Chemosphere 146 (2016) 497–502

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Estrogenic activities of diuron metabolites in female Nile tilapia (*Oreochromis niloticus*)

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HIGHLIGHTS

- Nile tilapia were exposed for 25 days to 100 ng/L diuron and three diuron metabolites.
- Diuron metabolites increased E_2 plasma levels, gonadosomatic indices and vitellogenic oocytes.
- Diuron and its metabolites caused a decrease in germinative cells.
- Concentrations of 17α-hydroxyprogesterone (17α-OHP) was not altered.

ARTICLE INFO

Article history:
Received 22 October 2015
Received in revised form
18 December 2015
Accepted 19 December 2015
Available online 30 December 2015

Handling Editor: Jim Lazorchak

Keywords:
Nile tilapia
Diuron
Diuron metabolites
Gametogenesis
Endocrine disrupting chemicals

ABSTRACT

Some endocrine disrupting chemicals (EDCs) can alter the estrogenic activities of the organism by directly interacting with estrogen receptors (ER) or indirectly through the hypothalamus-pituitary-gonadal axis. Recent studies in male Nile tilapia (*Oreochromis niloticus*) indicated that diuron may have anti-androgenic activity augmented by biotransformation. In this study, the effects of diuron and three of its metabolites were evaluated in female tilapia. Sexually mature female fish were exposed for 25 days to diuron, as well as to its metabolites 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 3,4-dichlorophenyl-*N*-methylurea (DCPMU), at concentrations of 100 ng/L. Diuron metabolites caused increases in E_2 plasma levels, gonadosomatic indices and in the percentage of final vitellogenic oocytes. Moreover, diuron and its metabolites caused a decrease in germinative cells. Significant differences in plasma concentrations of the estrogen precursor and gonadal regulator17α-hydroxyprogesterone (17α-OHP) were not observed. These results show that diuron metabolites had estrogenic effects potentially mediated through enhanced estradiol biosynthesis and accelerated the ovarian development of *O. niloticus* females.

1. Introduction

Endocrine disrupting chemicals (EDCs) are a class of environmental pollutants that can interfere with normal functions of the endocrine system (Tabb and Blumberg, 2006). Currently, the most studied are those that alter estrogenic functions of the organism by interacting with estrogen receptors (ER) (Sumpter and Jobling, 1995; Schlenk et al., 2012; Forsgren et al., 2014; Kroon et al., 2014). The interaction of EDCs with the specific nuclear or membrane receptors in target cells may alter the function of the hypothalamic-pituitary-gonadal (HPG) axis affecting synthesis and clearance of key sex steroid hormones and be a potential mechanism of endocrine disruption (Kroon et al., 2014; Sun et al., 2014). The biosynthesis of sex steroid hormones also provides enzymatic

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http://dx.doi.org/10.1016/j.chemosphere.2015.12.073
0045-6535/

targets for EDCs, especially the steps catalysed by cytochrome P450 aromatase (Sanderson and Van den Berg, 2003), the steroidogenic enzyme catalysing the final step in the conversion of androgens into estrogens (Simpson et al., 2002), which are important hormones involved in controlling the reproductive process in teleosts (Nagahama and Yamashita, 2008; Lubzens et al., 2010). In addition to 17β-estradiol, an additional steroid, 17α-hydroxyprogesterone mediates oocyte growth and ovulation (Nagahama and Yamashita, 2008). Consequently, disruption in the biosynthesis of either compound could have impacts on reproductive function in females.

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a substituted urea herbicide that has been identified in estrogenic fractions of water extracts (Schlenk et al., 2012) and caused indirect as well as sublethal effects on non-target species at environmentally relevant concentrations (Giacomazzi and Cochet, 2004; Cardone et al., 2008; Scheil et al., 2009). Following applications to soil, diuron has been shown to undergo run-off to rivers and lakes (Lamoree et al., 2002; Gooddy et al., 2002), potentially leading to negative effects to aquatic organisms such as teleosts (Nebeker and Schuytema, 1998; Mhadhbi and Beiras, 2012). Furthermore, diuron can also undergo biotransformation to 3,4-dichloroaniline (DCA), 3,4-dichlorophenylurea (DCPU) and 3,4-dichlorophenyl-N-methylurea (DCPMU) (Tixier et al., 2002; Hodge et al., 1967; Abbas et al., 2007). Some studies have shown that DCA may be more toxic than diuron (Giacomazzi and Cochet, 2004; Scheil et al., 2009; Da Rocha et al., 2013); however, studies regarding the toxicity of other metabolites (DCPMU, DCPU) are still limited. A recent study observed that diuron metabolites (mainly DCPMU, DCPU) have anti-androgenic activities in male Nile tilapia (Pereira et al., 2015), although no effect was observed for diuron, which is consistent with another recent study that did not observe estrogenic or anti-androgenic effects of diuron in juvenile barramundi (*Lates calcarifer*) (Kroon et al., 2015). Additional documented effects of diuron and its metabolites (DCA) in teleosts include morphological (Mhadhbi and Beiras, 2012; Gagnon and Rawson, 2009), biochemical (Sanchez-Muros et al., 2013), physiological (Vinggaard et al., 2000; Miranda et al., 2008; Scheil et al., 2009) and behavioral alterations (Saglio and Trijasse, 1998). However, studies evaluating potential steroidogenic activity associated with reproductive impacts of diuron and its metabolites are limited in teleosts.

Given the important role of sex steroid hormones in the regulation of reproduction in vertebrates and previous studies showing endocrine effects in male teleosts, the purpose of the present study was to evaluate the potential estrogenic effects of diuron and its metabolites on oogenesis of female *Oreochromis niloticus*. This work is the first to investigate these effects in teleost oogenesis providing useful data concerning the potential hazards of a widely used and frequently detected herbicide in the aquatic environment.

2. Materials and methods

2.1. Ethical note

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 Using Animal (CEUA), UNESP, São José do Rio Preto, SP, Brazil – permit 0715/2013.

2.2. Fish maintenance

Sexually mature female *O. niloticus* in pre-ovulatory stage (10.02 ± 1.17 cm, 71.15 ± 2.44 g) 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/5 L) during 30 days for acclimation before experiment began. Food (commercial pellets for tropical fish, 32% Crude Protein – Guabi-Pira/Brazil) was provided twice a day to satiation. External biological filters and constant aeration ensured water quality.

Water mean temperature was 26.6 ± 1.1 °C and photoperiod was 12L:12D (7:00–19:00 h). The water pH and NH3 levels during the exposure were 7.00 ± 0.40 and 0.55 ± 0.08 mg, respectively. Fish were fed with ration for tropical fish (Guabi-Pira/Brazil) corresponding to 3% of biomass, provided twice a day (at 8:00 h and 18:00 h). Water containing the respective compounds was 100% replaced every five days by static renewal to ensure water quality and compound concentrations.

2.3. Chemicals

All chemicals used were of analytical grade and purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA).

2.4. Exposures

After the acclimation period, the animals were exposed to diuron and its metabolites with subsequent measurements on oogenesis and plasma steroid levels. Six fish were used per treatment. Each fish was individually exposed in a glass aquarium of 17 L. One group remained in aquaria without contaminant (experimental controls) and the other groups were exposed to diuron, DCA, DCPU or DCPMU, at nominal concentrations of 100 ng/L for 25 days. This concentration was selected based in a previous study that found diuron at concentrations up to 200 ng/L in the San Francisco Bay Delta (Schlenk et al., 2012), and also based on the European Union legislation for unregulated herbicides, such as diuron, which establishes 100 ng/L as the permissible limit for individual herbicides in drinking water (Sanchis-Mallols et al., 1998). All chemicals were dissolved in a sock solution of 1 mL of acetone and then added (0.1 mL) into the aquariums. Control groups also received the same volume of acetone to avoid ambiguous interpretation of the results due to possible solvent effects. Selection of the exposure period was based on previous studies with other species which showed reproductive effects after chronic exposure to diuron (Cardone et al., 2008; Fernandes et al., 2007). The concentration of 100 ng/L was chosen based on mean values found in contaminated aquatic environments (up to 160 ng/L) (Köck-Schulmeyera et al., 2013; Masiá et al., 2015).

2.5. Chemical analyses

Water samples (10 mL) from the experimental aquaria were taken before adding the fish into the aquaria at the beginning of exposures and prior to each renewal, for the measurement of diuron, DCPMU, DCPU and DCA concentrations by HPLC. 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. Fifty microliters of the water were filtered and directly injected into the system, and the compounds were separated by a Shimadzu Shim-Pack XR-ODS column (2.0 × 100.0 mm, 2.2 μm particle size, 8 nm pore size). The PDA detector was set at 200–600 nm for all analytes, which were quantified at 250 nm. 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). The calculations

were based on a calibration curve previously constructed by injecting authentic standards into the HPLC system (10–1000 ng/L). The minimum detection levels for all compounds was 10 ng/L.

2.6. Blood sampling and steroid assays

At the end of the experimental period all animals were anesthetized with benzocaine (9 mg/L) for blood sampling. Blood was collected by puncturing the caudal vein with heparinized syringes (Liquemine, Roche, Rio de Janeiro, RJ, Brazil) and needles. Blood was centrifuged at 1300 *g* for 10 min. The plasma was separated into aliquots and frozen at −80 °C for the subsequent 17β-estradiol (E_2) and 17α-hydroxyprogesterone (17α-OHP) assays. The plasma steroid level was measured by ELISA (Enzyme Linked Immunosorbent Assay) (E_2 and 17α-OHP: Cayman Chemical, Michigan, USA). Plasma samples were run in duplicate with an acceptable limit of ≤20.0 for the intra-assay coefficients of variation (Brown et al., 2004). Absorbance measurements were collected using a microplate reader (Victor 2, Perkin–Elmer, Waltham, MA, USA).

2.7. Sample processing and histology

After blood collection, fish were killed with a lethal dose of benzocaine (28 mg/L) and their gonads were collected. The ovary were removed and weighed to calculate the gonadosomatic indices (GSI), which is the percentage of total body weight represented by the ovary. For histological evaluation, ovarian samples (cranial, middle and caudal regions) were collected, fixed in Bouin solution as described by Pereira et al. (Pereira et al., 2013). The fixed material was embedded in Historesin (Historesin Plus, Leica, Heidelberg, Germany), cut into 2 μm thick sections and stained with haematoxylin-fluoxin.

2.8. Histomorphometric analyses

Morphological analyses were performed on the ovary sections with ovarian lamellae that contained oocytes at various stages of development. Oocytes showing the nucleus in transversal sections were classified according to criteria given in Coward and Bromage (Coward and Bromage, 2005) and Pereira et al. (Pereira et al., 2013). The morphological changes were described using an Olympus BX41 microscope system (4x magnification) with an Olympus DP11 capture apparatus (with measurements performed using Image-Pro Plus Version 4.1.0.0 software).

2.9. Volume density of the gonads

Volume density of the gonad was determined using light microscopy and a 320-intersection grid. Three fields from each region of ovary (cranial, middle, and caudal) (9 fields total) were randomly selected, giving a total of 2.880 points scored for each animal at 4x magnification. For this analysis, it was used the method applied by Pereira et al. (2013). Cells were classified as one of the following: pre-vitellogenic (PV), cortical alveoli (CA), early vitellogenic with incomplete vitellogenesis and cytoplasm not filled with yolk (EV), final vitellogenic with cytoplasm filled with yolk (FV), atretic (AT), and interstitial tissue (IT). Artifacts were rarely observed and were not considered in the total number of cells used to obtain the percentages.

2.10. Statistical analysis

Data normality was evaluated using the Cramer von – Mises test and Homoscedasticity with the Fmax test. The plasma steroid level, GSI and volume density were analyzed by comparing different treatments with a one-way analysis of variance (ANOVA). The Tukey test was used in post hoc analyses. A threshold of $P \leq 0.05$ was set to infer statistical significance. All statistical analyses were based on Zar (Zar, 1999).

3. Results

3.1. Fish mortality, growth and exposure concentrations

Mortality was not observed in any of the experimental groups. Differences were not observed in food intake or growth among treatments and controls ($P = 0.35$, Table 1). Measured values for diuron, DCA, DCPU and DCPMU in water are shown in Table 1.

3.2. Steroid hormones and GSI

Female Nile tilapia exposed to diuron metabolites for 25 days had significantly altered sex steroid levels (Fig. 1) and GSI (Fig. 2). There was an increase of approximately 20% in E_2 plasma levels ($P = 0.03$) of fish exposed to DCPMU, DCPU and DCA compared to the control and diuron treatments (Fig. 1. A). However, there was no significant difference in 17α-OHP plasma levels ($P = 0.15$) among experimental groups (Fig. 1. B). There was an increase of approximately 30% of the GSI ($P < 0.0001$) in animals exposed to diuron metabolites compared to the control and diuron treatments (Fig. 2).

3.3. Histomorphometric analyses of the ovary

Ovarian sections of control Nile tilapia demonstrated a normal distribution of ovarian lamellae, with the presence of the following oocyte types: PV, CA, EV, FV and TI (Fig. 3A). In all treatments the oocytes are presented as follows: PV (showed a large nucleus, centrally positioned with numerous nucleoli and cytoplasm intensely basophilic), CA (oocytes had a large nucleus, slightly stained with numerous nucleoli and the cytoplasm contained cortical alveoli), EV (oocytes the nucleus remained centrally positioned, had an irregular shape, and a large number of cortical alveoli vesicles were observed), FV (the nucleus remained centrally positioned, the predominance of cortical alveoli vesicles was no longer observed and oocytes were at their maximum size and were filled with protein yolk granules) and AT (atretic oocytes often had broken or absent nuclei, fragmentation of the zone radiata and irregular yolk distribution). However, examination of the ovarian lamellae morphometry of Nile tilapia exposed to diuron and its metabolites showed a significant decrease (~10%, $P < 0.0001$) in the percentage of primary ovarian follicles (PV and CA oocytes) in comparison to the control group (Table 1 and Fig. 3B–C). Furthermore, exposure to diuron metabolites caused a decrease (~9%, $P = 0.0002$) in the percentage of EV oocytes in comparison to the control group (Table 2). On the other hand, following treatment with diuron metabolites, there was an increase of approximately 30% in FV oocytes ($P < 0.0001$) and 10% in AT oocytes ($P = 0.0073$). In particular, the greatest values were observed in animals exposed to DCPMU and DCA metabolites (Table 1 and Fig. 3 C–D). It is important to note that we did not observe changes in the morphological composition of PV, CA, EV, FV and AT oocytes between treatments, only variations in quantitative percentage of these oocytes.

4. Discussion

The results support our predictions that long term exposure to diuron and its metabolites caused alterations in plasma steroids and gonadal histology in adult females of Nile tilapia consistent with estrogenic activity. Diuron metabolites accelerated the

Table 1
Measured chemical concentrations of diuron (100 ng/L). Mean percentages (±S.E.M) of the mortality, food intake, body weight from *O. niloticus* females exposed to diuron and its metabolites during 25 days. (ANOVA, Tukey test, P < 0.05).

Parameters	Treatments				
	Control	Diuron	DCA	DCPU	DCPMU
Mortality (%)	0	0	0	0	0
Food intake (g)	10.84 ± 0.32	11.01 ± 0.23	10.55 ± 0.22	10.88 ± 0.17	10.79 ± 0.12
Body weigth (g)	70.96 ± 2.07	68.76 ± 1.96	71.68 ± 1.04	74.99 ± 0.57	69.38 ± 2.69
Measured conc. (ng/L)	<10	67.9 ± 29.7	61.67 ± 16.2	53.4 ± 6.9	53.5 ± 0.7

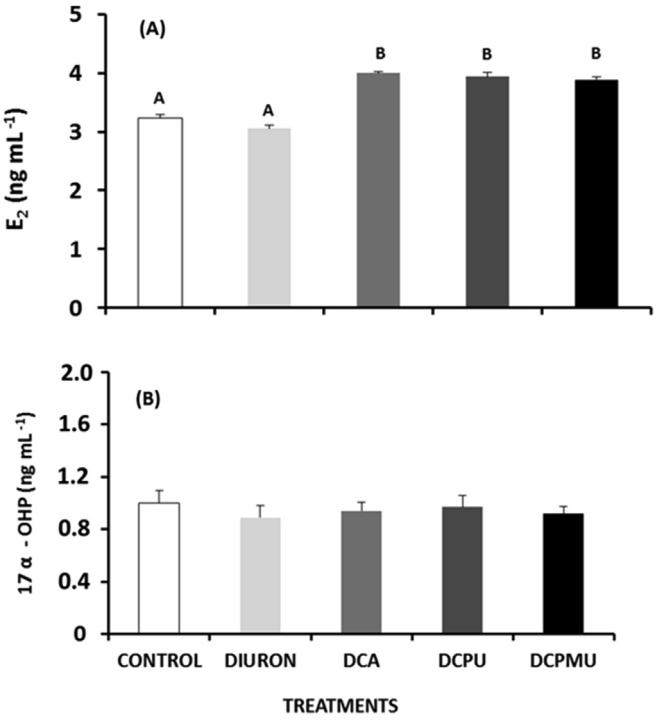

Fig. 1. (A) Mean (±S.E.M) plasma concentrations of E_2 in *O. niloticus* females exposed to 100 ng/L diuron and its metabolites during 25 days. Different letters indicate significant differences among treatments (ANOVA, Tukey test, P < 0.05). (B) Mean (±S.E.M) plasma concentrations of 17α-OHP in *O. niloticus* females exposed to diuron and its metabolites during 25 days.

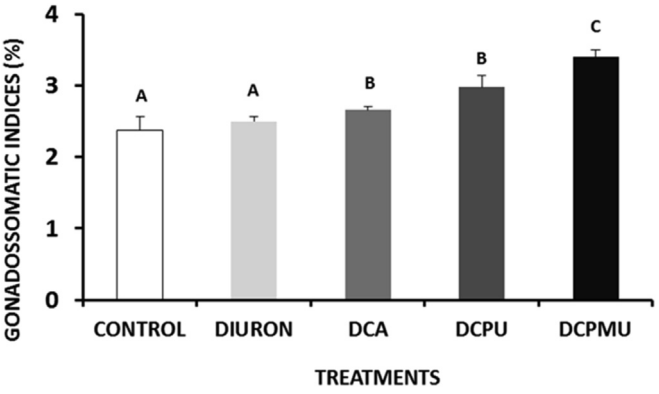

Fig. 2. Mean percentages (±S.E.M) of the gonadosomatic indices in *O. niloticus* females exposed to 100 ng/L diuron and its metabolites during 25 days. The diferente letters indicate significant differences among treatments. (ANOVA, Tukey test, P < 0.05).

oogenesis of Nile tilapia females causing increased gonadosomatic indices, and elevations in plasma concentration of the sex steroid E_2 and in the percentage of final vitellogenic oocytes.

In teleost fish, sex steroids are responsible for regulating gametogenesis (Nagahama and Yamashita, 2008; Haider, 2007; Lubzens et al., 2010). The primary role of E_2 is the stimulation of hepatically-derived vitellogenin (yolk protein) production, which is then incorporated into developing ovarian follicles (Nagahama and Yamashita, 2008; Lubzens et al., 2010). In this study, we observed that the exposure of female Nile tilapia to diuron metabolites significantly increased E_2 plasma levels, showing potential disruption of estrogen biosynthesis or clearance. Previous studies using in vivo bioassay guided fractionation in water extracts from the Central Valley and San Francisco Bay Delta in California (USA) indicated diuron in fractions with in vivo estrogenic activity, but not in vitro activity (Schlenk et al., 2012). As an individual compound, diuron failed to induce vitellogenin in male Japanese medaka (*Oryzias latipes*), but when the compound was combined with bifenthrin, several alkylphenol ethoxylates and alkylphenols at environmentally measured concentrations, vitellogenin expresssion was observed (Schlenk et al., 2012). EDCs can cause estrogenic activity through a number of mechanisms including direct interactions with specific nuclear or membrane receptors in target cells or indirectly by affecting synthesis and clearance of sex steroid hormones (Kroon et al., 2014; Sun et al., 2014). Bauer et al. (Bauer et al., 1998) showed that diuron and some of its metabolites antagonized androgen receptors and altered the synthesis, secretion, and/or metabolism of testosterone. The relationship between testosterone and E_2 is unclear, but reductions in testosterone may have feedback loop impacts on E_2 biosynthesis (Nagahama and Yamashita, 2008; Lubzens et al., 2010). Additional studies are needed to confirm this hypothesis.

Augmented E_2 can increase hepatically-derived vitellogenin production and/or incorporation into developing oocytes (Nagahama and Yamashita, 2008; Lubzens et al., 2010). The vitellogenic oocytes (characterized by the deposition in the cytoplasm exogenous yolk) significantly increased in size due to the occurrence of vitellogenin (Lubzens et al., 2010). Thus, the increase in the circulating E_2 after treatment with diuron and its metabolites might be causing an excessive release of hepatic vitellogenin and consequently promoting an increased GSI and in the percentage in final vitellogenic oocytes in the animals exposed to diuron metabolites. Furthermore, the greater percentage of vitellogenic follicles indicates that diuron metabolites may intensify and accelerate the follicle maturation process eventually damaging oogenesis. In contrast diuron and metabolites had no effect on the 17α-OHP activity in fish ovaries. The steroid 17 α-OHP is the main precursor of 17α, 20β-dihydroxy-4-prengnen-3-one (DHP), which is the most potent hormone inducing ovulation in fish (Nagahama and Yamashita, 2008). Failure to alter biosynthesis of DHP suggests a target for diuron or its metabolites down-stream of 17α-17α-OHP such as 17 Hydroxysteroid dehydrogenase or 5 α −reductase. Further study evaluating the expression and/or activity of these enzymes is warranted.

In conclusion, the results of this study reveal significant estrogenic activity caused by diuron herbicide metabolites in teleost oogenesis. These compounds are capable of increasing E_2 plasma

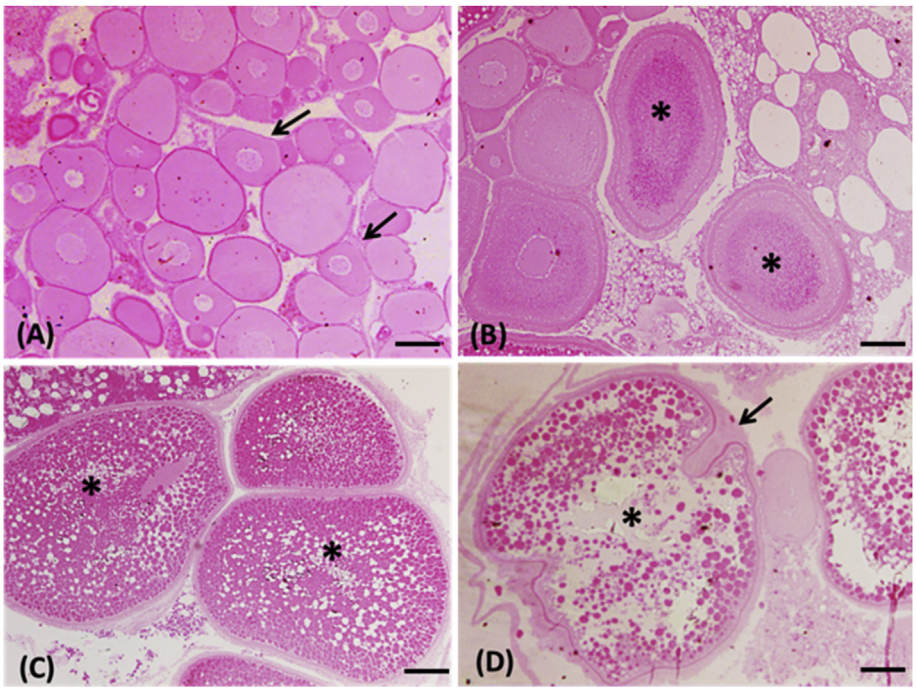

Fig. 3. Photomicrographs of cross sections of the ovary of *O. niloticus* females exposed to 100 ng/L diuron and its metabolites during 25 days. (A) Cross section of ovary of control Nile tilapia, demonstrating ovaries dominated by pre-vitellogenic oocytes (arrow). (B and C) Section of the ovary of Nile tilapia exposed to diuron metabolites shows high quantity of final vitellogenic oocytes (asterisk). (D) Section of the ovary of Nile tilapia exposed to metabolites of diuron (DCA) shows fragmented vitelline membrane (arrow) and a change in the appearance of the cytoplasm (asterisk). Hematoxylin-floxin. Scale bar = 100 μM.

Table 2
Mean percentages (±S.E.M) of different oocytes types from *O. niloticus* females exposed to 100 ng/L diuron and its metabolites during 25 days. Different letters indicate significant differences among treatments. (ANOVA, Tukey test, $P < 0.05$).

Germ and somatic cells	Treatments				
	Control (%)	Diuron (%)	DCA (%)	DCPU (%)	DCPMU (%)
Previtellogenic	13.23 ± 0.40^{a}	2.94 ± 0.38^{b}	1.02 ± 0.19^{b}	0.78 ± 0.18^{b}	0.34 ± 0.16^{b}
Cortical alveoli	4.08 ± 0.28^{a}	1.03 ± 0.22^{b}	0.86 ± 0.16^{b}	0.58 ± 0.15^{b}	0.45 ± 0.18^{b}
Early vitellogenic	10.43 ± 0.43^{a}	8.79 ± 0.48^{a}	2.05 ± 0.31^{b}	1.27 ± 0.25^{b}	1.40 ± 0.23^{b}
Final vitellogenic	53.12 ± 0.56^{b}	55.77 ± 0.59^{b}	79.42 ± 1.55^{a}	83.55 ± 0.45^{a}	85.89 ± 0.44^{a}
Atretic	0.25 ± 0.13^{b}	0.54 ± 0.22^{b}	9.36 ± 0.60^{a}	1.87 ± 0.28^{b}	1.62 ± 0.27^{b}
Interstitial tissue	12.13 ± 0.30^{a}	10.29 ± 0.41^{a}	10.40 ± 0.34^{a}	10.93 ± 0.35^{a}	10.26 ± 0.30^{a}

levels and quantity of vitellogenic oocytes causing enhanced ovarian development of *O. niloticus* females. In order to determine potential impacts of fishery reproduction and populations, further experiments are needed to evaluate the hatch and viability of the gametes from spawning animals and steroid biosynthesis following long term exposure to diuron and its metabolites.

Acknowledgements

The authors would like to acknowledge the “Programa Recém-Doutor”-PROPe- UNESP for Scholarship. This work has the financial support of FAPESP-BIOEN Program (2011/52061-8) and CNPq (401884/2012-0). EAA is a recipient of a CNPq productivity fellowship (307603/2014-8). The authors disclose any potential sources of conflict of interest.

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