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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.

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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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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