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Research paper

Estrogenic and anti-androgenic effects of the herbicide tebuthiuron in male Nile tilapia (*Oreochromis niloticus*)

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ABSTRACT

Tebuthiuron is a phenylurea herbicide widely used in agriculture that can reach the aquatic environments, possibly posing negative effects to the aquatic biota. Phenylurea herbicides, such as diuron, are known to cause estrogenic and anti-androgenic effects in fish, but no such effects were yet reported for tebuthiuron exposure. Thus, the aim of this study was to evaluate if tebuthiuron, at environmentally relevant concentrations (100 and 200 ng/L) and after 25 days of exposure have estrogenic and/or anti-androgenic effects on male of Nile tilapia (*Oreochromis niloticus*), through the evaluation of plasmatic testosterone (T) and estradiol (E_2) levels, brain aromatase (CYP19) levels (western-blot), and by evaluating the histology of the testicles. When compared to the control group, plasmatic T levels decreased about 76% in the animals exposed to 200 ng/L of tebuthiuron, while E_2 levels increased about 94%, which could be related to a significant increase (77%) in *CYP19A1* levels, an enzyme that catalyzes the conversion of androgens into estrogens. Histological analyses of the testicles also demonstrated that tebuthiuron at both tested concentrations caused a decrease in the diameter of the seminiferous tubules and in the diameter of the lumen. Therefore, the gonadosomatic index (GSI) was reduced by 36% % in the animals exposed 200 ng/L to tebuthiuron. Indeed, the relative frequency of spermatocytes and spermatids increased respectively 73% (200 ng/L) and 61% (100 ng/L) in the tebuthiuron exposed animals, possibly due to the impairment of sperm release into the lumen, that was decreased 93% (200 ng/L) in the treated animals compared to the control. These results confirm that tebuthiuron causes estrogenic and anti-androgenic effects in Nile tilapias at environmentally relevant concentrations.

1. Introduction

The amount and variety of agrochemicals applied in agriculture has increased significantly in the last decades. These substances have been widely used in pest and weed control, but can also affect negatively the physiology of non-target organisms (Barbieri, 2009; Kumar et al., 2011). Among the various pesticides used in agriculture stands out tebuthiuron (N-[5-(1,1-dimethylethyl)-1,3,4-thiadiazol-2-y1]-*N,N*'-dimethylurea, molecular weight 228.314 g/mol and log kow 1.79), a substituted urea herbicide that acts by inhibiting photosynthesis (Gomes, 2006). This herbicide is indicated for the control of weeds in many agricultural crops, especially in the sugarcane cultivation in Brazil, an activity with increasing demand due to the increasing interest for renewable fuels, such as ethanol (Rosseto, 2008).

Tebuthiuron has a relative long degradation half-life in soil (1–7 years) and high water solubility (2,500 $mg.L^{-1}$), thus being relatively persistent and having a high leaching potential (Kow = 1.8) (Johnsen and Morton 1989; Dam et al., 2004; Rodrigues and Almeida, 2011; Cerdeira et al., 2007; Mercurio et al., 2015). Photolysis has also been reported as a means of tebuthiuron degradation. Batterham (1992) reported the photodegradation half-lives of tebuthiuron under full sunlight in simulated northern Australian floodplain conditions to

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be 79 and 103 days in soil and water, respectively. The major pathway for microbial degradation of tebuthiuron in soils is demethylation of the terminal nitrogen to form one major and at least three minor metabolites (Morton and Hoffman 1976). According to Emmerich et al. (1984), microbial degradation of tebuthiuron in a loamy soil was slow, with 38% of the original tebuthiuron remaining after 21 months. The microbial degradation of tebuthiuron under simulated northern Australian floodplain conditions was also found to be slow, with over 95% remaining after 99 days (Batterham (1992)). These characteristics indicates that tebuthiuron have high potential to reach the aquatic environment, possibly causing harmful effects on exposed aquatic organisms, such as fish.

Despite the intense use of tebuthiuron in agriculture, studies regarding its negative effects at environmental concentrations on fish are scarce. Due to its relatively high LC50 value for fish (144 mg/L for rainbow trout, 112 mg/L for bluegill, (Hartley and Kidd, 1983; Meister, 1992), > 160 mg/L for goldfish and fathead minnow, (Worthing, 1983), tebuthiuron is considered not hazardous to aquatic organisms (WSSA, 1989). We found only one study regarding tebuthiuron sublethal effects in fish biochemical parameters, but using concentrations close to LC50 values for fish. Franco-Bernardes et al. (2014) reported that a commercial formulation of tebuthiuron at nominal concentrations of 62.5, 125 and 250 mg.L^{-1} can cause DNA damage and alteration in oxidative stress biomarkers in *Oreochromis niloticus*, being also lethal at the higher tested concentration.

Diuron, another urea-derived herbicide, was reported to cause morphological (Scheil et al., 2009; Mhadhbi and Beiras, 2012), biochemical (Sanchez-Muros et al., 2013) and physiological changes in fish (Miranda et al., 2008), besides causing also deficiency in fish growth (Nebeker and Schuytema, 1998). Diuron at concentrations similar to those commonly found in most contaminated aquatic environments (up to 200 ng/L; Schlenk et al., 2012; Köck-Schulmeyer et al., 2013; Masiá et al., 2015) have also been recently shown to alter the metabolism of sexual hormones in Nile tilapia, leading to alterations in gametogenesis and gonadosomatic index (Pereira et al., 2015, 2016; Felício et al., 2015) and aggressive behavior (Boscolo et al., 2017). Schlenk et al. (2012) first proposed that diuron in combination with alkylphenol ethoxylates at environmental concentrations would exert estrogenic effects by increasing vitellogenin levels in Japanese medaka (*Oryzias latipes)* using in vivo bioassays. After, Pereira et al. (2015) demonstrated that diuron, but especially its main biodegradation metabolites (3,4-dichloroaniline, 3,4-dichlorophenylurea and 3,4-dichlorophenyl-N-methylurea), at concentrations similar to those found in contaminated aquatic environments (200 ng/L), have anti-androgenic effects in male Nile tilapias, by decreasing testosterone (T) and 11-ketotestosterone (11KT) levels, which reflected in decreased gonadosomatic index, smaller diameter of seminiferous tubules, and causing a decrease in the mean percentages of spermatids and spermatozoa, after 25 days of exposure. Then, Pereira et al. (2016) demonstrated that diuron metabolites, but not diuron, have estrogenic effects in female Nile tilapias, evidenced by increased estradiol (E_2) plasma levels, gonadosomatic indices and elevated percentage of final vitellogenic oocytes, despite decreasing the amount of germinative cells. Felício et al. (2015) further showed that exposure of Nile tilapia to diuron and its metabolites at similar concentrations in combination with alkylphenols resulted in increases in vitellogenin levels and decreased testosterone levels, confirming the estrogenic and anti-androgenic effects of diuron and its metabolites in fish.

Being also a urea-derived herbicide, tebuthiuron would be expected to have similar estrogenic and anti-androgenic effects on fish as observed for diuron and diuron metabolites, but this hypothesis remains to be confirmed. Therefore, the aim of this study was to evaluate possible estrogenic and anti-androgenic effects of the herbicide tebuthiuron in male Nile tilapia (*Oreochromis niloticus*) exposed to environmental concentrations (100 and 200 ng/L) for 25 days. The histomorphology of the testis, quantification of the hormones T and estradiol E_2 in plasma and the analysis of brain aromatase levels (CYP19, which converts androgens into estrogens) by western blot were performed. Although Nile tilapia is not a native species in Brazil, it was chosen because it is the most cultivated freshwater species in the Brazil, being cultivated in areas close to sugarcane plantations, and thus being possibly affected by tebuthiuron contamination.

2. Materials and methods

2.1. Experimental design

Sexually mature male *O. niloticus* 150 days old posthatch were randomly selected from a stock culture maintained at the São Paulo State University (UNESP), São José do Rio Preto, Brazil. Fish were kept in 500 L indoor stock-tanks (ca. 1 fish 5L − 1) during 15 days for acclimation before experiment began. After the acclimation period the fish (49.4 ± 9.6 g and 12.0 ± 0.7 cm) were placed individually in 18 aquariums containing 17 L of dechlorinated wellwater (one fish per aquarium) in order to compose three groups of six aquariums (N = 6): one control group (without exposure to tebuthiuron), one group exposed to 100 ng/L of tebuthiuron and one group exposed to 200 ng/L of tebuthiuron. Tebuthiuron was added to the aquariums at indicated concentrations from a stock solution of tebuthiuron analytical standard (99.9% purity; Sigma-Aldrich) diluted in acetone. The volume of tebuthiuron solution used in each aquarium was 0.1 mL, and the same volume of acetone was added to the control aquariums to avoid misinterpretation of the results due to solvent effects. Fish were kept in these treatments for 25 days. The aquariums were covered with blue plastic on the side and back walls to prevent visual interactions between fish from different aquariums. Blue was used because it reduces stress levels in Nile tilapia (Volpato and Barreto, 2001). The animals were fed with tropical fish food (Guabi-Pirá/Brazil) corresponding to 3% of the biomass, supplied twice a day. The photoperiod was 12L: 12D (7:00–19:00 h). The water of the experimental aquariums was totally changed every five days to avoid excessive accumulation of ammonia. The concentration of unionized ammonia (NH_3) in the aquariums water after 5 days was 0.78 ± 0.16 mg/L, and the pH and temperature were maintained constant at 7.4 ± 0.5 and 25.6 ± 0.9° C, respectively, during the whole experiment.

The concentrations used were based on environmental concentrations recorded in the scientific literature. Actually, concentrations of tebuthiuron found in natural contaminated surface waters are very variable, ranging from 10 ng/L to 2 mg/L. According to Dam et al. (2004), tebuthiuron was found in surface waters of Northern Australia at concentrations ranging from 0.02 to 2.05 mg/L following application of tebuthiuron to a large Mimosa (*Mimosa pigra*) infestation. Tebuthiuron was still measurable in surface water three, four and five months following application, with the highest concentrations at these time points being 0.168, 0.037 and 0.034 mg/L. On the other hand, Embrey and Frans (2003) reported the detection of a maximum concentration of 90 and 25 ng/L of tebuthiuron in surface waters from the Duwamish River and the Thornton Creek, in the Puget Sound Basin, Washington. Moreover, a water quality study done in the Acadian-Pontchartrain Drainage systems of Louisiana and Mississipi revealed that thebuthiuron concentrations in surface waters is very seasonable, with concentrations varying from bellow the detection limit to about 1 μg/L (Demcheck et al., 2004). Diuron, another phenylurea herbicide extensively used worldwide for weed control was also found in surface waters at concentrations up to 200 ng/L in the San Francisco Bay–USA (Schlenk et al., 2012). Based on this information, studies regarding alterations in sexual hormones and gametogenesis in tilapias were previously done by our group, using 40, 100 and 200 ng/L od diuron (Pereira et al., 2015, 2016; Felício et al., 2015; Boscolo et al., 2017). Therefore, tebuthiuron concentrations used in this study was chosen considering realistic tebuthiuron concentration found in natural environments but also considering other studies previously published by

our laboratory with diuron, for comparative purposes.

This study was conducted in agreement with the precepts of National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Committee for Ethics on Animal Using (CEUA), UNESP, São José do Rio Preto, SP, Brazil − permission number 108/2015.

2.2. Quantitative analysis of tebuthiuron in water

At the beginning of the experiment, the concentration of contaminant in the water was checked by high performance liquid chromatography (HPLC). Total volume of the water with the respective treatments was renewed every 5 days during the exposure experiment and the amount of remaining tebuthiuron after 5 days were determined. A parallel experiment was carried out in aquariums without fish (water and contaminant only), to analyze the concentration of tebuthiuron along five days without the influence of the fish. The analysis of tebuthiuron in water was in accordance to the method proposed by Cerdeira et al. (2007) with modifications. An aliquot of 50 µL of the water sample was directly injected into a HPLC system after filtration (Millex GV in polyethylene with 0.22 µm durapore membrane) and the compounds were separated by a Shimadzu C18 column (150 × 4.6 mm, 5 µm). The HPLC system (Shimadzu Corporation, Kyoto, Japan) consisted of one CBM20A communication bus module, two LC20AD-XR pumps, one DGU20A3R degassing unit, one SIL20AC-XR autosampler, one CTO20AR column oven, and one SPDM20A photodiodearray (PDA) detector. The PDA detector was set at 200–600 nm for all analytes, which were quantified at 254 nm. The detection limit (DL) was 6.1 ng/L, the mobile phase was acetonitrile/water 40:60 (v/v) wich isocratic flow 1 mL/min, furnace temperature of 40° C and running time of 10 min. The calculations were based on an analytical curve constructed by injecting analytical standards (tebuthiuron > = 100%, Sigma-Aldrich, St. Louis, MA, USA) at the concentrations of 10, 50, 100, 200 and 300 ng/L.

The mobile phase consisted of acetonitrile and water (40:60, v/v), and it was isocratically pumped in a flow rate of 0.5 mL/min. The column oven temperature was set to 40 °C. Chromatogram was monitored during 5 min and peaks were identified and quantified using LAB Solutions 5.71 software (Shimadzu Corporation).

2.3. Analysis of hormones

After the exposure period (25 days), the fish were anesthetized by immersion in a benzocaine solution (9 mg.L^{-1}) as recommended by Gontijo et al. (2003) for blood collection. Blood was collected using hypodermic needles and heparinized syringes (Liquemine, Roche, Rio de Janeiro, Brazil), punctured from the caudal vein and centrifuged at 3000 rpm for 10 min. Plasma was separated and frozen at −80° C for further analyses of estradiol (E2) and testosterone (T) by enzyme linked immunosorbent assay (ELISA) without any previous protein-bound extraction procedure (free plasmatic hormone levels) (E_2 and T: Cayman Chemical, Michigan, USA) validated for *O. niloticus*, following the method described by Brown et al. (2004). Plasma samples were analyzed in duplicate and the validations of the kits (E2 and T) were determined by calculating the intra and variation coefficients and the recovery peak (%). The acceptable limit for the intra and inter-assay coefficients was ≤ 20% and for the peak recovery 90–110%. Absorbance measurements were performed using a microplate reader (Victor 2, Perkin-Elmer, Waltham, MA, USA).

2.4. Gonadosomatic index (GSI)

After blood collection, the animals were euthanized by immersion in 5 L of water containing benzocaine at 28 mg/L for the collection of the gonads (testicles) and the calculation of the GSI. The GI was compared between the treatments and obtained by the following formula: GSI = (gonad weight/total fish weight X 100), as proposed by Romagosa (2010).

2.5. Morphological analyses

After calculating the GSI, fragments of the cranial, middle and caudal regions of the testis were removed for histomorphometric analyses. The testis samples were fixed in Bouin for 24 h and submitted to histological routines for histomorphometric analysis. Fixed material was included in historesin (Historesin Plus, Leica, Heidelberg, Germany), cut to a thickness of 2 µm and stained with hematoxylin and floxin (Gurr, 1971). The relative frequencies of spermatogonia, spermatocytes, spermatids and spermatozoa were analyzed, and the diameter of the lumen and seminiferous tubules were measured. The volume of each cell was evaluated by light microscope in a grid with 320 points. Three microscopic fields from each region of the testis (cranial, middle and caudal) were randomly selected for each animal. The points on the different types of spermatogenic cells were computed. The percentage of each component of the testicles was calculated using the formula: (n° points X 100/total of points). The method used for counting was the same used by Alvarenga and França (2009) and Pereira (2013). Histological slides of the testis were observed using an Olympus BX41 microscope (4X objective) with capture system (Olympus DP11).

2.6. Analysis of CYP19A1

CYP19A1 was quantified in the brain by western blotting. The tissues were homogenized (1:4, m:v) in Tris-HCl buffer (0.2 mM, pH 7.5). The homogenized samples were centrifuged at 10,000 *g* for 30 min at 4 °C. The supernatant fraction was then collected and centrifuged again at 50,000 *g* for one hour to obtain the cytosolic and pellet fractions. The pellets were resuspended in 0.1 mM Tris-HCl buffer and pH 7.5. For protein quantification, the method of Bradford (1976) was used with bovine serum albumin as standard.

The amount of *CYP19A1* in the brain was estimated in duplicate by western blotting as described by Lavado et al. (2004), with minor modifications. The pellet fraction (10 µg of protein) was heated at 95° C for 5 min in SDS-PAGE buffer (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and was separated by electrophoresis in a 10% SDS-polyacrylamide gel for 30 min at 60 V followed by 80 min at 150 V. After the electrophoresis run the gel was transferred to nitrocellulose membrane (Bio-Rad) using *trans*-Blot® Semi-Dry Transfer Gel (Bio-Rad Laboratories, Hercules, CA, USA) for 30 min at 15 V. After transfer, the membrane was immersed in mouse monoclonal anti-*CYP*19 primary antibody (1:200 dilution) and incubated overnight at 25 °C. The membrane was then washed three times with buffered saline containing 0.2% Tween 20 (v/v) and 0.5% (m/v) gelatin and incubated for 1 h with goat anti-mouse IgG (H + L) AP conjugate, then the bands were developed with the “Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad Laboratories)." Quantification was performed on a Bio-Rad photo documentation system using Image Lab software (Bio-Rad).

2.7. Statistical analyses

The outliers were identified by mean ± 2 SD and these values were replaced by the mean (as recommended by Cousineau et al., 2010). Initially the normality of the data was verified through the Shapiro Wilk test and the homoscedasticity of the data through the Levene test. For parametric data, one-way ANOVA was used followed by the Tukey post-hoc test. For nonparametric data, the Kruskal–Wallis test was used, followed by multiple comparisons of mean ranks (Zar, 1999; Ha and Ha, 2007).

Table 1
Tebuthiuron concentrations in water (mean ± SD) at the beginning of the experiment and after 5 days, with or without fish.

Parameters	Control (0 ng/L)	TBH (100 ng/L)	TBH (200 ng/L)
Initial concentration with fish	< DL	122.3 ± 10.1	204.4 ± 8.5
Concentration after 5 days with fish	< DL	104.5 ± 23.3	130.6 ± 16.6
Initial concentration without fish	< DL	99.9 ± 11.1	204.1 ± 7.0
Concentration after 5 days without fish	< DL	98.2 ± 4.2	201.7 ± 9.4

< DL = Bellow detection limit. Concentrations in ng/L. N = 6.

3. Results

3.1. Tebuthiuron concentration in water

There was no mortality in any of the experimental groups. The concentrations of the herbicide analyzed in the water at the beginning of the experiment and after 5 days of exposure in the presence or the absence of fish are shown in Table 1. In those aquariums without fish, tebuthiuron concentrations after 5 days were practically unchanged, being decreased from 99.9 ± 2.0 to 98.2 ± 1.1, and from 204.1 ± 3.9 to 201.7 ± 5.4 in the aquariums spiked with 100 and 200 ng/L, respectively. On the other hand, the concentrations of tebuthiuron in the water of the aquariums containing fish decreased from 122.3 ± 6.5 to 104.5 ± 3.9 ng/L (14.5%) and from 204.4 ± 0.9 to 130.6 ± 4.2 ng/L (36%) in the groups exposed to 100 and 200 ng/L, respectively.

3.2. Hormone levels

Levels of plasmatic T decreased 76% in the fish treated with 200 ng/L in comparison to the control group (P = 0.014) (Fig. 1A). Plasmatic E2 levels increased 94% in the animals exposed to 200 ng/L of tebuthiuron when compared to the control group (P = 0.002) and 61% in relation to the group that was exposed to 100 ng/L of the herbicide (P = 0.0002) (Fig. 1B).

3.3. Gonadosomatic index (GSI)

The GSI presented was reduced (30%) in the animals exposed to 200 ng/L of tebuthiuron compared to the control group (P = 0.011) (Fig. 1C).

Histomorphometric analysis of the testis

The diameter of seminiferous tubules of fish exposed to both concentrations of tebuthiuron was 43% reduced (p = 0.0001) compared to the control group (Fig. 2A). The diameter of the lumen also decreased 96% (p < 0.001) in the animals exposed to the concentration of 200 ng/L, and 77% (p = 0.0003) in animals exposed to concentration of 100 ng/L, compared to the control group (Fig. 2B). Also, the diameter of the lumen in animals exposed to 200 ng/L were 81% lower (p = 0.0038) compared to those of animals exposed to 100 ng/L. The control group presented all phases of gametogenesis: spermatogonias, spermatocytes, spermatids and spermatozoa (Fig. 2C). There were no significant differences in the relative frequency of spermatogonia between the experimental groups. There was a 73% increase (P = 0.0001) in the relative frequency of spermatocytes of the group exposed to 200 ng/L compared to the control group. The relative frequency of spermatids increased 61% (P = 0.0007) in fish exposed to tebuthiuron 100 ng/L in relation to the control group (Fig. 2C). The relative frequency of spermatozoa released in lumen decreased by 93% (P = 0.0001) in fish treated with the concentration of 200 ng/L compared to control group. Photomicrographs of cross sections of male testis of *O. niloticus* exposed to tebuthiuron for 25 days can be seen in Fig. 3.

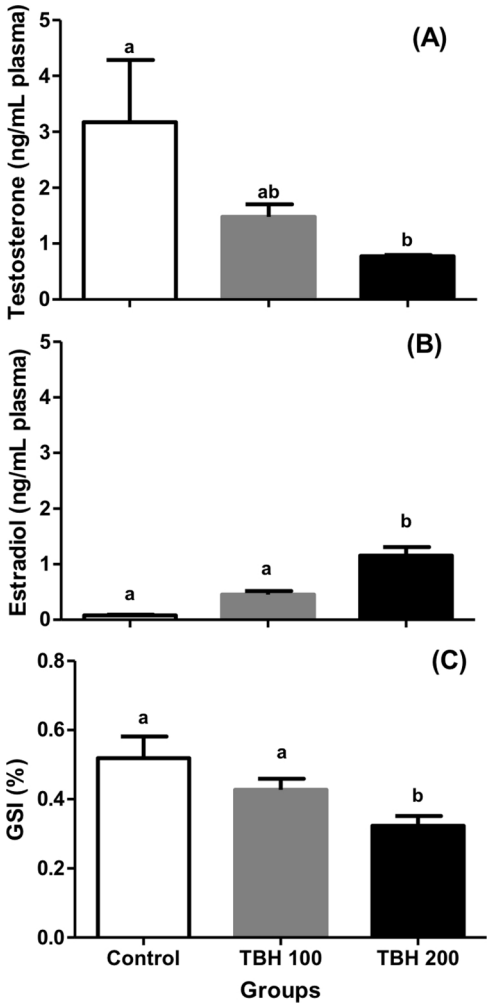

Fig. 1. Plasma testosterone concentrations (A), plasma estradiol concentrations (B) and gonadosomatic index (C) in male *O. niloticus* exposed to tebuthiuron for 25 days. Different letters indicate significant difference between groups (ANOVA, Tukey, P < 0.05). Data are expressed as mean ± S.E. N = 6.

3.4. CYP19A

CYP19A protein levels were increased in the brain of animals exposed to tebuthiuron at 200 ng/L (77% increase, Fig. 1A) compared to the control group (p = 0.0242) and to the group exposed to the concentration of 100 ng/L (p = 0.0365) (Fig. 4A). A picture of the nitrocellulose membrane can be seen in Fig. 4B.

4. Discussion

This study aimed on evaluating the effect of the herbicide tebuthiuron on sexual hormones and histopathology of the gonads of male Nile tilapias. In order to verify if tebuthiuron was absorbed from water by the fish, tebuthiuron concentrations were checked by HPLC along five days in the presence or the absence of the fish into the aquariums. The decrease in the tebuthiuron concentrations in the aquariums along five days of exposure indicated that the herbicide was absorbed by the fish, since no significant decrease in tebuthiuron concentration was observed in the aquariums without fish. Tebuthiuron absorption by fish from water was already previously demonstrated

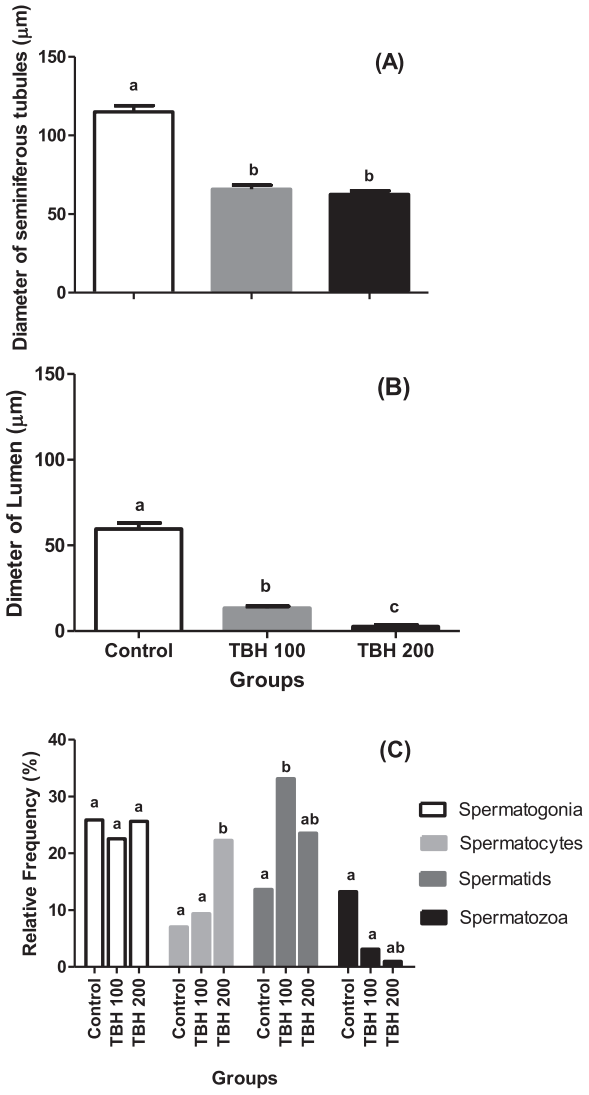

Fig. 2. Diameters of the seminiferous tubules (A) and of the testis lumen (B) (mean ± S.E.), and the relative frequency of spermatogonia, spermatocytes, spermatids and spermatozoa (C) of male *O. niloticus* exposed to 100 and 200 ng/L of tebuthiuron for 25 days. Different letters indicate statistical differences (ANOVA, Tukey, $P < 0.05$). N = 6.

(Morton and Hoffman, 1976).

In this study, levels of plasmatic T decreased about 76% in fish treated at the higher tebuthiuron concentration, indicating anti-androgenic effect of this herbicide, with potential negative effects on gametogenesis. One of the main regulators of development and maintenance of testicular spermatogenesis in teleosts is T, produced by the Leydig cells (Miura and Miura, 2003; Kaptaner and Kankaya, 2013). T also assists in spermatogonial proliferation (Schulz and Miura, 2002; Almeida et al., 2009; García-Lopez et al., 2009). Therefore, compounds that affects T production generally causes alteration in gonad morphology and fuction (Tilton et al., 2003; Katsiadaki et al., 2012; Forsgren et al., 2014), such as reduction in testicular mass (Tilton et al., 2003), in the diameter of seminiferous tubules (Dutta and Meijer, 2003) and in the percentage of germ cells (Kaptaner and Kankaya, 2013), which is in accordance with our results. Similarly, Pereira et al. (2015) observed anti-androgenic effects and delayed development of male sexual organs and germ cells in Nile tilapia exposed to diuron and diuron metabolites. Indeed, Boscolo et al. (2017) observed that diuron decreased testosterone levels leading to a decrease in the aggressive behavior in Nile tilapias. Thus, the effects of these two urea-derived compounds seem to act similarly, causing anti-androgenic effects.

In fish, reductions in T levels may affect E_2 biosynthesis (Nagahama and Yamashita, 2008; Lubzens et al., 2010). In this study, there was a strong negative relationship between T and E_2 levels: plasma E_2 increased 94% while T decreased 76% in the animals exposed to the highest tebuthiuron concentration. E_2 is the main hormone involved in sexual differentiation of females, being responsible for stimulating the development of female ovaries (Guiguen et al., 2010), and because of that E_2 is widely used in sexual reversion of fish in aquaculture (Pandian and Sheela, 1995; Piferrer, 2001). The E_2 increase observed in male Nile tilapia exposed to tebuthiuron suggests estrogenic effect, which combined to the anti-androgenic effects (T decrease) is probably impairing reproductive-related organs.

Studies have shown that exposure to estrogenic compounds can induce numerous adverse effects in fish such as hermaphroditism, reduction of normal testis size, impairment of normal Leydig cell function and reduction of spermatozoa quality and quantity (Lathers, 2002). The significant alteration of the sex hormones T and E_2 in the present study are related to the observed reduction of diameter of seminiferous tubules and the diameter of lumen. In this context, it is known that an increase in seminiferous tubule diameter during the fish testes maturation occurs first due to an increased proliferation of Sertoli and germ cells and also due seminal fluid production (Schulz et al., 2005; Nóbrega et al., 2009; Schulz et al., 2010) It is also known that each Sertoli cell can support a fixed number of germ cells, so the number of Sertoli cells per testis ultimately dictates testis size (GSI) (Grier and Uribe-Aranzábal, 2009; Schulz et al., 2010). Taken together the reduced seminiferous tubule diameter, and the GSI values for treated males are probably a consequence of the accumulation of immature germ cells in treated animals (spermatocytes for tebuthiuron at 200 ng/L and spermatids for tebuthiuron at 100 ng/L). In the control group the relative frequency of spermatozoa (differentiated mature cells) was higher than those observed in animals exposed to 200 ng/L of tebuthiuron, characterizing a less intense spermatogenesis for the later. Consequently, GSI decreased by 36% in the highest concentration, causing a decrease in size of the gonads in treated male tilapia evaluated in the present study.

With respect to gametogenesis, spermatogonia after a species-specific number of mitotic divisions differentiate into spermatocytes, initiating the meiotic or spermatocyte phase. After the first meiotic division, they originate the haploid spermatids, through the second meiotic division. Then, the spermiogenic or differentiation phase occurs in which spermatids form spermatozoa, which are released in lumen (Silva, 1987; Schulz et al., 2010).The lack of significant difference in the relative frequency of spermatogonia in the testes of treated fish together with the fact that the relative frequencies of spermatocytes and spermatids were significantly increased after exposure to the higher and the lower tebuthiuron concentrations suggest that tebuthiuron decreased the rate of meiosis and spermatid differentiation causing an accumulation of spermatocytes and spermatids in treated animals. Similarly, accumulation of immature germ cells has been reported in male fish submitted to diverse type of adverse conditions, such as hypoxia (Wu et al., 2003; Shang et al., 2006; Thomas et al., 2007) exposition to diuron and its metabolites (Pereira et al., 2015), wrong management conditions (De Souza et al., 2015) and low temperature (Alvarenga and França, 2009).

One of the physiological mechanisms that results in the production of estrogen in fish is the conversion of androgens into estrogens through the action of the microsomal enzyme aromatase (*CYP*19) (Murphy, 1998; Huber et al., 2002; Ghosh et al., 2009). Aromatase is expressed mainly in testes, brain, ovary and adipose tissue, and endogenous estrogens originate from the conversion of androgens by the enzymatic aromatase complex (Bulun et al., 1994; Stauffer et al., 2012; Jeng et al., 2012; Tzchori et al., 2004). Changes in aromatase activity have previously been suggested to be a possible indicator of endocrine disruption or reproductive dysfunction in fish, since increases in aromatase levels are related to increased estrogen levels (Lyssimachou et al., 2006;

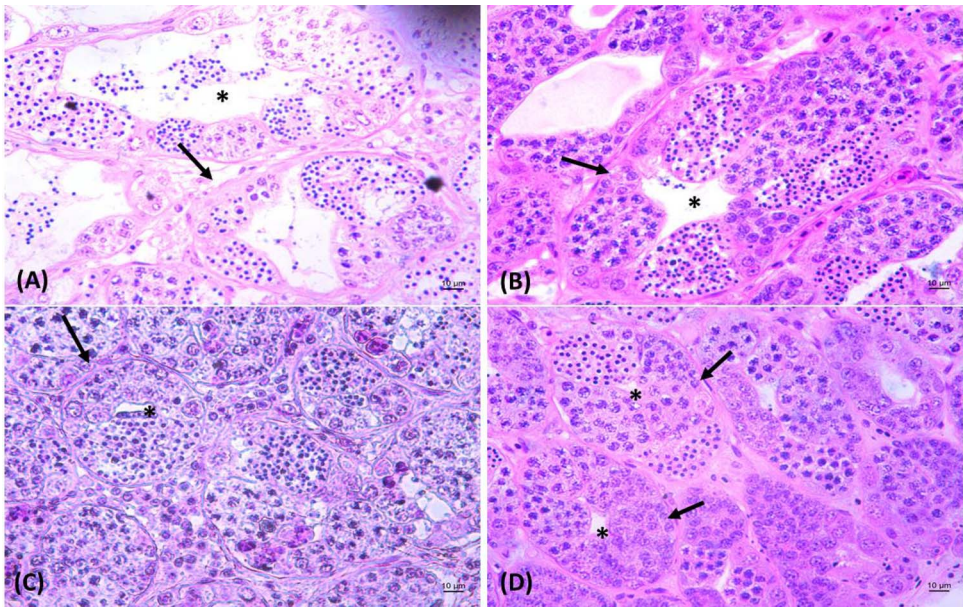

Fig. 3. Photomicrographs of cross sections of testis of *O. niloticus* males exposed to tebuthiuron for 25 days. (A) Section of testis of the control group indicating a regular shape and size seminiferous tubule (arrow) lumen (asterisk). (B) Section of testis of animals exposed to tebuthiuron at concentrations of 100 ng/L showing seminiferous tubules with different degenerative degrees changes in addition to a relative reduction of diameter (arrow), reduction of sperm numbers in lumen and decrease in diameter of lumen (asterisk). (C) and (D) Testicular section of animals exposed to tebuthiuron at concentrations of 200 ng/L showing extreme reduction of seminiferous tubule diameter (arrow), disappearance of the spermatozoa released in the lumen and almost disappearance of the lumen diameter (asterisk). Hematoxylin-floxin. Scale bar = 100 µm. N = 6.

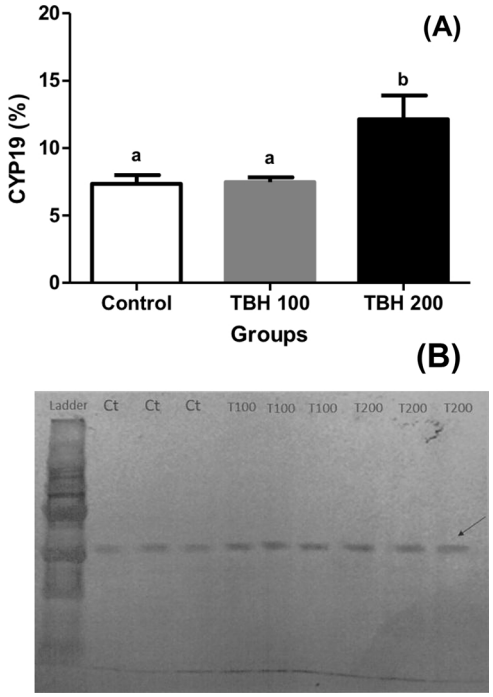

Fig. 4. Brain CYP19A protein levels of male *O. niloticus* exposed to 100 and 200 ng/L of tebuthiuron for 25 days (A). Nitrocellulose membrane showing the brain CYP19A bands (arrow) (B). Ct: control group; T100: treated with 100 ng/L of tebuthiuron; T200: treated with 200 ng/L of tebuthiuron. N = 6.

Sanderson, 2006). In this work, the levels of brain aromatase as indicated by western-blot analysis was increased about 77% in fish exposed to of 200 ng/L when compared to the control group and the group exposed to 100 ng/L of tebuthiuron. Since aromatase is involved in the conversion of androgens into estrogens, the increased aromatase in animals exposed to tebuthiuron could be related to the decreased T and increased E_2 levels in plasma.

In general, our results showed anti-androgenic effects of tebuthiuron, in accordance with similar effects observed in Nile tilapias exposed to diuron, another phenylurea herbicide. However, while diuron at 200 ng/L for 25 days caused only a 10% decrease in plasmatic T levels (Pereira et al., 2015), tebuthiuron at same concentration and experimental conditions decreased T levels by 76%. Also, diuron at these experimental conditions did not alter the GSI or any of the histomorphological parameters (Pereira et al., 2015), while tebuthiuron at 200 ng/L lead to a decrease in the GSI value and all of the histomorphometric markers. Moreover, while diuron at 100 ng/L for 25 days was not estrogenic to female Nile tilapia (Pereira et al., 2016), tebuthiuron at same concentration and exposure time in this study increased E_2 levels in male Nile tilapia, demonstrating estrogenic activity. These results suggest that tebuthiuron is more potent in causing anti-androgenic and estrogenic effects in Nile tilapia than diuron, thus possibly representing a higher risk for fish. However, a comparative estimation of environmental risks of diuron and tebuthiuron for fish is something difficult, considering that diuron sales in Brazil is the double (8580 tons in 2014) of tebuthiuron sales (3950 in 2014) (IBAMA, 2014). Also, tebuthiuron has a larger half-life than diuron, but the lower half-life of diuron lead to the generation of metabolites with more anti-androgenic and estrogenic activities than the parental compound. Nevertheless, it is clear that the increasing use of both diuron and tebuthiuron in Brazil would represent an important risk to be considered for the aquatic life.

5. Conclusion

The results of this study reveal that the herbicide tebuthiuron, at environmentally relevant concentrations, causes deregulation of hormonal biosynthesis and loss in the spermatogenesis (meiotic and spermiogenic phases) in male Nile tilapias, causing estrogenic and anti-androgenic effect in Nile tilapia. Taken together the increase in CYP19A levels and the alterations in T (decrease), E_2 (decrease), GSI (decrease) and alterations in gametogenesis, we can hypothesize that increased aromatase levels due to tebuthiuron exposure are causing excessive conversion of T to E_2, leading to increased estrogen levels and decreased testosterone, causing changes in spermatogenesis and leading to reduced gonad size. Our study clearly contributes to a better understanding of the negative impacts of tested herbicide on fish reproduction.

Conflict of interest

None.

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