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Fate of Endogenous Steroid Hormones in Steer Feedlots Under Simulated Rainfall-Induced Runoff

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Supporting Information

ABSTRACT: Steroid hormones pose potential risks to fish and other aquatic organisms at extremely low concentrations. To assess the factors affecting the release of endogenous estrogenic and androgenic steroids from feedlots during rainfall, runoff, and soil samples were collected after simulated rainfall on a 14-steer feedlot under different rainfall rates and aging periods and analyzed for six steroid hormones. While only 17α-estradiol, testosterone, and progesterone were detected in fresh manure, 17β-estradiol, estrone, and androstenedione were present in the surficial soil after two weeks. In the feedlot surficial soil, concentrations of 17α-estradiol decreased by approximately 25% accompanied by an equivalent increase in estrone and 17β-estradiol. Aging of the feedlot soils for an additional 7 days had no effect on estrogen and testosterone concentrations, but androstenedione concentrations decreased substantially, and progesterone concentrations increased. Androstenedione and progesterone concentrations in the surficial soil were much higher than could be accounted for by excretion or conversion from testosterone, suggesting that other potential precursors, such as sterols, were converted after excretion. The concentration of androgens and progesterone in the soil were approximately 85% lower after simulated rainfall, but the estrogen concentrations remained approximately constant. The decreased masses could not be accounted for by runoff, suggesting the possibility of rapid microbial transformation upon wetting. All six steroids in the runoff, with the exception of 17β-estradiol, were detected in both the filtered and particle-associated phases at concentrations well above thresholds for biological responses. Runoff from the aged plots contained less 17α-estradiol and testosterone, but more estrone, androstenedione, and progesterone relative to the runoff from the unaged plots, and most of the steroids had a lower particle-associated fraction.

INTRODUCTION

During the past decade, estrogens, androgens, and progestogens have been detected in surface waters at concentrations high enough to affect fish and other aquatic organisms. In particular, attention has been focused on the feminization of fish in surface waters receiving discharges of steroid-containing municipal wastewater effluent.[1−3] Exposure of sensitive species of fish to estrogenic hormone concentrations as low as 1−10 ng/L can result in biological changes, decreased reproductive success and alterations in species composition in receiving waters.[1,4] Androgens and progestogens have also been detected in surface waters subjected to municipal wastewater effluent discharges[5−7] at concentrations known to elicit behavioral and biochemical responses in fish.[7−9]

In addition to wastewater effluent, animal husbandry activities in which large numbers of animals are maintained in a small area may be important sources of steroid hormones in some watersheds. Concentrated animal feeding operations,[6,10,11] grazing cattle,[12] dairies,[13] and aquaculture facilities[13] all have the potential to release steroid hormones to surface waters. In watersheds where runoff from feedlots, pastures, or land to which manure has been applied is not sufficiently treated or diluted, steroid hormones could reach concentrations that pose risks to aquatic ecosystems. However, the diffuse or intermittent nature of agricultural runoff coupled with the transport of hormones in association with particles and organic matter make it difficult to predict steroid hormone concentrations in surface waters impacted by agriculture.

Previous studies have demonstrated the presence of steroid hormones in runoff and waste storage lagoons at dairies and beef cattle feedlots at concentrations up to several orders of magnitude higher than reported thresholds for biological responses.[13−15] However, these wastes often receive some form of treatment before reaching surface waters. The most common forms of treatment

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include application to cropland and pastures, infiltration through soil, composting, and treatment in ponds or wetlands. Irrespective of whether or not runoff is treated prior to release, management decisions require an understanding of the fraction of the steroid hormones excreted by animals that is released to surface waters.

In cattle feedlots, steroid hormones in runoff only account for a small fraction of the mass of the excreted steroids.[16,17] The steroid hormones that are not transported in runoff are assumed to be transformed by microbes in the soil and waste. Results from lab experiments in which manure and/or soil were stored or incubated under moist, aerobic conditions indicate that most endogenous steroids are rapidly transformed to other biologically active steroids or compounds with little affinity for steroid receptors with half-lives on the order of hours to days.[10,18−21] However, some studies indicate that the steroid hormones can persist much longer during manure storage.[16] In addition, most steroids exhibit a relatively high affinity for soils, retarding their movement in runoff and groundwater and increasing the importance of transport of particle-associated steroids in surface waters.[18,21,22]

As a result of the important role of natural attenuation and particle-associated transport in the release of steroid hormones to surface waters, it is important to understand the role of factors such as soil moisture, aging, and phase partitioning on the transformation and transport of steroids in feedlots. Because some transformation products exhibit biological activity, it is also important to identify steroids formed in animal wastes after excretion. To gain insight into these processes, field studies were conducted using rainfall simulators in beef cattle feedlot pens under conditions representative of commercial animal feeding operations.

MATERIALS AND METHODS

Materials. All reagents except acetonitrile were purchased from Fisher Scientific (Fairlawn, NJ) at the highest possible purity. Acetonitrile (99.8%), steroids, and heptaflourobutyric anhydride were purchased from Sigma Aldrich (St Louis, MO) also at the highest possible purity. Deuterated steroids were purchased from CDN isotopes (Quebec, Canada) except for estrone-2,4,16, 16-d_4 which was purchased from Isotec (Miamisburg, OH).

Experimental Design. Experiments were conducted during the summer and fall of 2009 at the University of California's Animal Science Feedlot in Davis, CA. Weather conditions for this period are summarized in the Supporting Information. Fourteen steers were housed in two, 190 m^2 pens with 33% shade cover for 14 days prior to initiating the experiments. The pens were scraped to the clay layer and graded at 3% slope prior to the introduction of animals. The animals were implanted with Revalor S (Intervet Inc. Millsboro, DE), an implant which contains 120 mg of trenbolone acetate and 24 mg estradiol, either 15 or 8 days before they were placed in the pens, providing a steady release of steroid hormones to the animals. Data on the concentration of trenbolone and its metabolites in the animal waste and runoff will be reported elsewhere (Webster et al., manuscript in preparation).

After 14 days, the cattle were removed from the pens where a new soil layer of mostly dried manure solids (mixed with soil particles from the dense clay layer below) had developed. Rainfall was simulated on eight, 3-m^2 subplots within each pen using a rainfall simulator described previously.[23,24] Briefly, a $^1/_2$ in. nozzle (Spraying Systems Co, Carol Stream, IL) held on an aluminum frame 3 m above the ground provided uniform rainfall over a 2 m × 1.5 m plot (see Supporting Information Figures SI1 and SI2). A solenoid valve and timer were used to control the intensity of the rainfall. The simulator provided a drop size, velocity, and uniformity close to that of rainfall.[23,24] Reverse osmosis water (Applied Membranes Inc., Vista, CA) used in the simulator was equilibrated overnight in an open 500 gallon (1890 L) container prior to rainfall simulation. The simulator was operated at a rainfall intensity of either 15 mm/h (low rate) or 25 mm/h (high rate). Four plots received the low rainfall rate and four received the high rate within each pen. Experiments in which simulated rainfall was applied immediately after the cattle were removed from the pen were repeated twice. After these two experiments, an additional experiment was conducted on the same pens in which the wastes were aged an additional 7 days after removing the cattle before simulating rainfall. The pens were scraped to the packed clay layer between each experiment.

Prior to rainfall application, an aluminum frame was placed in the ground around each plot to capture runoff and channel it into an aluminum pipe where samples were collected. Runoff samples were collected 5, 20, 40, and 60 min following initiation of runoff. The runoff flow rate was measured by timing flow into a 250 mL container. The samples at each time point from four replicate plots were composited. This process was repeated at both rainfall rates in both pens.

For each experiment, 10−12, 100 g (wet weight) grab samples of the topsoil and the clay layer were collected in plastic bags and composited prior to and immediately following simulated rainfall. A grab sample of fresh manure from one of the pens was also collected. Runoff samples were transported on ice to UC Berkeley for analysis. All liquid samples were extracted within 48 h of collection. Solids samples were frozen, homogenized at UC Davis, and shipped on ice to UC Berkeley where they were kept frozen until extraction which occurred within 3 months.

Chemical Analysis. Total suspended solids (TSS) (2540 D), fraction organic matter of the suspended solids (f_{om}) (2540E), moisture content and fraction organic matter of the soil (2540 G), dissolved organic carbon (DOC) (5310 B), chloride (4500-Cl^- F), and nitrate (4500-NO_3^- C) were analyzed using standard methods.[25] DOC was analyzed using a Shimadzu TOC 5000A analyzer. Chloride and nitrate concentration were analyzed using a Dionex DX-120 ion chromatograph with an Ionpac AS14A 4 × 250 mm column.

Steroid Hormone Analysis. Steroid hormones were analyzed using solid phase extraction, Florisil cleanup, derivatization, and gas chromatography tandem mass spectrometry (GC/MS/MS) with isotope dilution. A surrogate standard consisting of 100 ng each of 17α-estradiol-2,4-d_2, 17β-estradiol-2,4,16,17,17-d_5, estrone-2,4,16,16-d_4, 4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7, testosterone-16,16,17-d_3, progesterone-2,2,4,6,6,-17α-21,21,21-d_9 and mesterolone in acetonitrile was added to each runoff sample, suspended solids sample, and soil sample.

Runoff samples (1 L) were centrifuged at 5000 rpm (4620g) for 10 min to remove suspended solids. The pellet was frozen until extraction. The supernatant was filtered through a 1 μm, glass fiber filter (Millipore, Bellerica, MA), the surrogate standard was added, and the sample was extracted on a Supelco ENVI-18 solid phase extraction cartridge (Sigma Aldrich, St. Louis, MO). The cartridge was eluted with 12 mL methanol and dried at 60 °C in a vacuum oven. The sample was resuspended in 2 mL 95% dichloromethane/5% methanol and passed through a Supelco

LC-Florisil cartridge (Sigma Aldrich, St Louis, MO) to remove polar organic matter. The cartridge was eluted with 20 mL of 95% dichloromethane/5% methanol which was collected, added to the 2 mL of solvent which had already passed through the cartridge, and dried in a vacuum oven. When the solvent remaining was less than 1 mL, the sample was transferred to a 2 mL gas chromatography vial (Grace, Deerfield, IL), dried under vacuum, and resuspended in 200 μL acetonitrile.

The extracts were derivatized using 50 μL heptaflourobutyric anhydride followed by heating at 80 °C for 90 min. The samples were then dried under a gentle stream of nitrogen and resuspended in 100 μL isooctane with 1 ng/μL hexachlorobenzene as an internal standard. Steroids were analyzed by GC/MS/MS as described previously.[12] The method was modified to include 17α-estradiol-2,4-d_2, 4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7, and testosterone-16,16,17-d_3 as additional surrogate standards. The parent ion used to identify 17α-estradiol-2,4-d_2 was 666, and the two daughter ions were 239 and 452. The parent ion used to identify 4-androsten-3,17-dione-2,2,4,6,6,16,16-d_7 was 488, and the two daughter ions were 473 and 275. The parent ion used to identify testosterone-16,16,17-d_3 was 683, and the two daughter ions were 668 and 320. The parent and daughter ions for all of the measured steroids are listed in Supporting Information Table SI1.

For soil-associated steroids, approximately 1−2 g (wet weight) of soil was weighed into a 15 mL centrifuge tube and the surrogate standard was added. A 10-mL aliquot of methanol was then added and the tubes were shaken by hand for 20 s followed by sonication for 15 min. They were then centrifuged at 5000 rpm (3836g) and the supernatant was decanted. A 10 mL aliquot of methanol was added and the extraction process was repeated two more times. 70 mL of deionized water was added to the 30 mL of methanol and the solution was filtered through an AP40 glass fiber filter. The solution was then extracted on a C-18 SPE cartridge followed by Florisil cleanup, derivatization, and GC/MS/MS analysis as described above. Particle-associated steroids were analyzed in a similar manner except that the whole pellet after centrifugation of the runoff was extracted in 500 mL centrifuge bottles using three times the amount of methanol and deionized water.

Quality Assurance/Quality Control. To ensure accurate and precise analysis in the complex matrix, blanks and matrix recoveries were included with each set of samples. Steroids were never detected above quantification limits in blanks. For one time point per hydrograph, triplicate samples were collected and analyzed. Two samples were analyzed as duplicates, and the third was used as a matrix spike. Matrix spikes of filtered and suspended phases after separation were amended with 100 ng of 17α-estradiol, 17β-estradiol, estriol, estrone, testosterone, androstenedione, and progesterone in 100 μL acetonitrile prior to analysis. One of every six soil samples also served as matrix spikes. Despite all precautions, the complex matrix and multiple sample handling steps occasionally resulted in incomplete recovery of matrix spikes or altered instrument responses. The mean spike for each compound for each round was calculated from all the individual spikes, and, in approximately 11% of these, exceptionally high or low recoveries were observed, most likely due to the presence of high concentrations of NOM or colloids. To avoid systematic errors, only data from groups of samples where mean spike recoveries between 75 and 140% were observed were used for all compounds except 17α-estradiol. For this compound, the deuterated surrogate standard was not added to any of the runoff samples or the first round of soil samples, so 17β-estradiol-2,4,16,17,17-d_5 was used as a surrogate. As a result, spike recoveries for 17α-estradiol exhibited greater variability and spike recoveries between 75 and 180% were considered to be acceptable.

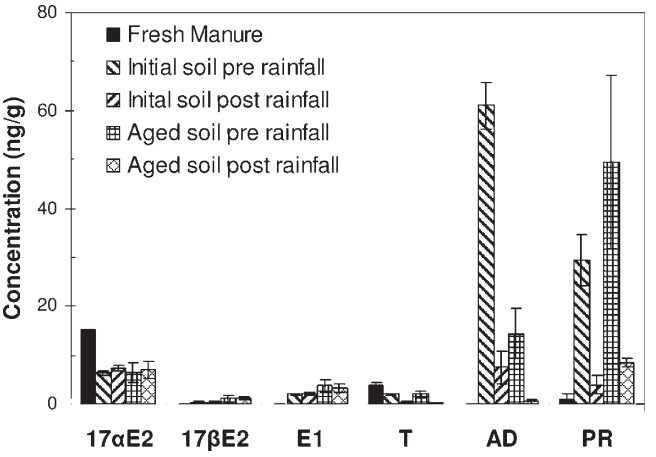

Figure 1. Mean steroid hormone concentrations (ng/g dry weight) in fresh manure and surficial soil before and after aging 7 days and after simulated rainfall. 17αE2 = 17α-estradiol, 17βE2 = 17β-estradiol, E1 = estrone, T = testosterone, AD = androstenedione, and PR = progesterone. Error bars represent ± standard error.

The relative percent difference (RPD) of the duplicate samples was calculated as the difference between the two samples divided by the mean multiplied by 100. The RPD varied with the measured steroid concentration, with higher RPD values at lower concentrations (Supporting Information Figure SI3). Median RPDs were 18%, 21%, and 7% for filtered samples with steroid concentrations above 10 ng/L and suspended solids and soil samples with steroids concentrations above 10 ng/g. However, median RPDs were 17%, 31%, and 21% for filtered, suspended, and soil samples below those concentrations, respectively.

The quantification limit was between 1 and 10 ng/L for filtered runoff, 0.5 and 10 ng/g for suspended solids, and 0.5 and 5 ng/g for soil and manure depending on the steroid (see Supporting Information Table SI1 for a summary). For samples with detectable steroid concentrations below the quantification limits, half the limit of quantification was used in calculations.

RESULTS

The concentrations of steroid hormones in the fresh manure ranged from below detection limit to 15 ng/g (dry weight) (Figure 1). The concentration of testosterone in fresh manure (2 ng/g) agreed with published values for median steroid excretion rates for veal calves[17] (2.8 ng/g) which has been used to estimate endogenous steroid excretion by steers.[16,26] Progesterone was detected in the fresh manure, but below the limit of quantification. While Arts et al. did not report the presence of progesterone in manure, they did detect the steroid in the urine of veal calves.[17] The concentration of 17α-estradiol (15 ng/g) was approximately five times higher than the median reported value in Arts et al. (3 ng/g), presumably because some of the 17β-estradiol in the implants was metabolized to 17α-estradiol by the cattle.[27,28] The water content of the fresh manure was 79%.

The surficial soil sampled at the initiation of the experiment consisted of manure deposited during the 14-day period in which

Table 1. Mean Total Suspended Solid (TSS), Percent Organic, Dissolved Organic Carbon (DOC), and Chloride Concentrations for the Runoff Collected. Average ± Standard Error. For Changes with Time, See Supporting Information Figure SI4

	TSS (g/L)	percent organic in TSS (%)	DOC (mg/L)	chloride (mg/L)
From initial plots, high rainfall rate	5.9 ± 0.4	34 ± 1	498 ± 48	155 ± 26
From initial plots, low rainfall rate	4.6 ± 0.3	36 ± 2	636 ± 70	215 ± 36
From aged plots, high rainfall rate	3.3 ± 0.2	31 ± 2	509 ± 52	398 ± 45
From aged plots, low rainfall rate	2.4 ± 0.1	29 ± 1	645 ± 55	464 ± 44

the cattle were present mixed with clay and feed by the action of the animals walking in the pen. The top 3 cm of surficial soil above the packed clay layer had a water content between 3 and 6% and was between 19 and 25% organic matter on a dry weight basis. After aging, the organic matter content was between 25 and 32%. The water content of the soil did not change significantly during the 7-day period after the animals were removed, so all drying must have taken place during the 14-day period in the which the cattle were present.

All six steroids were detected in the surficial soil. Concentrations of 17α-estradiol and testosterone were lower in the soil than in the manure, but concentrations of all other hormones were substantially higher (Figure 1). In particular, androstenedione and progesterone increased from concentrations near or below detection limits to concentrations up to approximately 60 ng/g. The mean concentration of androstenedione decreased by approximately 70% during the 7-day period after the cattle were removed whereas the mean concentration of progesterone increased by approximately 30% (Figure 1).

Samples collected from the clay layer had concentrations of steroid hormones near or below the limit of quantification at all times with concentrations never exceeding 1 ng/g with the exception of androstenedione and progesterone, which were detected at concentrations up to 11 ng/g and 6 ng/g, respectively (Supporting Information Table SI2).

Soil samples collected after simulated rainfall showed little or no change in concentration of estrogens (i.e., 17α-estradiol, 17β-estradiol, and estrone) while concentrations of androstenedione, progesterone, and testosterone decreased by approximately 85% after simulated rainfall (Figure 1). The water content of the soil increased from 3 to 6% before rainfall to 44% ± 3% after simulated rainfall.

In the runoff, concentrations of chloride and dissolved organic carbon decreased with runoff duration during the experiments while suspended solids concentrations remained approximately constant (Supporting Information Figure SI4). The higher rainfall rate resulted in lower chloride and dissolved organic carbon concentrations, but higher suspended solids concentrations (Table 1 and Supporting Information Figure SI4). The organic matter content of the suspended solids was similar to that of the soil (i.e., the suspended solids consisted of 23 to 48% organic matter compared to 19−32% for the soil). While the relationship between the suspended solids concentration and runoff rate was weak ($r^2 = 0.32$), the suspended solids concentrations were higher from plots receiving the higher rainfall rate ($p = 0.005$). Aging caused a decrease in the average suspended solids concentration in the runoff from approximately 5 g/L to 3 g/L ($p = 0.007$). Aging also increased the concentration of chloride in the runoff at both rainfall rates. However, this may be because the same pens were used for each experiment and excess salts were not removed when the pens were scraped between experiments.

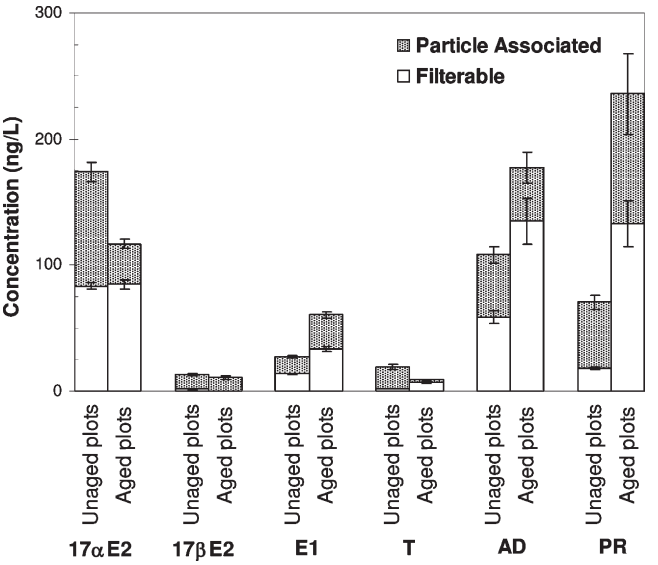

Figure 2. Mean steroid hormone concentrations in runoff from unaged and aged plots after simulated rainfall. 17αE2 = 17α-estradiol, 17βE2 = 17β-estradiol, E1 = estrone, *T* = testosterone, AD = androstenedione, and PR = progesterone. Error bars represent ± standard error.

All six steroids were detected consistently in the runoff with concentrations remaining approximately constant throughout the hydrograph and showing no significant correlation with rainfall rate (Supporting Information Figure SI5). Therefore, comparisons among treatments were made by averaging all time points in the hydrograph at both rainfall rates (Figure 2). Concentrations of 17α-estradiol, androstenedione, and progesterone ranged from 50 to 250 ng/L whereas concentrations of 17β-estradiol, estrone, and testosterone were usually less than 50 ng/L. With the exception of 17β-estradiol, a significant portion of each steroid was detected in both the filtered and particle-associated phases. Aging affected both the concentrations and partitioning of each steroid. For most of the steroids, a higher fraction of the steroids were present in the filtered phase of the runoff from the aged plots where TSS concentrations were approximately 45% lower. The runoff from the aged plots contained less 17α-estradiol and testosterone, but more androstenedione, progesterone, and estrone relative to the unaged plots.

DISCUSSION

Mass Balance. A mass balance estimate for the plots was made using published data on hormone excretion rates,[17,26,29] along with measured hormone concentrations in manure, soil, and runoff (see Supporting Information for details). The mass balance calculation suggests that approximately a quarter of the

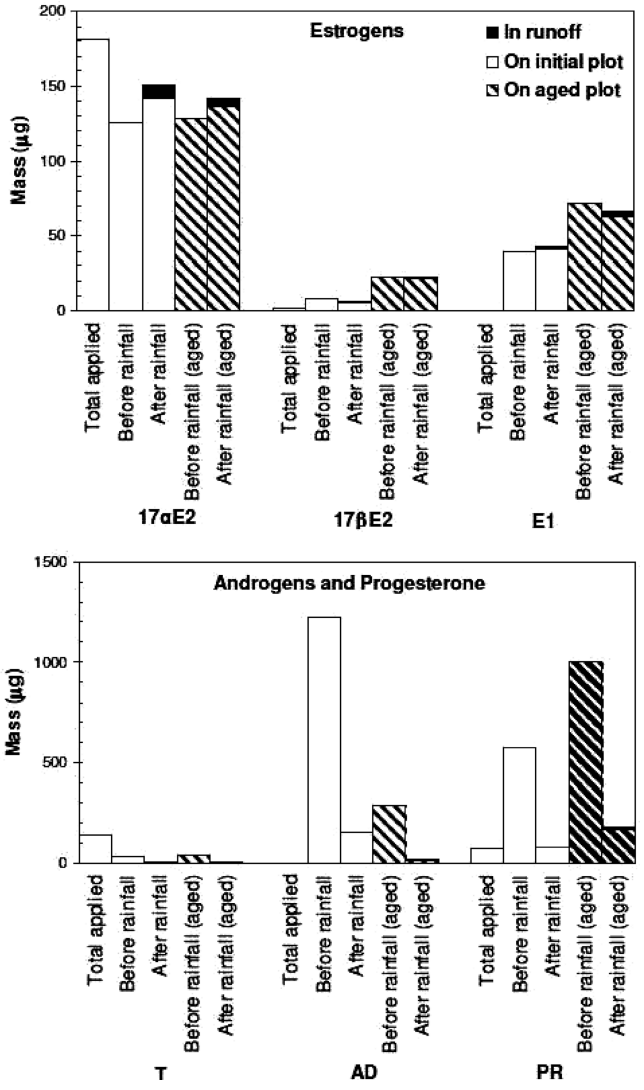

Figure 3. Mass balance estimate on steroid hormones. 17αE2 = 17α-estradiol, 17βE2 = 17β-estradiol, E1 = estrone, *T* = testosterone, AD = androstenedione, and PR = progesterone.

17α-estradiol excreted by the cattle was converted into estrone and 17β-estradiol during the 14-day period in which the cattle were present in the pen (Figure 3). Aging of soil after the animals were removed resulted in a small increase in the mass of estrone and 17β-estradiol, and simulated rainfall had little effect on the total mass of estrogens in the soil (Figure 3).

The mass of testosterone decreased by approximately 70% during the 14-day period in which the cattle were present. No further loss was observed during the 7-day period after the animals were removed (Figure 3). After rainfall, approximately 5% of the testosterone excreted by the cattle remained in the soil. Removal of testosterone from the plot via runoff accounted for less than 1% of the missing mass (Figure 3).

Unlike the estrogens and testosterone, androstenedione and progesterone exhibit a different pattern. Androstenedione was not detected in the fresh manure, but had the highest mass of any of the steroids in the soil after the 14-day period in which the cattle were present. During the 7-day period after the cattle were removed, the mass decreased by approximately 75% (Figure 3). The mass of progesterone increased by roughly an order of magnitude while the cattle were present, and increased further during the aging period after the animals were removed (Figure 3). Although 83−97% of the masses of these two steroids were lost after simulated rainfall, only a small fraction of the masses of these two steroids was recovered in the runoff.

Estrogens. Under the relatively dry conditions of the feedlot soil, the estrogens were surprisingly stable. While there was an initial decrease in the concentration of 17α-estradiol accompanied by an increase in the concentration of estrone and 17β-estradiol during the 14-day period when the cattle were present, the concentrations remained essentially unchanged during the 7-day aging period and in the brief period after water was added in the simulated rainfall (Figure 1). Estrone is a metabolite of 17α-estradiol, and interconversion of 17β-estradiol and 17α-estradiol through estrone has been observed in sediments under anaerobic conditions.[30] It is also possible that the 17β-estradiol was produced when conjugated forms of the steroid in the urine were deconjugated in the soil. The total concentration of estrogens in the soil was approximately constant throughout the 14−21-day experimental period (Figure 1).

Other researchers have reported rapid transformation of 17α-estradiol and 17β-estradiol to estrone in dairy manure and waste lagoons.[15] 17β-estradiol has also been observed to undergo conversion to estrone in dairy waste solids,[20] soils,[19,21] river water,[31] and wastewater treatment plants[32] on the time scale of hours to days. However, the storage conditions of the manure appear to play a role in the rate of transformation. For example, Lorenzen et al.[33] observed that under some storage conditions, the estrogenicity of manure remained constant or even increased by several orders of magnitude. However, they were unable to identify the conditions responsible for the stability of the compounds.

One possible explanation for the enhanced stability of estrogens in the feedlot soils is decreased microbial activity in the relatively dry soils. Microbial activity in soil typically increases with moisture content until aeration becomes insufficient and is lowest under air-dried conditions.[34] Transformation rates of 17β-estradiol[19] and trenbolone[35] in soil also have been reported to decrease steadily as soil moisture is reduced, so it is possible that the dry conditions were responsible for the high stability of the estrogens. However, the estrogen concentrations also remained constant upon wetting of the soil by the simulated rainfall while the androgens and progesterone concentrations decreased substantially (Figure 1). Also, androstenedione and progesterone concentrations changed substantially during the dry, 7-day aging period suggesting there was some microbial activity in the soil (Figure 1). It is possible that other factors, such as location of the steroid within the manure also protected the estrogens from biotransformation. Only a small fraction of the mass of the estrogens was released into the runoff, suggesting that they were not easily removed from the soil (Figure 3). This close association of these steroids with the soil could also have limited their bioavailability, increasing their persistence.

Androgens and Progesterone. Although the cattle excreted little androstenedione and progesterone, relatively high concentrations of the steroids were observed in the soil after the 14-day period when the cattle were present on the feedlot (Figure 1). In previous experiments in which manure from pregnant cattle was aged 0−14 days and applied to pasture plots prior to irrigation, progesterone concentrations in the runoff also increased as the manure aged (Supporting Information Figure SI7). Zheng et al. also observed an increase in progesterone concentrations when

dairy manure was aged, with concentrations increasing from below detection limits to approximately 200 ng/g over a 2-week period.[15] Androstenedione and progesterone have also been detected concurrently in streams at relatively high concentrations downstream of grazing cattle with concentrations up to 44 ng/L and 27 ng/L, respectively. While only detected in 5% of the samples, when present, progesterone concentrations were usually higher than those of the other steroids measured.[12]

Androstenedione is produced from testosterone in soils with half-lives on the order of several hours to a few days depending on the soil.[18] However, even if all the testosterone excreted by the cattle were converted to androstenedione, it would only account for approximately 10% of the measured concentration. An alternative explanation is production of androstenedione and progesterone by the transformation of sterols in manure. Soil bacteria and *E. coli* isolated from human feces have been shown to convert cholesterol, sitosterol, and stigmasterol into androstenedione.[36−39] Reported yields for these reactions varied from 1% to 39% depending on the sterol, the organism, and the medium used for incubation. Cholesterol and stigmasterol have been measured in cow manure at concentrations of 3770 ± 250 μg/g and 490 ± 20 μg/g (mean ± standard deviation), respectively.[40] If the steer manure contained similar concentrations of these sterols and the lowest reported yield for conversion of these two sterols into androstenedione (i.e., 1%) is used, the calculated mass of androstenedione would be between 30 and 250 times higher than those measured in the soils under the conditions observed in our experiments. Cholesterol has been shown to form oxidation products in frozen food even while frozen at −20 C.[41] Therefore, it is possible that some of the additional androstenedione and progesterone were formed in the frozen soil and manure during storage. However, it is more likely that they were formed at the ambient temperatures on the pen.

Progesterone and androstenedione have also been detected in surface waters subject to discharges from sterol-rich pulp and paper mill effluents where masculinization of fish was observed.[42] Lab experiments verified the release of progesterone from pine wood used for pulp followed by conversion to androstenedione by soil bacteria.[43,44] While our findings suggest that a similar process may also be occurring in manure-containing soils, additional research is needed to determine which sterols are responsible for progesterone and/or androstenedione formation and to better understand the factors affecting their formation.

When simulated rainfall was applied to the plots, soil concentrations of the androgens and progesterone decreased substantially within a very short period. The loss of steroid mass from the soil was not accounted for by the runoff (Figure 3). It is also unlikely that the steroids were leached through the soil; the clay layer under the soil was very dense, and the soil was only wetted to a depth of 2−3 cm. Furthermore, concentrations of steroids detected in the clay layer before and after simulated rainfall were near or below quantification limits (Supporting Information Table SI2). One possible explanation for the rapid loss is stimulation of microbial activity in the brief period between wetting and extraction. For example, in aged feedlot runoff, Havens et al. reported rapid transformation of estrogens and androgens within 3 h of spiking if the spiked samples were not acidified.[45] The soil samples from our feedlot were placed on ice immediately after collection and were kept frozen until extraction. Runoff samples were also placed immediately on ice after collection and were extracted within 2 days. While a similar rapid disappearance was not observed for the estrogens, previous research has demonstrated that testosterone is more labile than estradiol in soils.[18,46] While data on the transformation of androstenedione and progesterone in soil or agricultural runoff are limited, Chang et al. reported higher removal efficiencies for these compounds (96−97%) than for the estrogens (67−80%) in municipal wastewater treatment systems, implying they are more labile in the presence of aerobic bacteria.[5] Further research is needed to determine if wetting the soil could induce rapid degradation of the androgens and progesterone, but not the estrogens.

Partitioning and Transport of Steroids to Surface Waters. Because the centrifuged and filtered runoff contained dissolved organic carbon concentrations as high as 1400 mg/L, it is likely that a significant portion of the steroids measured in the filtered fraction of the runoff were not truly dissolved, but were bound to colloidal organic matter. Reported partition coefficients for association of steroids with colloidal organic matter are typically between 10^3 and 10^6.[47] Using these values along with the mean filtered organic carbon concentration of 570 mg/L we estimate that between 37% and 99% of the steroids could have been associated with organic matter that was not removed by centrifugation and filtration. Colloidal organic matter potentially enhances transport,[21,48] increases the fraction of steroids in the filtered fraction,[49−51] and slows biotransformation of steroids.[52] Management practices, such as settling basins and lagoons, which rely on gravitational settling to remove suspended solids from feedlot runoff will remove steroid hormones associated with large particles, but may have little effect on steroids associated with colloidal organic carbon.

The partitioning of the steroids between the filtered and particulate phases did not fit equilibrium hydrophobic partitioning models, with or without consideration of colloids. There also was no relationship between the fraction of steroids associated with particles and the concentration of suspended solids as would be predicted from equilibrium partitioning. Equilibrium partitioning models assume a matrix where hydrophobic partitioning yields nearly linear partitioning provided sufficient time is allowed for equilibration. The matrix in this runoff was complex and heterogeneous and may not have reached equilibrium prior to sample collection. Equilibration times for 17β-estradiol and estrone to soils and sediments vary greatly with some researchers reporting equilibrium partitioning after 5−24 h,[22] while others report a range from 3 to 14 days.[49,51] Previous studies have also indicated that steroid sorption to soils was not correlated with log K_{ow} values.[18,53] Thus, equilibrium hydrophobic partitioning models may not accurately predict observations for conditions encountered in feedlots.

The concentrations of steroids measured in the runoff exceed reported thresholds for end points related to feminization, masculinization, and interfere with pheromonal responses for fish.[1,8,54,55] The estrogenicity of estrone and 17α-estradiol relative to 17β-estradiol depends upon the assay used,[54,56] but assuming the lowest and highest values reported (0.08 and 0.8 for 17α-estradiol, 0.01 and 1 for estrone relative to 17β-estradiol, the total estrogenicity expressed in 17β-estradiol equivalents in the runoff was between 1 and 2 orders of magnitude higher than the 1 ng/L threshold for biological response and was not significantly different after the plots were aged for 7 days. Androstenedione concentrations in the runoff from the initial and aged plots were approximately 3−4 times higher than the reported threshold for masculinization of 40 ng/L.[8] Androstenedione also elicits odorant responses in goldfish at concentrations as low as 0.3 ng/L,[9] and testosterone can elicit pheromonal response in Atlantic salmon

parr at 0.003 ng/L.[57] Thus the runoff concentrations are 2−3 orders of magnitude above thresholds for odorant responses in fish. While progesterone has been shown to induce vitellogenin expression in crayfish at concentrations of approximately 30 μg/L,[58] no data on threshold concentrations for endocrine disruption or pheromonal activity in aquatic life are available. However, other progestogens, such as 17-α,20-β-dihyroxy-4-pregnen-3-one have been shown to illicit pheromonal and odorant responses in goldfish at 3 ng/L and 0.03 ng/L, respectively.[59] Further research is needed to determine if progesterone also poses a threat to aquatic life at similarly low concentrations.

In applications where treatment or dilution is insufficient, estrogens and androgens could pose threats to aquatic life. Microbial transformation reactions may reduce the concentrations of some steroid hormones, but aging of wastes under dry conditions will have little effect on the mass of estrogens and may even result in a substantial increase in the mass of androgens or progestogens. As a result, caution must be exercised in the management of waste discharges from animal feeding facilities and pastures.

■ ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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