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The effects of the urea-based herbicide linuron on reproductive endpoints in the fathead minnow (*Pimephales promelas*)

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ABSTRACT

Linuron is a widely used urea-based herbicide that has anti-androgenic activity in both fish and rodents. To further elucidate the potential mode of action (MOA) of linuron on the vertebrate endocrine system, adult male and female fathead minnows were exposed for 21 days to dechlorinated water, a solvent control, 17β-estradiol (E2; 0.1 μg/L), dihydrotestosterone (DHT; 100 μg/L), linuron (1, 10, 100 μg/L) and one co-treatment of DHT (100 μg/L) and linuron (100 μg/L). There were no effects of linuron on egg hatching, 7 day egg survival, nuptial tubercle formation or gonadal histopathology. Administration of DHT and 1 and 100 μg/L linuron reduced plasma vitellogenin in females, while male plasma vitellogenin were induced after E2 exposure and co-exposure of DHT and linuron. Ovarian mRNA levels were examined for several genes involved in steroidogenesis (e.g. *p450scc*, *cyp19*a, *star*, *tspo*, *hsd17b* and *hsd11b*) and estrogen-mediated responses (*esr1*, *esr2b*, *esr2a*). Only *p450scc* mRNA was significantly decreased with DHT + linuron co-treatment. Clustering of steroidogenic mRNA transcript expression patterns revealed that patterns for linuron were more similar to E2 compared to DHT. Collectively, this study supports the hypothesis that linuron may not be a pure anti-androgen and may have multiple MOAs that affect vertebrate reproduction.

1. Introduction

The widespread use of pesticides worldwide has resulted in heightened public concern regarding the health risks of pesticide exposure in humans and wildlife (Cerejeira et al., 2003; Nakano et al., 2004; Bocquene and Franco, 2005; Du Preez et al., 2005; Sankararamakrishnan et al., 2005; Konstantinou et al., 2006). One class of widely used herbicides in North America and Europe for control of broadleaf and grass weeds during pre- and post-emergent crop production is the urea-based herbicides (e.g. linuron and diuron). Their high water solubility has resulted in concentrations in aquatic environments in the ng/L to μg/L levels (Frank et al., 1987; O'Neill and Bailey, 1987; Berryman and Giroux, 1994; Kotrikla et al., 2006; Gatidou et al., 2007) posing a potential hazard to non-target organisms (Struger et al., 2011). In Canada, recent studies have reported linuron levels of 1.05 μg/L in surface waters from agricultural sites in the Lower Fraser Valley region of British Columbia (Woudneh et al., 2009) and 0.86 μg/L in surface waters in Ontario (Berryman and Giroux, 1994). The Canadian Water Quality Guideline for the protection of aquatic life of 7 μg/L linuron was derived from acute toxicity tests on fish and plants (Canadian_Council_of_Ministers_of_the_Environment, 1999). However, limited information exists on its sub-lethal effects or mode of action (MOA) in non-target aquatic organisms.

Several studies have shown that urea-based herbicides are endocrine disrupting chemicals (EDCs) in vertebrates based on demonstrable effects on steroid production and sexual differentiation. *In vitro* studies in mammals (rat and human) have demonstrated that linuron competitively inhibits the binding of androgens to the androgen receptor (AR) with a Ki of 100 μM (Cook et al., 1993; Waller et al., 1996; Bauer et al., 2001), and acts as a weak AR antagonist in transcriptional activation assays (Lambright et al., 2000; McIntyre et al., 2000). In addition, prenatal *in vivo* exposure experiments using rodents showed that high concentrations of linuron caused reduced testosterone production, altered gene expression patterns involved in tissue morphogenesis, and morphological disruptions to androgen-organized tissues (Turner et al., 2003; Hotchkiss et al., 2004; Wilson et al., 2009). Interestingly, the pattern of malformations of androgen-dependent tissues in adult male rats exposed *in utero* resembles that produced by some phthalate esters (i.e. high rates of epididymal and testicular malformations) rather than other known AR

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antagonists (i.e. high rates of hypospadias and vaginal pouches for the pesticides, vinclozolin and procymidone;(Kelce et al., 1994; Ostby et al., 1999; Wilson et al., 2004a, 2005). However, recent experiments conducted by Wilson et al. (2009) have shown the MOA of linuron to be different than that of phthalate esters. In particular, in fetal rats linuron does not alter expression of key steroidogenic enzymes (i.e. *stAR, cyp11a* and *cyp17a*) or insulin-like hormone 3, but this has been reported for phthalate esters (Lehmann et al., 2004; Wilson et al., 2009). Currently, it is hypothesized that linuron acts via a mixed MOA including both AR antagonism and reduced testosterone production resulting in a cumulative effect in the male rodent model, however the relative contribution of each mechanism is not fully understood (Wilson et al., 2009).

The literature to date provides limited evidence of the effects of linuron on androgen-mediated processes in fish. Linuron was shown to bind to the fathead minnow (FHM; *Pimephales promelas)* AR *in vitro* (Wilson et al., 2004b) and anti-androgenic behavior has been reported in female three-spined stickleback, via reduction of dihydrotestosterone-mediated spiggin production (Jolly et al., 2009; Katsiadaki et al., 2006). Further investigations into the anti-androgenic effects of linuron and the potential for multiple MOAs involving the teleost reproductive axis are required to fully characterize its impacts on fish.

In the current study, the effects of linuron on the reproductive endocrine axis of the FHM were investigated by exposing adult male and female fish to waterborne concentrations of 17β-estradiol (E2), dihydrotestosterone (DHT), linuron and one co-treatment of DHT and linuron. E2 and DHT are model vertebrate estrogenic and androgenic endogenous steroids, respectively. DHT was used because unlike other androgens, DHT cannot be converted into E2 by aromatase, therefore any androgen-induced responses could be distinguished from E2-mediated responses caused by elevations in E2. The MOA of linuron in males and females was examined using the biomarker vitellogenin, nuptial tubercle formation, gonadosomatic index, egg hatching and 7 day egg survival. The expression patterns for a number of key genes involved in steroid production in the female ovary were also examined and gene clustering was performed to better elucidate the MOA of linuron compared to the model androgen DHT and model estrogen E2.

2. Materials and methods

2.1. Chemicals

Linuron (99.7% purity, CAS#330-55-2), E2 (≥98% purity, CAS#50-28-2) and methanol (MeOH; ≥99.9% purity, CAS#67-56-1) were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). DHT (98% purity, CAS#521-18-6) was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). For convenience, nominal concentrations of chemicals in each treatment are reported in tables, figures, and throughout the text.

2.2. Test organisms

Adult fathead minnows (*P. promelas;* FHM) (20 ± 2 weeks of age) were purchased from Aquatic BioSystems Inc. (Fort Collins, CO, USA) and housed at Nautilus Environmental (Burnaby, BC, Canada) throughout the pre-exposure (4 weeks) and exposure period (3 weeks). At the onset of the pre-exposure period, sexually mature adult FHMs were housed in single sex glass tanks (6 adults / 14 L volume) for two weeks. At this point and for the remaining two weeks of the pre-exposure period, the fish were re-distributed so that 2 males (3.87 ± 0.11 g [n = 60]) and 4 females (1.89 ± 0.04 g [n = 125]) were present in each tank. Fish were maintained in de-chlorinated municipal tap water under a 16 h light:8 h dark photoperiod, and were fed 2% body weight divided into two feedings daily (Finfish Starter #1, Aquatic Biosystems Inc.). The following parameters were monitored in each tank every 48 h (measured range provided in parentheses): dissolved oxygen (6.5–8.2 mg/L); pH (6.9-7.5); temperature (23.0–24.5 °C). Ammonia was measured once per week during exposure and ranged from 0.17 to 0.25 mg/L. Animal care protocols were approved by the Simon Fraser University Animal Care Committee according to guidelines outlined by the Canadian Council on Animal Care.

2.3. Experimental design

The experimental design and exposures were performed according to Test No. 229: Fish Short Term Reproduction Assay (OECD, 2009), with the exception of use of a static-renewal exposure, rather than flow-through. Briefly, adult FHMs (4 replicate test vessels per treatment) were exposed for 21 days in 14 L test volumes in glass aquaria to the following treatments: dechlorinated water; solvent control (0.01% MeOH); E2 (0.1 μg/L); DHT (100 μg/L); linuron (1, 10, 100 μg/L); and one co-treatment of DHT (100 μg/L) and linuron (100 μg/L). Water renewals of 80% were performed every 48 h. Each test vessel contained two spawning substrates (semi-circular polyvinyl chloride tiles with no base), and eggs adhering to a tile were quantified daily. For each treatment, 10 eggs were cultured in 100 mL of dechlorinated water in triplicate and survival, number of days to hatch and deformities at hatching were assessed (Environment_Canada, 2008).

Concentrations of linuron were measured at day 14 and day 16 (immediately after and before a water renewal, respectively) of the exposure period using an Enzyme-Linked Immuosorbent Assay for the Determination of Diuron in Water Samples (Abraxis, Warminster, PA, USA). According to the manufacturer's protocol, antibodies employed in this immunoassay demonstrate 25% cross-reactivity with linuron. The manufacturer's protocol was adhered to with the exception of using linuron (and not diuron) standards for the standard curve. A diuron positive control (0.3 μg/L) and negative control (assay buffer only) were included in each immunoassay. A total of two composite water samples for each treatment were obtained. Both composite samples for each treatment were tested in duplicate on the day of collection. Logit/Log analyses were performed to evaluate the data and estimate average linuron water concentrations in two composite samples for each treatment (Microsoft Office 2007, Redmond, WA, USA).

2.4. Fish sampling

After the exposure period, adult fish were sacrificed using MS222 (tricaine methanesulphonate [Syndel Laboratories Ltd., Vancouver, BC, Canada]). Body lengths and wet weight were recorded and blood was collected from the caudal vasculature using a heparinised microhematocrit capillary tube and centrifuged at 12,700 *g* for 3 min. The plasma was removed and stored with a protease inhibitor (aprotinin, 0.13 units/tube; G-Biosciences, Saint Louis, MO, USA) at −80 °C until vitellogenin analysis. Gonadal tissue was removed and weighed to determine the gonadosomatic index (GSI = gonad weight/body weight × 100). The head was removed and preserved in 10% neutral buffered formalin (Sigma-Aldrich Canada Ltd.) for subsequent tubercle counts. The number of tubercles on the snout of each fish was evaluated using a 3× illuminated dissecting scope and their size was quantitatively ranked as follows: 1, present; 2, enlarged; and 3, pronounced (Jensen et al., 2001). Plasma vitellogenin was measured using the FHM Vitellogenin Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Biosense, Bergen, Norway) according to the manufacturer's protocol. All vitellogenin standards and diluted plasma samples were tested in duplicate.