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Article

Androgen Diffusion Patterns in Soil: Potential Watershed Impacts

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SUMMARY

Androgens are natural or synthetic steroid hormones that control secondary male sex characteristics. Ranchers use FDA-approved androgens to increase livestock growth rates and size. In concentrated animal feeding operations (CAFOs), androgens are excreted in cattle urine and feces. Once excreted, androgens can run off or seep into nearby waters, negatively impacting aquatic life and potentially polluting human water sources. This study aimed to determine the extent to which soil is a barrier to androgen flow, thus protecting waterways. Due to prohibitive costs and purchasing regulations of androgens, luminol, a chemical analogous to androgens in both polarity and organic makeup, was used to mimic androgen diffusion patterns. We formulated two hypotheses: first, that soil would be a poor barrier to the luminol, and second, that the luminol would have a greater vertical diffusion than horizontal diffusion. Diluted luminol was added to soil plots and the diffusion was measured by analyzing soil plot layers. We extracted moisture from the soil layers and used a Woods lamp to detect luminol, which diffused up to 22.5 cm vertically and 26 cm horizontally. Diffusion patterns indicated that soil was a poor luminol barrier. If androgens reach the soil's water table, they can potentially diffuse through the local watershed and into the surrounding waterways. This preliminary research indicates the need for further testing of androgen diffusion patterns to ensure the safety of waterways for aquatic life and for human water consumption.

INTRODUCTION

Endocrine-disrupting chemicals (EDCs) are especially disconcerting, as they disrupt endocrine system development and the processes that regulate human growth and metabolism, tissue functioning, reproductive systems, and sleep (1-2). Studies show that even at low concentration levels, EDCs can have negative impacts on fish and aquatic invertebrates (3-4). For example, male-exclusive appendages, such as the fathead minnow's tubercles, can form on female fish as a result of increased EDC concentrations (5). EDCs include androgens, which are natural or synthetic steroid hormones. Androgens control the development of male secondary sex characteristics by binding to androgen receptors.

Androgens are used in cattle farming. Trenbolone acetate, an anabolic-androgenic steroid, is widely used by ranchers to promote efficient feeding and mineral absorption, as well as to stimulate growth and increase muscle mass in cattle (6). Many cattle are fed in large CAFOs (concentrated animal feeding operations), where by definition no crops or vegetation are grown and at least 1,000 animals are confined (7). Trenbolone is administered to cattle subcutaneously, using slow-release ear implants. Roughly 8% of each dose of 17α-trenbolone (the most abundant metabolite) is excreted by the animal (8). Given the density of cattle in CAFOs, large amounts of trenbolone are excreted in cattle urine and feces. CAFOs' lack of grasses and plant root systems that could potentially filter chemicals leaves soil as the lone barrier between excreted trenbolone and nearby waters (7).

Trenbolone is not FDA approved for human use, as anabolic steroids can interfere with endocrine and hormonal processes. Negative effects of anabolic steroids for humans include mood alterations, kidney damage, heart attack, stroke, and both lung and deep-vein embolisms (9). Research shows that trenbolone acetate is toxic for aquatic life as well: *in vitro* exposure to 17-beta-trenbolone caused changes in both male and female reproductive systems of fathead minnows (5). Female minnows exposed to trenbolone developed nuptial tubercles (appendages on the snout) normally exclusive to males (5; **Figure 1**). Male reproductive structures were also altered, although at higher trenbolone concentrations than females (5). This outcome is due to naturally higher amounts of androgens in males (5). In addition to *in vitro* findings, research shows that the reproductive and endocrine systems of wild fish can be adversely affected by CAFO pollutants (10). Exposure to androgens has been shown to impact the embryonic development of the offspring of exposed fish as well (11). These findings indicate a need to understand the mechanism of androgen diffusion through CAFO soil in order to inform solutions to the negative physiological impacts of CAFO runoff. This project takes a first step in that research by examining potential trenbolone diffusion patterns in soil.

In light of the negative impacts of trenbolone and the potential for excreted steroids to enter waterways near CAFOs, this project aimed to determine whether soil is an effective barrier for androgen diffusion. We hypothesized that soil would act as a poor barrier, and androgens would flow through the soil. Secondly, we hypothesized that the androgens would flow through the soil from their entry point

Figure 1. Occurrence of nuptial tubercles in fathead minnows exposed to β-trenbolone. (a) Control male, (b) control female, and (c) female. Image from (5). The photos demonstrate that exposure to anabolic-androgenic steroids can result in important physical and physiological changes in aquatic life that warrant further investigation of how these chemicals diffuse through soil.

into the soil plot, with gravitational forces creating vertical diffusion greater than the horizontal diffusion caused by water flow through the soil. To investigate our hypotheses, we added diluted luminol to soil plots and inspected for the presence of luminol below the surface. In separate experiments, we examined soil core samples and soil layers.

The results of this experiment across three trials supported our hypothesis that androgens would diffuse through the soil. An androgen-analogous compound, luminol, diffused vertically and horizontally through the soil plot. The hypothesized shape of the diffusion pattern was not supported. Horizontal diffusion was more pronounced than vertical diffusion and the horizontal diffusion pattern appeared random between layers. Given the observed movement of luminol through the soil, it is reasonable to think that its analog, trenbolone, could reach local waters by contaminating groundwater if it seeps deep enough into the earth's soil.

RESULTS

Initial Trial: Core analysis

To detect whether and how luminol diffused through an organic soil plot, we implemented an initial experiment using coring analysis. We added luminol dissolved in isopropyl alcohol to the dirt, and after an hour, removed nine cores with a coring tool (**Figure 2**). We ejected the dirt onto paper towels and inspected the towels under a Woods lamp for fluorescence, which indicates the presence of luminol. In core 5, we detected luminol throughout the extracted soil core. In cores 2, 3, 4, 6, and 8, we detected luminol in various areas through the extracted cores, with no clear pattern. These results suggested that the luminol diffused both vertically and horizontally, indicating that it could move through the soil. However, when the loosely packed, dry soil was ejected from the core, the coring brush compacted the soil and disturbed the representation of the soil depth within the sample plot. As a result, the measurements of horizontal and vertical diffusion patterns with coring were unreliable and the diffusion patterns in this initial experiment were unclear. This indicated the need for an additional experiment using layer analysis to further evaluate the diffusion patterns of luminol.

Layer analysis

To more efficiently assess luminol diffusion through soil, we added luminol to isopropyl alcohol and then soil. After

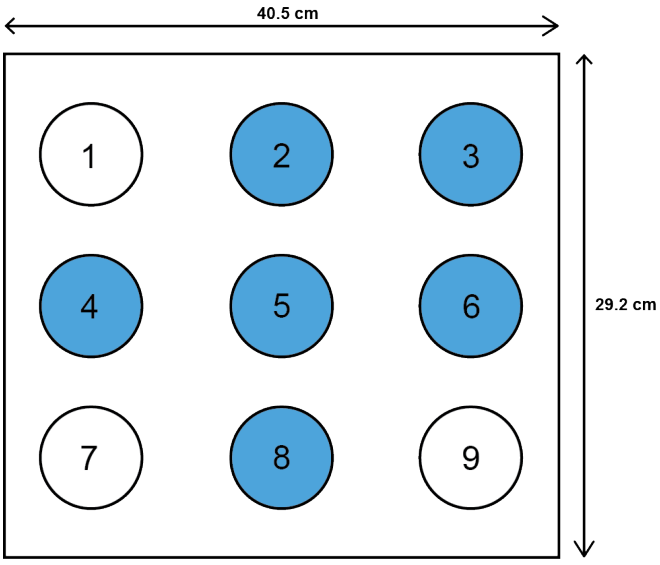

Figure 2. Illustration of core positions during coring analysis experiment. Luminol was detected in cores 2, 3, 4, 5, 6, and 8.

one hour, we transferred 13 soil layers of approximately 2.5 cm each from the container onto paper towels. We applied pressure to the soil to transfer moisture from the soil to the paper towels, which we then inspected for presence of luminol. Luminol, detected by its fluorescence under a Woods lamp, was present at a depth of 22.5 cm below the soil surface (**Table 1**). Lateral diffusion was observed through layer 9, with a maximum lateral diffusion distance of 26 cm from the solution's entry point into the soil (**Table 2**).

The alcohol appeared to move randomly through layers, depositing luminol in its path (**Figure 3**). For example, in layer 1, luminol was present between y = 3 cm and y = 13 cm on the graphed observations. In layer 2, luminol was present in a much wider horizontal range, from 3 cm to 21 cm, with a few dots at 27 cm from the side of the container. By the 4th layer, the horizontal diffusion range was lower, reaching the 16 cm mark, with two dots observed at the 20 cm mark.

DISCUSSION

This project is significant in that it provides preliminary evidence that soil is a poor barrier to androgen diffusion. The combination of vertical and horizontal diffusion of androgen-analogous luminol within the soil sample indicates the soil's potential to be contaminated with endocrine-disrupting compounds. The findings, however, do not support our hypothesized diffusion pattern. These findings do support our first hypothesis that soil is a poor barrier to the chemical diffusion and suggest that trenbolone from CAFOs may have the capability of moving both across and through soil to infiltrate neighboring water systems. The widespread use of trenbolone in CAFO cattle farming may increase human and wildlife exposure to EDCs if the excreted chemicals infiltrate water systems. Aquatic animals exposed to EDCs are likely to experience adverse health outcomes, which may extend to non-aquatic animals higher up in the food chain. Humans may

be exposed to EDCs if androgens move far enough through subsurface soils to reach groundwater. This contaminated groundwater may eventually discharge into local ponds, lakes, or streams that are human drinking water sources (12). In consuming this water, humans could be exposed to negative EDC effects. Complex and expensive drinking water filtration systems may not be available or feasible for many communities. Even if these filtration systems were available, they would not protect aquatic animals or those who consume them.

We took experimental design measures to ensure the validity of this research. However, the project does have limitations. Luminol, which we chose for its accessibility and affordability, may not have exactly the same diffusion patterns as excreted trenbolone. Since trenbolone has a greater molecular mass than luminol, there may be even more pronounced diffusion patterns. Future researchers could apply for funding for and permission to use trenbolone itself.

The use of alcohol may have influenced the rate of diffusion, as alcohol may differ from water in carrying EDCs through soil. In addition, when we extracted moisture from soil onto paper towels, moisture may have diffused through the towels themselves, creating the illusion of additional diffusion. To estimate the impact of this diffusion, we dropped one droplet of solution onto the paper towels. The droplet diffused in a circle of approximately 1 cm diameter within the paper towel, indicating a small but acceptable margin of error. As luminol is water insoluble, isopropyl alcohol was a readily available solvent. When the androgens enter the CAFO soil, they will likely be in cow urine, but since we were unable to procure cow urine for a trial, we used alcohol instead. In addition, we could not test the diffusion of alcohol without luminol, giving little insight into the estimation of the diffusion of cow urine without the presence of androgens. Lastly, we carried out this experiment using one type of soil that was likely not as densely packed as soil underneath a CAFO.

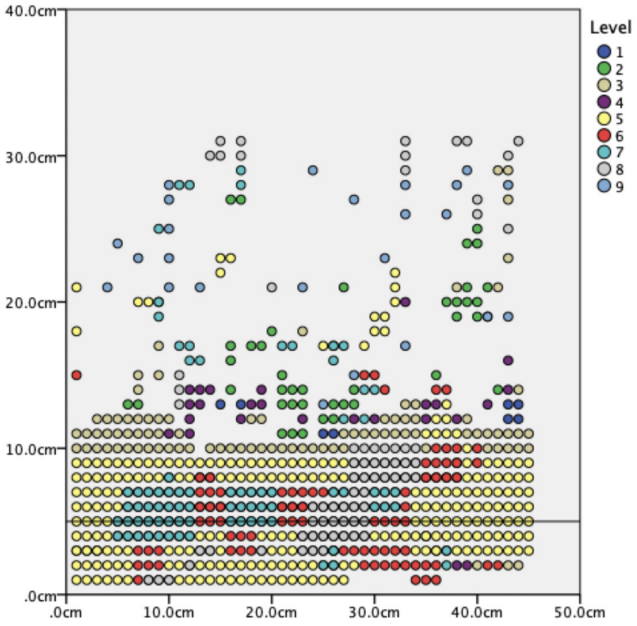

Figure 3. Scatterplot of luminol diffusion for all layers showing horizontal diffusion patterns of luminol during layer analysis process. Luminol was added to the plot in a line at y=5 cm. Colors indicate the deepest layer of luminol detection.

Diffusion patterns may vary based on soil characteristics such as structure, water content, or chemistry. Larger-scale research projects might test several types of soil in large plots that resemble commercial CAFO sites. If these drugs do leach into water sources, future research could focus on filtration systems that can eliminate these harmful substances. Filtration methods would need to be affordable and practical for diverse communities.

MATERIALS AND METHODS

Luminol as an analog for trenbolone acetate

To test the hypothesis that soil would be a poor barrier,

Table 1. Vertical luminol diffusion through soil layers.

Layer	Depth (cm)	Trial 1	Trial 2	Trial 3
1	2.5	X*	X	X
2	5.0	X	X	X
3	7.5	X	X	X
4	10.0	X	X	X
5	12.5	X	X	X
6	15.0	X	X	X
7	17.5	X	X	
8	20.0	X	X	
9	22.5	X		
10	25.0			
11	27.5			
12	30.0			
13	31.0			

*X indicates luminol detected.

Table 2. Horizontal luminol diffusion through soil layers.

	Maximal luminol horizontal diffusion distance (cm)		
Layer	Trial 1	Trial 2	Trial 3
1	8.0	5.0	9.0
2	22.0	19.0	19.0
3	24.0	22.0	25.0
4	15.0	22.0	26.0
5	18.0	17.0	14.0
6	10.0	9.0	23.0
7	25.0	13.0	-
8	26.0	16.0	-
9	24.0	-	-
10	-	-	-
11	-	-	-
12	-	-	-
13	-	-	-

Note: Distance measured from line (y=5 cm) of luminol entry into container. For layer depth, see Table 1.

an androgen analog, luminol, was added to an organic soil plot to measure the diffusion of the chemical through the soil. The original research plan included adding androgens to soil to measure their diffusion. However, in the United States, androgens are classified as Schedule III controlled substances (13). Even though some androgens are available for research purposes, the cost and lack of permission to obtain trenbolone made the drug inaccessible for this project. As a result, we looked for a compound with the same polarity and similar molar mass as trenbolone acetate. Calcium sulfate was initially considered as it approximated the polarity and molar mass of trenbolone, but sulfates in the soil precluded that option. Due to the difficulties with trenbolone and calcium sulfate, luminol was assessed as an analog for trenbolone. Both luminol and trenbolone are polar, indicating that both compounds would be likely to move through soil in a similar manner. Luminol is a relatively light compound in terms of molar mass (177.2 g/mol vs. 270.4 for 17 α-Trenbolone) (14-15), but it has a unique feature: its fluorescence, making it possible to visualize under a Woods lamp, which emits long-wave UV radiation (i.e. black light). Luminol, while polar, is water insoluble, as is trenbolone acetate. Both have carbon rings in their structures, potentially contributing to this attribute. Because of its practicality and accessibility, luminol was chosen as the trenbolone analog for this experiment. To avoid artificial inflation of observed luminol concentration, we used organic soil. If we had used regular soil, there is a chance that artificial chemicals in the soil may also have fluoresced under the Woods lamp, yielding a false positive. CAFO soils may contain synthetic chemicals that interact with androgens, but with our available resources, we were not able to observe this possibility.

Initial trial: Core analysis

To test the hypothesis that soil was a poor barrier to luminol diffusion, we conducted an initial trial with the purpose of detecting luminol below the surface of the soil. To begin, we crushed 0.2214 g of luminol (Flinn Scientific; 3-aminophthalhydrazide $C_8H_7N_3O_2$) and then added it to a glass beaker containing 250 mL of 91% isopropyl alcohol. This created a .01 M solution which is a common concentration, and strong enough to be detected under the Woods lamp. We used alcohol as the solvent due to luminol's water insolubility. The solution was gently shaken and stirred until the luminol was fully dissolved, with no solid luminol left in the bottom of the container. We added the solution to the center of a plot of organic soil (Nature's Care brand) in a clean shallow container (40.5 cm x 29.2 cm x 16 cm) in a radius of 5 cm. We extracted cores from the plot after one hour and ejected them from the tool onto Bounty paper towels in a room that had no source of natural light. The paper towels were labeled to indicate the portion of the soil that was closest to the surface. We had previously inspected the paper towels under the Woods lamp and they were not fluorescent. We used the back of a clean shovel to firmly press down on the cored samples to extract the moisture and transfer it to the paper towel. We removed the soil from the towels, and shone a Woods lamp on the towels to inspect for fluorescence. We recorded any instance of luminol detection.

Layer Analysis

We scanned a clean bin measuring 45 cm x 31 cm x 31 cm with the Woods lamp to ensure no preexisting fluorescence and marked it with north orientation to facilitate layer analysis. Then we added 66.25 L (280 cups) of organic soil to the bin. To mimic compacted earth as much as possible, we applied pressure to layers of dirt as they were added, using a flat piece of board and two 18 kg weights. The compacted dirt measured 31 cm deep. A level was used to ensure uniformity of depth across the sample.

Wearing safety glasses, we used an analytical balance to measure 0.4429 g of luminol into a glass beaker, an amount that would create a .01 M solution. To facilitate dissolution, we crushed any dry luminol clumps with a stirring rod prior to adding 500 mL of 91% isopropyl alcohol to the beaker. We used alcohol as the solvent due to luminol's water insolubility. The solution was stirred until the luminol was completely dissolved, with no solid luminol left in the bottom of the container. To mark the pouring point for the solution, a 35 cm length of nonabsorbent dental floss was placed 5 cm in from the south edge of the soil plot and 5 cm from each end of plot. The 5 cm border served as a buffer to prevent the solution from seeping down the sides of the container. Using a plastic container with a small opening to pour slowly and evenly, we added the solution to the south edge of the soil plot using the floss as a pouring guide. Adding soil to one side of the bin allowed more space to measure horizontal diffusion within the container. The solution was allowed to diffuse for one hour.

Given the soil depth of 31 cm, the planned analysis was to inspect layers of approximately 2.5 cm each. A clean bank of paper towels was labeled for each of the 13 layers and marked with north orientation to mimic the soil's orientation within the plot. We shone the Woods lamp on the prepared paper towels to ensure that no areas were fluorescent prior to the experiment. We slid a flat, thin sheet of Plexiglas horizontally through the soil to capture each layer. The soil was transferred with proper orientation to the corresponding paper towel bank. We marked the soil plot's edges on each sheet to facilitate measurement of fluorescent areas. We used the clean side of the Plexiglas sheet to press another set of paper towels (marked with the layer number and 'top' to indicate the side of the soil they represented) down onto the layer of soil to transfer the moisture to the paper towels, and the soil was removed from the towels. The two sets of paper towels were taken into a room with no natural light and inspected under the Woods lamp. As alcohol is not fluorescent, areas that glowed indicated the presence of luminol. Areas of luminol were marked on the paper towels with a permanent marker. Marked observations from both the top and bottom set of towels for each layer were recorded

on pre-prepared grid sheets (one sheet per layer) to indicate diffusion throughout the layer from the (0, 0) origin point of the soil. We washed and dried the Plexiglas sheet between layers to avoid cross-contamination. This process was repeated until the bottom-most layer was analyzed.

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REFERENCES

1. Colborn, T., *et al*. "Developmental Effects of Endocrine-Disrupting Chemicals in Wildlife and Humans." *Environmental Health Perspectives*, vol. 101, no. 5, 1993, pp. 378–384, doi:10.1289/ehp.93101378.
2. Zimmerman, K. A. "Endocrine system: Facts, functions and diseases." *Live Science*. February 15, 2018, www.livescience.com/26496-endocrine-system.html.
3. Dietrich S., *et al*. "Single and Combined Toxicity of Pharmaceuticals at Environmentally Relevant Concentrations in Daphnia Magna – A Multigenerational Study." *Chemosphere*, vol. 79, no. 1, 2010, pp. 60–66.
4. Vajda, A. M., *et al*. "Reproductive Disruption in Fish Downstream from an Estrogenic Wastewater Effluent." Environmental Science & Technology, vol. 42, no. 9, 2008, pp. 3407–14.
5. Ankley, G. T., *et al*. "Effects of the Androgenic Growth Promoter 17-β-Trenbolone on Fecundity and Reproductive Endocrinology of the Fathead Minnow." *Environmental Toxicology and Chemistry*, vol. 22, no. 6, 2003, pp. 1350–1360.
6. United States. "Title 21, Chapter I, Subchapter E: Animal Drugs, Feed, and Related Products." *Electronic Code of Federal Regulations*. November 8, 2018. www.ecfr.gov. Accessed 12 November 2018.
7. Massachusetts Executive Office of Energy and Environmental Affairs. "Concentrated Animal Feeding Operations (CAFOs): Definitions." *Massachusetts Department of Agricultural Resources*. www.mass.gov/eea/agencies/agr/about/divisions/concentrated-animal-feeding-ops-definitions-generic.html. Accessed 01 June 2017.
8. Webster, J. P., *et al*. "Occurrence of Trenbolone Acetate Metabolites in Simulated Confined Animal Feeding Operation (CAFO) Runoff." *Environmental Science & Technology*, vol. 46, no. 7, 2012, pp. 3803–10.
9. U.S. Food and Drug Administration. "Caution: Bodybuilding products can be risky." *Consumer Updates*. www.fda.gov/ForConsumers/ConsumerUpdates/ucm173739.htm. Accessed 20 June 2017.
10. Orlando, E., *et al*. "Endocrine-Disrupting Effects of Cattle Feedlot Effluents on an Aquatic Sentinel Species, the Fathead Minnow." *Environmental Health Perspectives*, vol. 112, no. 3, 2004, p. 353-358.
11. Schultz I., *et al*. "Effect of Parental Exposure to Trenbolone and the Brominated Flame Retardant BDE-47 on Fertility in Rainbow Trout (Oncorhynchus Mykiss)." *Marine Environmental Research*, vol. 66, no. 1, 2008, pp. 47–49.
12. U.S. Department of the Interior. "What is groundwater?" *The USGS Water Science School*. www.water.usgs.gov/edu/earthgw.html. Accessed 19 January 2017.
13. United States. "Title 21, Chapter II, Part 1308: Schedules of Controlled Substances." *Electronic Code of Federal Regulations*. November 8, 2018. www.ecfr.gov. Accessed 12 Nov. 2018.
14. National Center for Biotechnology Information. "Luminol." *PubChem Compound Database*; CID=10638. https://pubchem.ncbi.nlm.nih.gov/compound/10638. Accessed 12 Nov. 2017.
15. National Center for Biotechnology Information. "17alpha-Trenbolone." *PubChem Compound Database*; CID=149836. https://pubchem.ncbi.nlm.nih.gov/compound/149836. Accessed 12 Nov. 2017.