível constatar que os hormônios e as concentrações hormonais avaliadas afetaram distintamente os parâmetros de desempenho zootécnico. Foi observada diferença significativa ($p < 0,05$) no fator de condição entre o controle e os tratamentos. Indivíduos intersexo foram observados nos tratamentos de ambos os hormônios, com exceção do controle. Além disto, foram observadas malformações nos indivíduos dos tratamentos, principalmente na região da cabeça. Com os dados coletados na presente pesquisa é possível concluir que a presença dos compostos químicos 17 β -estradiol e 17 α -etinilestradiol afetam severamente o desenvolvimento e a diferenciação sexual da tilápia do Nilo.

Palavras-chave: Poluentes emergentes. *Oreochromis niloticus*. 17 α -etinilestradiol. 17 β -Estradiol.

DOI: 10.5935/1806-6690.20190048

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Received for publication 02/07/2018; approved on 21/09/2018

¹Parte da Tese de Doutorado do primeiro autor em Engenharia Civil (Recursos Hídricos) - Departamento de Engenharia Hidráulica e Ambiental - Pesquisa Financiada pelo CNPq - Edital MCTI/CNPQ N° 14/2012 - Universal

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INTRODUCTION

The first indications that substances with the potential for endocrine disrupters affected wildlife were observed in the 1980s from observations of female characteristics in male bird specimens colonizing the Great Lakes region, which occupies part of the United States and Canada (REIS FILHO; ARAÚJO; VIEIRA, 2006). Ever since, researchers around the world have observed similar effects on other animal taxa such as amphibians (PHUGE; GRAMAPUROHIT, 2015), reptiles (CZARNY *et al.*, 2017), mollusks (BORYSKO; ROSS, 2014), and fishes (WOODS; KUMAR, 2011).

Endocrine disrupters are still poorly studied and their synergistic actions in the environment are totally unknown to most compounds since they are part of a group of compounds collectively known as emerging pollutants (NIEMUTH; KAPER, 2015). Among these endocrine disrupters, the natural estrogenic steroid 17 β -estradiol (E2) and the synthetic estrogenic steroid 17 α -ethinylestradiol (EE2) are the most studied since they are among the most widespread substances in the environment (CZARNY *et al.* 2017). According to these authors, some of these compounds (E2, for example) are present in the urine of animals and their concentration depends on gender, hormonal status, the existence of pregnancy, the phase of the menstrual cycle, in addition to synthetic forms used as contraceptive pills (EE2) and hormone-based medicines.

The feminizing action of endocrine disrupters of estrogenic action has been observed in fishes that inhabit rivers near large urban centers in several places of the world (DAMMANN *et al.*, 2011; MEIJIDE *et al.*, 2016; TETREAULT *et al.*, 2011). In addition, laboratory tests have confirmed that considerably lower hormone doses, in the orders of microgram ($\mu\text{g L}^{-1}$) and nanogram per liter (ng L^{-1}), are likely to impair the development of various fish species (LANGE *et al.*, 2001; LEI *et al.*, 2014; NIEMUTH; KLAPER, 2015).

Pessoa *et al.* (2012) found E2 and EE2 concentrations of the order of ng L^{-1} in a sewage treatment plant (STP) located in the Metropolitan Region of Fortaleza. However, studies conducted in other Brazilian states have shown that such concentrations can reach the order of $\mu\text{g L}^{-1}$ in the surface water bodies close to large urban centers (CAMPANHA *et al.*, 2015; MACHADO *et al.*, 2014).

Therefore, studies that assess the effects of endocrine disrupters in a controlled environment are necessary since they allow assessing these compounds in isolation. The main goal of this study was to evaluate the effect of the endocrine disruptors 17 β -estradiol (E2) and 17 α -ethinyl estradiol (EE2) on the sexual differentiation of Nile tilapia during spawning. Besides that, the occurrence

of malformations and zootechnical performance (weight, length, allometric condition factor, and survival) were also evaluated.

MATERIAL AND METHODS

The present study was developed at the Laboratório de Recursos Aquáticos (LARAq) of the Departamento de Engenharia de Pesca (Laboratory of Aquatic Resources of the Department of Fisheries Engineering), the Laboratório de Saneamento (LABOSAN) of the Departamento de Engenharia Hidráulica e Ambiental (Laboratory of Sanitation of the Department of Hydraulic and Environmental Engineering), and the Laboratório de Histologia e Reprodução Animal (LAHRA) of the Departamento de Biologia (Laboratory of Histology and Animal Reproduction of the Department of Biology) of the Universidade Federal do Ceará-UFC (Federal University of Ceará).

Newly hatched larvae of Nile tilapia (*Oreochromis niloticus*) were obtained from the Aquaculture Station Professor Doctor Raimundo Saraiva da Costa affiliated to DEP/UFC and derived from the same spawning.

Six treatments and a control with three replications were assessed, totaling 21 experimental units with a useful volume of 40 L each. Ten larvae of Nile tilapia were stored in each aquarium. The experimental design was completely randomized design.

The hormones 17 β -estradiol (E2) and 17 α -ethinylestradiol (EE2) were purchased in a lyophilized form from the company Sigma-Aldrich. The effects of these hormones were assessed in three different concentrations: 250, 500, and 1,000 ng L^{-1} (treatments E2-250, E2-500, E2-1,000, EE2-250, EE2-500, and EE2-1,000).

A stock solution with a concentration of 20 mg L^{-1} was prepared by diluting 10 mg of each hormone in 500 mL ethyl alcohol PA (solvent). These solutions were stored in an amber bottle and maintained in a refrigerator at 3-4 °C. The experimental aquaria (40 L) were dosed with 0.5, 1.0, and 2.0 mL of stock solution in order to obtain the experimental concentrations of 250, 500, and 1,000 ng L^{-1} , respectively. In order to ensure that all treatments received equal solvent volumes, 2.0, 1.5, and 0.5 mL alcohol was added to the control and treatments of 250 and 500 ng L^{-1} , respectively.

The individuals were exposed to hormone doses during the first 28 days of life. Weekly, the water from each tank was 100% renewed, and hormone doses replaced to the same dosages.

After 28 days of treatment, the water in each tank was completely renewed without the addition of

hormones, and the fish were kept in tanks for another 60 days, so that the mean weight of the individuals in all groups reached at least 10 g, totalizing 88 days. The water was drained from the tanks and treated with ultraviolet radiation before disposal, as described by Sornalingam, McDonagh and Zhou (2016).

Each experimental unit was covered by a glass cover and equipped with a mechanical filter driven by an air compressor under the air lift system, which remained on for 24 h. Mechanical filters were removed weekly for cleaning. Individuals were fed *ad libitum* four times a day at 9:00, 12:00, 14:00, and 16:00 h, with powder feed containing 45% crude protein.

Temperature ($^{\circ}\text{C}$) and dissolved oxygen concentration (mg L^{-1}) were measured daily by using a digital oximeter. In addition, pH, alkalinity ($\text{mg CaCO}_3 \text{ L}^{-1}$), and total ammoniacal nitrogen (mg L^{-1}) were measured every five days by using colorimetric and titrimetric tests according to the instructions of the manufacturer.

Two biometry assessments of the whole lot, the first 40 days after hatching (DAH) and the second 88 DAH, were carried out over the experimental period by measuring the total length (cm) and live weight (g) using a caliper (0.002 cm precision) and a digital scale (0.01 g precision), respectively.

After the second biometric evaluation, the fish were euthanized by ice desensitization, the gonads were removed, stored in histological microcassettes, labeled, and fixed in 10% formalin solution for 24 hours. Histological sections were stained with Harris hematoxylin and Gomori trichrome, as the method adapted by Tolosa *et al.*, (2003).

The allometric condition factor (KA) was calculated with the data of weight (W) and length (L) by means of the equation $\text{KA} = \text{W}/\text{L}^b$, where b is the allometric coefficient obtained by the regression equation of the weight-length relationship (LE CREN, 1951).

Data analysis was performed for water physical and chemical parameters (dissolved oxygen, temperature, pH, alkalinity, and total ammonia), zootechnical variables (weight, length and survival), and allometric condition factor. For this purpose, analysis of variance (ANOVA) at a level of significance of 5% was used considering the primary effect of two variables: type of hormone and hormone concentration. In cases of statistical significance, Tukey's test was applied to compare the means using systematic component 1.

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk} \quad (1)$$

Where: Y_{ijk} is the type of hormone i, hormonal concentration j, and repetition k ($i = 1, 2; j = 1, 2, 3; k = 1, 2, 3$); μ is the population mean; α_i is the effect of hormone type i; β_j is the effect of hormone concentration j; and ε_{ijk} is the residual error.

The values expressed as percentages were transformed into arcsine for applying the statistical test. The data were subjected to the Lilliefors test to verify normality.

All statistical analyses were performed by using the Programa de Aplicações Estatísticas nas Áreas das Ciências Biomédicas (BioEstat, version 5.0) at a 5% significance level (AYRES; AYRES Jr., 2007).

Regression analysis equations of the weight-length relationship were determined by using spreadsheets for each treatment and control through the power model $y = \text{Ax}^b$, where $\ln y = a + b \ln x$ is the linearized form of this curvilinear model.

RESULTS AND DISCUSSION

Water quality parameters assessed during the experimental period (Table 1) were within the comfort range for *O. niloticus*, according to Kubitz (2011). Dissolved oxygen, temperature, pH, alkalinity, and total ammoniacal nitrogen presented average values respectively between 7.37 ± 0.42 and $6.97 \pm 0.35 \text{ mg L}^{-1}$ (treatments E2-500 and EE2-250), 26.24 ± 0.76 and $25.93 \pm 0.81 \text{ }^{\circ}\text{C}$ (treatments EE2-250 and E2-250), 7.49 ± 0.25 and 7.61 ± 0.11 (treatments EE2-500 and E2-1,000), 80.55 ± 9.80 and $68.62 \pm 7.31 \text{ mg CaCO}_3 \text{ L}^{-1}$ (treatment E2-500 and control), and 0.92 ± 0.66 and $0.54 \pm 0.51 \text{ mg L}^{-1}$ (treatments E2-1,000 and EE2-500).

There was no significant difference ($p > 0.05$) in fish weight among the groups (Table 2). However, the analysis of the other parameters of zootechnical performance indicated that the effects of the type of hormone and hormone concentration were independent. Total length and allometric condition factor were affected by the hormone concentration whereas survival was affected by the type of hormone.

There was a significant difference ($p < 0.05$) in total length between treatments E2-250 ($5.88 \pm 1.56 \text{ cm}$) and EE2-500 ($7.07 \pm 1.90 \text{ cm}$).

For both hormone types, the allometric condition factor tended to decrease as hormone concentration was increased. There was a significant difference ($p < 0.05$) in this variable in all cases, except in the group treated with EE2-500 when compared to the control group and the group treated with E2-1,000.

Table 1 - Dissolved oxygen (DO), temperature (Temp.), pH, alkalinity (Alk.) and total ammoniacal nitrogen (TAN) in the water of juvenile Nile tilapia (*Oreochromis niloticus*) under different hormonal concentrations, 17 β -estradiol e 17 α -ethinylestradiol, during the first 28 days of life

Treatment	DO (mg L ⁻¹)	Temp. (°C)	pH	Alk. (mg CaCO ₃ L ⁻¹)	TAN (mg L ⁻¹)
Control	6.98 ± 0.43	26.13 ± 0.67	7.52 ± 0.22	68.62 ± 7.31	0.58 ± 0.49
E2-250	7.26 ± 0.44	25.93 ± 0.81	7.50 ± 0.14	77.57 ± 9.24	0.75 ± 0.69
E2-500	7.37 ± 0.42	26.03 ± 0.89	7.58 ± 0.08	80.55 ± 9.80	0.75 ± 0.76
E2-1,000	7.04 ± 0.38	26.09 ± 0.61	7.61 ± 0.11	71.0 ± 11.32	0.90 ± 0.74
EE2-250	6.97 ± 0.35	26.24 ± 0.76	7.60 ± 0.21	74.58 ± 7.31	0.92 ± 0.66
EE2-500	7.04 ± 0.40	26.08 ± 0.68	7.49 ± 0.25	74.58 ± 7.31	0.54 ± 0.51
EE2-1,000	7.08 ± 0.42	25.96 ± 0.71	7.53 ± 0.07	71.60 ± 2.55	0.83 ± 0.68

E2-250 – 17 β -estradiol, hormonal concentration 250 ng L⁻¹; E2-500 – 17 β -estradiol, hormonal concentration 500 ng L⁻¹; E2-1,000 – 17 β -estradiol, hormonal concentration 1,000 ng L⁻¹; EE2-250 – 17 α -ethinylestradiol, hormonal concentration 250 ng L⁻¹; EE2-500 – 17 α -ethinylestradiol, hormonal concentration 500 ng L⁻¹; EE2-1,000 – 17 α -ethinylestradiol, hormonal concentration 1,000 ng L⁻¹

Table 2 - Zootechnical performance parameters in the first biometry of juvenile Nile tilapia (*Oreochromis niloticus*) under different hormonal concentration, 17 β -estradiol e 17 α -ethinylestradiol, during the first 28 days of life. Body weight (BW), total length (TL), allometric condition factor (KA) and survival (S). Mean±standard deviation. Values in the same column with different letters superscripts indicate statistical differences (p<0.05)

Treatment	BW (g)	TL (cm)	KA	S (%)
Control ¹	5.59 ± 3.93	6.60 ± 1.50 ab	1.06 ± 0.08 a	80.00 ± 10.00
E2-250 ¹	4.47 ± 4.10	5.88 ± 1.56 a	1.62 ± 0.15 c	80.00 ± 0.00
E2-500 ¹	4.79 ± 3.18	6.23 ± 1.29 ab	1.41 ± 0.13 b	73.33 ± 5.77
E2-1,000 ¹	5.48 ± 3.97	6.51 ± 1.37 ab	0.85 ± 0.08 e	73.33 ± 11.55
EE2-250 ¹	5.64 ± 6.64	6.10 ± 1.70 ab	1.24 ± 0.19 f	66.67 ± 15.28
EE2-500 ¹	7.82 ± 7.64	7.07 ± 1.90 b	0.96 ± 0.07 ae	66.67 ± 11.55
EE2-1,000 ¹	6.35 ± 4.61	6.66 ± 1.76 ab	0.49 ± 0.12 d	60.00 ± 0.00
Concentration(F;p)	1.6780; 0.1987	3.1573; 0.0356	129.2885; <0.0001	1.7091; 0.1911
Control ²	5.59 ± 3.93	6.60 ± 1.50	1.06 ± 0.08	80.00 ± 10.00 A
E2 ²	4.90 ± 3.75	6.19 ± 1.42	1.30 ± 0.35	75.56 ± 7.26 AB
EE2 ²	6.57 ± 6.36	6.59 ± 1.80	1.11 ± 1.05	64.44 ± 10.14 B
Hormone (F;p)	2.4135; 0.1165	1.5145; 0.2458	3.4754; 0.0518	4.6972; 0.0223

¹Hormonal concentration, ²Hormone, E2-250 – 17 β -estradiol, hormonal concentration 250 ng L⁻¹; E2-500 – 17 β -estradiol, hormonal concentration 500 ng L⁻¹; E2-1,000 – 17 β -estradiol, hormonal concentration 1,000 ng L⁻¹. EE2-250 – 17 α -ethinylestradiol, hormonal concentration 250 ng L⁻¹; EE2-500 – 17 α -ethinylestradiol, hormonal concentration 500 ng L⁻¹; EE2-1,000 – 17 α -ethinylestradiol, hormonal concentration 1,000 ng L⁻¹

No significant difference (p>0.05) was observed for survival when comparing E2 treatment to control and to EE2 treatment, whose values were, respectively, 75.56 ± 7.26%, 80.00 ± 10.00%, and 64.44 ± 10.14%. However, a statistical difference (p<0.05) was observed between control and EE2 treatment.

As in the first biometric analysis, the effects of hormone type and hormonal concentration were different on the parameters of zootechnical performance in second biometrics (Table 3).

No significant differences (p>0.05) were found among the evaluated groups for total length and survival.

However, when considering the effect of the type of hormone, a significant difference (p<0.05) in animal weight was found when comparing the control group (22.56 ± 19.83 g) with E2 (13.75 ± 8.04 g) and with EE2 (15.32 ± 11.76 g).

In turn, the allometric condition factor was significantly affected by hormone concentration in the two treatment groups. For hormone type, no significant difference (p>0.05) was observed between the control group and the two treatments. However, a significant difference (p<0.05) was found between the two treatments. In the group treated with EE2, the increase in hormone

Table 3 - Zootechnical performance parameters in the second biometry of juvenile Nile tilapia (*Oreochromis niloticus*) under different hormonal concentration, 17 β -estradiol e 17 α - ethinylestradiol, during the first 28 days of life. Body weight (BW), total length (TL), allometric condition factor (KA) and survival (S). Mean \pm standard deviation. Values in the same column with different letters superscripts indicate statistical differences (p<0.01)

Treatment	BW (g)	TL (cm)	KA	S (%)
Control ¹	22.56 \pm 19.83	9.91 \pm 2.57	1.10 \pm 0.08 ab	80.56 \pm 17.35
E2-250 ¹	14.79 \pm 9.16	9.12 \pm 2.01	1.57 \pm 0.20 c	63.69 \pm 7.22
E2-500 ¹	11.24 \pm 5.36	8.34 \pm 1.48	1.42 \pm 0.11 c	64.05 \pm 9.59
E2-1,000 ¹	15.26 \pm 9.02	8.94 \pm 1.79	0.85 \pm 0.08 d	66.83 \pm 14.37
EE2-250 ¹	15.04 \pm 13.11	8.58 \pm 2.71	1.13 \pm 0.08 a	72.38 \pm 13.20
EE2-500 ¹	17.43 \pm 9.67	9.36 \pm 2.20	0.95 \pm 0.07 bd	81.11 \pm 20.09
EE2-1,000 ¹	12.89 \pm 13.22	8.01 \pm 2.81	0.50 \pm 0.11 e	68.33 \pm 16.07
Concentration (F;p)	1.7832; 0.1744	1.2107; 0.3570	103.2084; <0.0001	0.9170; 0.5118
Control ²	22.56 \pm 19.83 A	9.91 \pm 2.57	1.10 \pm 0.08 AB	80.56 \pm 17.35
E2 ²	13.75 \pm 8.04 B	8.81 \pm 1.77	1.30 \pm 0.3 4A	64.85 \pm 9.48
EE2 ²	15.32 \pm 11.76 B	8.71 \pm 2.54	1.19 \pm 1.43 B	73.94 \pm 15.53
Hormone (F;p)	4.8398; 0.0204	2.2254; 0.1355	4.4154; 0.0269	2.0229; 0.1599

¹Hormonal concentration, ²Hormone, E2-250 – 17 β -estradiol, hormonal concentration 250 ng L⁻¹; E2-500 – 17 β -estradiol, hormonal concentration 500 ng L⁻¹; E2-1,000 – 17 β -estradiol, hormonal concentration 1,000 ng L⁻¹. EE2-250 – 17 α -etinilestradiol, hormonal concentration 250 ng L⁻¹; EE2-500 – 17 α -etinilestradiol, hormonal concentration 500 ng L⁻¹; EE2-1,000 – 17 α -etinilestradiol, hormonal concentration 1,000 ng L⁻¹

concentration resulted in a significant decrease (p<0.05) in the condition factor, as observed in the first biometric evaluation.

The mean weight and body length in the control group in both biometric analyses were lower than those found by Massago *et al.* (2010) and Santos, Mareco and Silva (2013) for different tilapia strains.

The highest allometric condition factor values found in this study (treatment E2–250) are still considered as low when compared to those found by Tachibana *et al.* (2008), who studied Nile tilapia during the period of sexual reversion, and Passos-Neto *et al.* (2015), who studied red tilapia during the early development stages. Niemuth and Klaper (2015) found significantly lower condition factor values for *Pimphales promelas* exposed to the endocrine disruptor metformin at a concentration of 40 μ g L⁻¹. Dammann *et al.* (2011), on the other hand, found no significant difference in the condition factor value for *P. promelas* exposed to different concentrations of estrone (10, 50, and 100 ng L⁻¹) and 17 β -estradiol (5, 25, and 50 ng L⁻¹).

According to Vazzoler (1996), the condition factor is a quantitative indicator of the degree of healthiness or welfare of fishes, reflecting recent food conditions and/or reserve expenditure in cyclical activities, influenced by environmental conditions and behavioral aspects of the species. According to the data obtained in our study,

juveniles from control and treatments presented different conditions.

Control and treatments E2–250, E2–500, E2–1,000, EE2–250, EE2–500, and EE2–1,000 presented allometric regression coefficients (b) equal to 3.2371, 3.0424, 3.1123, 3.3633, 3.2176, 3.2995, and 3.6457, respectively. Table 4 shows the equations defining the weight \times length ratios and their respective determination coefficient values (R²).

The values of b found are within the range of 2.5 to 4.0 mentioned by Le Cren (1951). Values of b higher than 3 indicate a positive allometric growth, with a higher increase in length when compared to weight, while values of b lower than 3 indicate a negative allometric growth, which means that weight is the predominant factor. In addition, values of b close to 3 characterize an isometric growth (b=3), which indicates an equal contribution of both biometric variables.

The gonads of the individuals were histologically analyzed from control (n=16) and treatments E2–250 (n=10), E2–500 (n=9), E2–1,000 (n=13), EE2–250 (n=9), EE2–500 (n=14), and EE2–1,000 (n=10). In some cases, the gonads could not be removed successfully because of their small size. Woodling *et al.* (2006) also demonstrated an atrophy in gonadal development in fish collected downstream from the discharge point of an STP. These authors reported difficulty in removing the gonads and in the visual identification of the individual's sex. Dammann *et al.* (2011) reported a decrease of the gonadosomatic

Table 4 - Equations and determination coefficient (R^2) of the weight x length ratios of juvenile Nile tilapia (*Oreochromis niloticus*) under different hormonal concentration, 17 β -estradiol e 17 α -ethinylestradiol, during the first 28 days of life

Treatment	Equation	R^2
Control	$Y = 0.0107X^{3,2371}$	0.9940
E2-250	$Y = 0.0159X^{3,0424}$	0.9862
E2-500	$Y = 0.0141X^{3,1123}$	0.9884
E2-1,000	$Y = 0.0085X^{3,3633}$	0.9892
EE2-250	$Y = 0.0119X^{3,2176}$	0.9865
EE2-500	$Y = 0.0095X^{3,2995}$	0.9943
EE2-1,000	$Y = 0.0047X^{3,6457}$	0.9218

E2-250 – 17 β -estradiol, hormonal concentration 250 ng L⁻¹; E2-500 – 17 β -estradiol, hormonal concentration 500 ng L⁻¹; E2-1,000 – 17 β -estradiol, hormonal concentration 1,000 ng L⁻¹. EE2-250 – 17 α -etinilestradiol, hormonal concentration 250 ng L⁻¹; EE2-500 – 17 α -etinilestradiol, hormonal concentration 500 ng L⁻¹; EE2-1,000 – 17 α -etinilestradiol, hormonal concentration 1,000 ng L⁻¹

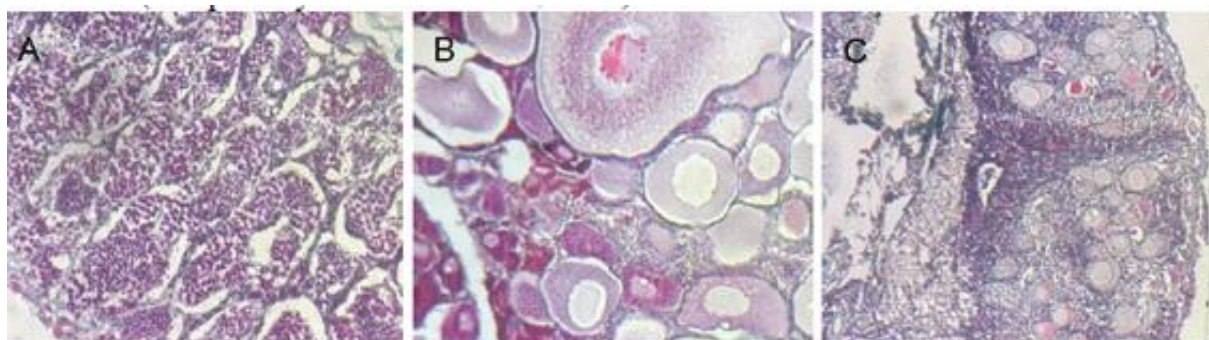
index in *P. promelas* as estrone (10 to 100 ng L⁻¹) and E2 concentrations (5 to 50 ng L⁻¹) increased.

Intersex individuals were submitted to treatments with both hormones (Figure 1) at the lowest concentrations of 250 ng L⁻¹. Reis Filho, Araújo and Vieira (2006) showed that endocrine disrupters are substances that although presented in small concentrations, are capable of triggering effects on the systems in which they are introduced. Campanha *et al.* (2015) also stated that during the degradation process, some of these compounds generated byproducts that may be more toxic than the original compound. On the other hand, laboratory studies at higher concentrations should also be conducted. In this sense, Machado *et al.* (2014) found E2 concentrations of the order of $\mu\text{g L}^{-1}$ in surface water of rivers in southern Brazil.

The scientific interest in quantifying endocrine disrupters in the environment arose due to an increase in

the relationship between the detection of abnormalities in human health (PESSOA *et al.*, 2012) in the presence of these micropollutants and the finding of intersex fish downstream from discharge points of STP close to large urban centers (TETREAUULT *et al.*, 2011). In addition, laboratory analyses of their effects have been observed and documented for specimens of different taxa such as fishes (*Melanotaenia fluviatilis*) (WOODS; KUMAR, 2011), mollusks (*Nassarius burcardi* and *N. jonisii*) (BORYSKO; ROSS, 2014), and amphibians (*Euphlyctis cyanophlyctis*) (PHUGE; GRAMAPUROHIT, 2015).

In treatment E2-500, only one intersex individual was found, while in treatments E2-250 and E2-1,000 no intersex individual was observed. In these last two treatments, however, a considerable number of situations (7 and 5 individuals, respectively) was observed in which the gonads could not be removed due to their small size and fragility. Treatments EE2-250, EE2-500, and

Figure 1 - Photomicrography of juvenile Nile tilapia (*Oreochromis niloticus*) gonads under different hormonal concentration, 17 β -estradiol e 17 α -ethinylestradiol, during the first 28 days of life. Testicle (A), ovary (B) e intersex (C). Harris hematoxylin and Gomori trichrome (Adapted by TOLOSA *et al.*, 2003)

EE2-1,000 presented 2, 4, and 2 intersex individuals, respectively.

According to Czarny *et al.* (2017), in addition to reproductive dysfunctions such as the appearance of intersex individuals, abnormal production of vitellogenin, and a low sperm count, endocrine disrupters may still act negatively on nervous and immune systems, cause behavioral disorders, and affect the homeostasis of organisms in a way general. It is, therefore, imperative that studies in this area be developed as a basis for discussions that may result in future public policies on water quality regarding the presence of these emerging pollutants.

According to Devlin and Nagahama (2002), sex determination in fishes is controlled by a series of biochemical reactions involving different proteins. In order to change the sexual differentiation of males and females, the acting factor must cause structural and functional alterations in such proteins.

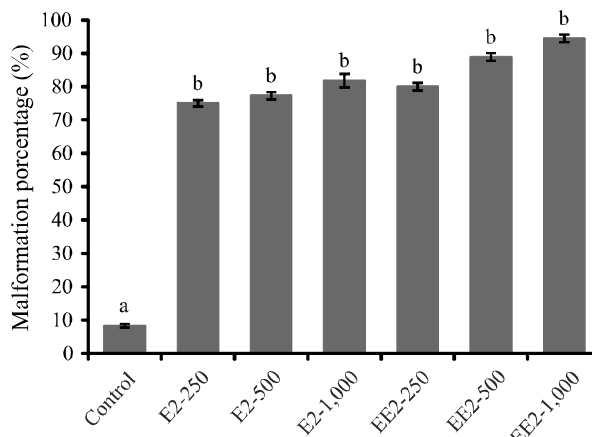
In addition to anthropogenic factors, some environmental factors can influence, on their own, sex determination in fishes. As an example is the temperature, which influences sexual differentiation in tilapias (AZAZA; DHRAÏEF; KRAÏEM, 2008; ROUGEOT *et al.*, 2008) and in other fish species such as the rainbow trout (*Oncorhynchus mykiss*) (MAGERHANS; MÜLLERBELECKE; HÖRSTGEN-SCHWARK, 2009). It is not yet clear whether the influence of anthropogenic (endocrine disrupters) and natural factors (temperature) and future researches may clarify this issue.

A great difference was found between the number of individuals who presented some type of malformation when comparing control (two individuals) with hormonal treatments ($F=7.1771$; $p=0.0015$). However, no significant difference ($p>0.05$) was observed for the number of malformed individuals when treatments were compared to each other.

The number of individuals with some type of malformation was 18 (75.00% of the total individuals that survived until the end of the experiment), 17 (77.27%), 18 (81.82%), 16 (80.00%), 16 (88.89%), and 17 (94.44%) for treatments E2-250, E2-500, E2-1,000, EE2-250, EE2-500, and EE2-1,000, respectively (Figure 2).

Lange *et al.* (2001) found severe malformations in *P. promelas* exposed to low concentrations of 17 α -ethinylestradiol (16 and 64 ng L⁻¹) during their life cycles, such as atrophy, hemorrhage, and distended abdomen. Lei *et al.* (2014) observed mainly scoliosis and dilated

Figure 2 - Percentage of malformations observed in juvenile Nile tilapia (*Oreochromis niloticus*) under different hormonal concentrations, 17 β -estradiol e 17 α -ethinylestradiol, during the first 28 days of life. Different letters superscripts indicate statistical differences ($F=7.1771$; $p=0.0015$). Vertical bars indicates standard error of the mean



E2-250 – 17 β -estradiol, hormonal concentration 250 ng L⁻¹; E2-500 – 17 β -estradiol, hormonal concentration 500 ng L⁻¹; E2-1,000 – 17 β -estradiol, hormonal concentration 1,000 ng L⁻¹. EE2-250 – 17 α -ethinylestradiol, hormonal concentration 250 ng L⁻¹; EE2-500 – 17 α -ethinylestradiol, hormonal concentration 500 ng L⁻¹; EE2-1,000 – 17 α -ethinylestradiol, hormonal concentration 1,000 ng L⁻¹

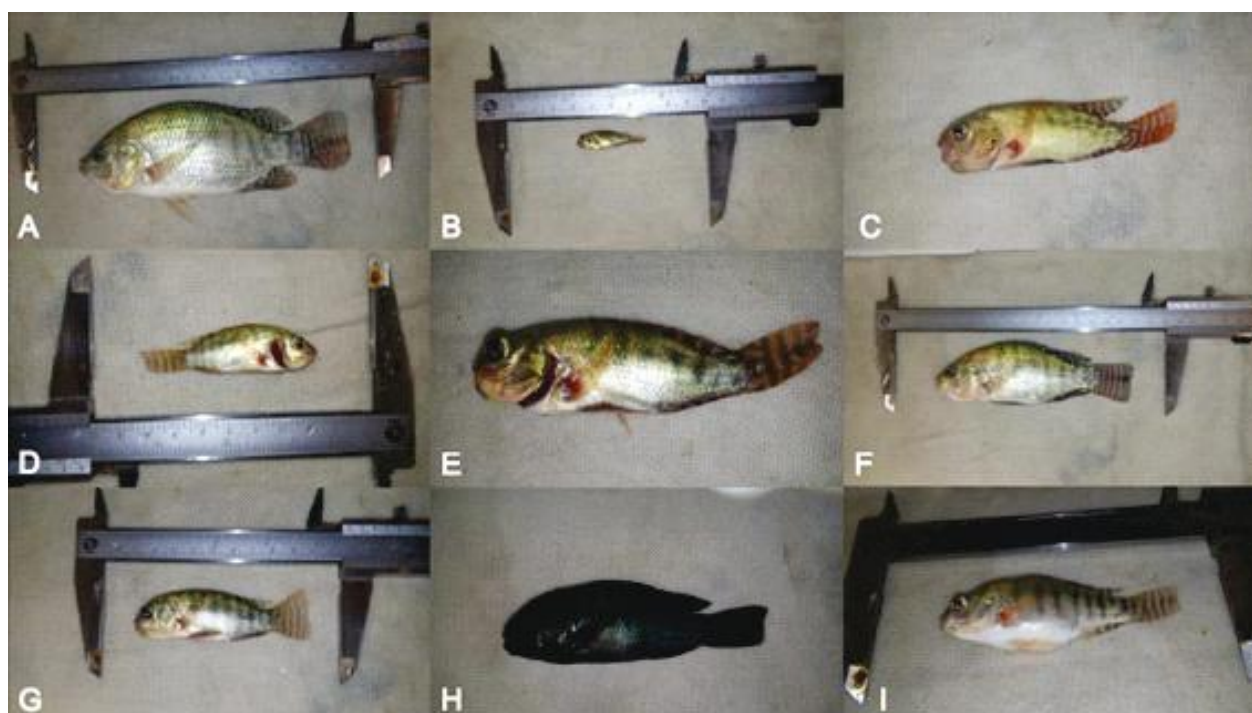
abdomen in *Oryzias latipes* exposed to estriol (E3) concentrations ranging from 5 to 5,000 ng L⁻¹.

Figure 3 shows characteristic examples of malformations found in our study. The most observed deformity was the retracted belly. This condition is often associated with low-quality feed or nutritional deficiency of physiological origin. A retracted belly gives the individual low weight, resulting in an allometric coefficient higher than 3 and hence a low allometric condition factor, as shown in Figure 3 and Tables 2 and 3.

We also observed a severe developmental delay, deformities in the mouth, deformities in the shape of the head, exophthalmos (bulging eyes), distended abdomen with fluid accumulation (ascites), lowering of the mouth floor, exposed gills due to operculum deformities, change in natural color, and signs of hemorrhages in the base of the fins, especially in the pectoral fin.

In addition, we observed individuals with erratic and unbalanced swimming and buoyancy problems since they were most of the time standing at the bottom of the aquarium, which does not correspond to the natural behavior of Nile tilapia.

Figure 3 - Types of malformations found in juveniles Nile tilapia (*Oreochromis niloticus*) submitted to different hormonal concentrations of 17β -estradiol and 17α -ethinylestradiol during the first 28 days of life



Normal individual (A); growth retardation (B); malformation in the shape of the head (C, E, G, and H); oral malformation (C, E, and G); Lowering of the mouth floor (C); malformation of the operculum (D and E); belly retraction (C, D, E, F, G, and H); distended abdomen with fluid accumulation (ascites) (I); exophthalmos (E); abnormal color (H); signs of hemorrhages in the pectoral fin (C, D, and E). All individuals are the same age

CONCLUSIONS

1. Both 17β -estradiol and 17α -ethinyl estradiol are endocrine disruptors and impair the sexual differentiation of Nile tilapia (*Oreochromis niloticus*), causing the development of intersex fish;
2. The hormones 17β -estradiol and 17α -ethinyl estradiol cause malformations (particularly in the head), erratic swimming, problems of buoyancy, and reduce zootechnical performance;
3. The zootechnical performance parameters (weight, body length, survival, and allometric condition factor) were negatively affected by both hormones 17β -estradiol and 17α -ethinyl estradiol.

ACKNOWLEDGEMENTS

The authors would like to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq (National Council for Scientific

and Technological Development) for financial support to perform this research (MCTI/CNPq 14/2012-Universal).

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