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ASSESSING THE POTENTIAL OF HORMONES FROM LIVESTOCK OPERATIONS TO CONTAMINATE GROUNDWATER

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INTRODUCTION

Concern has recently emerged about a group of trace organic compounds identified in the aquatic environment which might affect reproduction and development of wildlife species and humans due to endocrine disruption (Colborn et al., 1996; Roefer et al., 2000). Studies in recent years have documented a high occurrence of these endocrine disrupting compounds (EDCs) in aquatic ecosystems (e.g. Kolpin et al., 2002), although the impact of EDCs in groundwater is not well understood. An important source of EDCs which may potentially impact groundwater is manure from animal feeding operations (Schiffer et al., 2001; Lange et al., 2002). Animal feeding operations generate more than 500 million tons of manure annually (USEPA, 2003) which can enter the environment in a variety of ways including runoff into surface waters after field applications or leaching from holding tanks and composting facilities. Livestock excretions can contain a mixture of natural and/or synthetic steroid hormones (Lange et al., 2002) which have the potential to act as EDCs. Some of these hormones, such as the synthetic steroid hormones used in the cattle industry, have been found to be persistent in the soil (Schiffer et al., 2001) increasing the chance for movement into groundwater.

Hormones in Livestock Production

The hormones of concern are the female sex steroids (e.g., estrogens and progestogens) and the male sex hormones (androgens--e.g., testosterone). These steroid hormones have important roles in development and reproduction. Steroid hormones exert their effects by interacting with specific hormone receptors which bind to steroid response elements of DNA in the nucleus, altering gene expression. Currently, hormones cannot be given to poultry or swine; however farm animals naturally produce and excrete steroid hormones. For example, manure from dairy cows, liquid swine manure and poultry litter have been found to contain estrogenic and androgenic compounds

(Lorenzen et al., 2004). Certain natural and synthetic hormones are FDA approved for use in cattle. These include estradiol (often in the form of estradiol benzoate), zeranol (an estrogen mimic), testosterone (often in the form testosterone propionate), trenbolone acetate (a testosterone mimic), progesterone and melengestrol acetate (MGA- a progesterone mimic). The hormones are delivered via ear implants (except MGA which is a food additive) and are mainly used to increase growth rates. Male cattle used for meat production are castrated and hormones are used to replace the androgens that would normally be produced in the testes. Some implants are also used to control reproduction. For example MGA is also used to synchronize estrus to aid in breeding programs (Schiffer et al., 2001).

The estrogens are the best studied hormones with respect to potential for endocrine disruption. 17β-estradiol is the most potent endogenous form. Other estrogens naturally produced include estrone, estriol and 17α-estradiol. The main forms of estrogens excreted vary depending on the species of animals (Hanselman et al., 2003 and references therein). 17α-estradiol is found mainly in cattle excrement, not in poultry or swine, but the remaining estrogens are excreted by all livestock species, with quantities varying depending on sex and reproductive status. Although 17β-estradiol is the most potent form, concentrations of the other estrogens can be higher. Other than estradiol benzoate, the only other synthetic estrogen used with FDA approval is zeranol (α-zearalanol) and is found, for example, in the cattle implant Ralgro®. Zeranol is related to a suite of estrogenic compounds, (including e.g., α-zearalenol zearalanone and zearalenone) produced by the fungus *Fusarium*. It is also possible for zeranol, zearalanone or zearalenone to be found in animal excrement if the animals have eaten food contaminated by *Fusarium*.

In regards to the androgens, testosterone is the primary form secreted by the testes in mammals, but it is converted to the more potent dehydrotestosterone (DHT) at target cells. Less is known regarding the specific metabolites of testosterone likely to be in livestock manure. In previous work, we found high levels of androgenic activity in primary treated WWTP effluent, but the only androgen measured was testosterone, which was present, but only accounted for a small fraction of androgenic activity (Drewes et al., 2005). Thomas et al., (2002) identified six other androgens contributing to androgen activity in WWTP effluent. Animal operations with intact males or those using implants containing testosterone propionate will likely have testosterone metabolites such as those found by Thomas et al., (2002). However, manure from steer or heifers implanted with trenbolone acetate will include the metabolites α-trenbolone, β-trenbolone and trenedione (Durhan et al., 2006; Schiffer et al., 2001).

Goals of the Research

Project Objectives

The original goals of this project were to: 1) Identify and quantify endocrine active compounds (specifically hormones that will activate the estrogen or androgen receptors) in samples from livestock operations; 2) Identify and quantify endocrine active compounds in samples collected from tiles draining a variety of farm sites (e.g. under paddock, under field) with special emphasis on fate after land application of manure; 3) Assess the differences between hormone profiles to help begin understanding which hormones are likely to be most persistent and identify

based on bioassay activity which ones will likely have greatest potential to affect organisms based on their potencies.

Problems were encountered in this work relating to: 1) stability of the samples and 2) ability to collect tile samples under the conditions originally proposed. First, with respect to sample stability, we discovered that runoff samples collected with automated samples from field edges during rain and snowmelt events, while being held at 4 ^{o}C, did not inhibit bacterial growth enough to prevent degradation of the deuterated internal standards needed to measure hormones. The microbial community in the samples degraded both hormones and internal standards on the order of hours to days, making preservation (sulfuric acid to pH < 2) during sample collection necessary. This work was previously published (Havens et al., 2010). Second, although our proposed sampling strategy was to collect samples from tile drains before, during and after manure applications, sites with tiles flowing during these conditions were not found. We were able to have tile samples collected from three dairies with tiles flowing during rain events or snowmelt. Due to this limited sampling ability, we also found UW-Platteville Pioneer Farms was willing to allow us to analyze samples from their Farm.

MATERIALS AND METHODS

Site Description

University of Wisconsin Extension – Discovery Farms, the University of Wisconsin--Platteville Pioneer Farm and United States Geological Survey (USGS) identified sites for use in this study. Farms A, B and C are privately owned farms and Farm D (Pioneer Farms) is UW-Plattville's research farm (Table 1). Farm A is a dairy that milks approximately 320 cows and manages about 1000 acres using a no-till farming system. Semi-solid dairy manure is used as fertilizer and is surface applied on fields that will be used for corn. Farm B is a pasture based dairy where livestock graze 600 acres under an intensive rotational grazing system. About 50% of the manure produced on the farm is deposited by the cows in the paddock. Most of the remaining manure is stored and hauled away, although a small percentage is piled and spread using a manure spreader. The cows are housed in free stalls during the winter when grazing is not possible. Shallow (0-8") soil cores were collected from the monitored field and was characterized as a clay loam with 34:37:30 (% sand:silt:clay). Farm C is a dairy that has approximately 1400 cows. It is a permitted confined animal feed operation (CAFO) that uses approximately 1600 acres for crops to feed the dairy. Manure from the dairy is used as fertilizer to grow feed for the dairy.

Farm D is the University of Wisconsin-Platteville's Pioneer Farm (http://www.uwplatt.edu/pioneerfarm/). It is a 430 acre production farm located in Lafayette County. The farm is comprised of 330 acres of cropland and 73 acres of pasture. The seven-year crop rotation includes one year of oats followed by three years of alfalfa and three years of corn. Livestock enterprises include a Holstein dairy cow herd (125 milking), a farrow-to-finish swine herd, and a beef cow-calf herd. Manure from the dairy herd is collected from alleyways with mechanical scrapers, stored in an earthen basin, and land-applied in the fall using a towed-hose system. Manure from beef and swine facilities are handled as a solid which is composted and land applied year-round. We collected soil from two fields and it was characterized as silty clay loam with 8:64:29 (% sand:silt:clay).

Sample Collection

Edge-of-field surface and tile drainage water monitoring stations (Teledyne Isco automated sampler, water level/stage and temperature monitoring) were placed at Farms A-C by United States Geological Survey (USGS) and University of Wisconsin Extension – Discovery Farms staff to facilitate studies on nutrient and sediment loss. The sampling infrastructure and methods are described in Stuntebeck et al., 2008. Because of the difference in holding time and sample preservation requirements between the ongoing studies at those farms and the needs for hormone analyses, we were not able to take advantage of the monitoring structure as originally planned. Instead, grab samples were collected when staff had the time and conditions were conducive for grab sampling to occur. The events that were sampled are listed in Table 1. One exception to the samples being collected as grab samples was the surface water snow melt samples in January 2010 at Farm B (surface site 5). Those samples were collected as composites by a Teledyne Isco Avalanche® automated sampler used for hormone sample collections. Sample 5-1 was a composite sample collected over approximately three hours that was then split to be analyzed as a duplicate. Sample 5-2 was the subsequent composite collected during the next three hours of the event. Samples were collected in silanized glass bottles containing sulfuric acid to preserve the hormones. Samples were shipped to the lab where they were held at 4 ^{o}C until extraction. Field blanks and method blanks were generated for most events and a duplicate sample was generated for at least every 20 samples.

Table 1. Description of Farms and Samples Collected

Farm ID	Size	Animals	Samples Collected
A	1000 acres	320 dairy cows (confined)	• March 2008 Snow melt:2 tile drains • June 2008 Rain event: surface and tile site
B	900 acres	400 cows, 200 steers (grass-based rotational grazing dairy)	• March 2008 Snow melt: surface and tile site • January 2010 Snow melt: surface and tile site • Soil
C	1600 acres	1400 dairy cows (permitted CAFO)	• March 2009 Rain event: 2 tile drains
D	430 acres	UW-Platteville Research Farm: Pioneer Farms. 200 dairy cow + 200 steer + 100 swine capacity. (Confined)	• November-December 2009: 13 piezometer samples • Soil

UW-Platteville had nested piezometers installed in 2005 and 2006 for groundwater studies. Further information about the hydrogeology of Pioneer Farms can be found in Macholl and Kraft (2010). Samples were collected from six different wells ranging in depth from 22-101 ft (Table 2, Figure 1). Depending on depth, piezometer sampling was done using either a Wattera Hydrolift II (wells LF 462, 463, 470) or a Spectra Field Pro peristaltic pump (wells LF 467, 469, 471). The samples were not collected until after water was purged for a period of time based on the depth of the well. The groundwater samples for hormone analyses and bioassays were collected in silanized glass bottles, filtered with a muffled Whatman GFF/B filter on site to remove any particles, acidified and returned to the lab. A field blank was generated on site by running type 1 water through a peristaltic pump prior to filtration and preservation of the sample. One duplicate sample was generated on this sampling event.

Hormone Analyses

Target compounds

The hormones selected for analyses are estrogens, androgens and progestogenic compounds (Table 2). All of the analytical standards were of >98% purity and were obtained from Sigma-Aldrich (St. Louis, MO) except 17α-trenbolone, which was purchased from Hayashi Pure Chemical Inc. (Osaka, Japan). The isotopically (*deuterium-d*)-labeled standards (ISTDs) 17β-estradiol-*d*5, estriol-*d*3, testosterone-*d*5, and progesterone-*d*9 were obtained from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) and melengestrol-*d*3, melengestrol acetate-*d*3, 17β-trenbolone-*d*3 and α-zearalenol-*d*4 were obtained from the European Union Reference Laboratory at the National Institute for Public Health and the Environment (RIVM; Bilthoven, The Netherlands).

Chemical Analyses

Hormones were extracted from the filtered water samples (~225 mL) using Isolute® ENV+ polypropylene solid phase extraction (SPE) cartridges as described in Havens et al., 2010. The hormones were eluted from the cartridge with methanol followed by methanol-ethyl acetate (1:1; v:v). The extracts were concentrated with a gentle stream of nitrogen gas to a volume of approximately 100 µl and reconstituted to a final volume of 1.0 ml using methanol.

The hormone concentrations in the extracts were analyzed using high-performance liquid chromatography (Agilent Technologies 1100 HPLC, Santa Clara, California) with tandem mass spectrometric detection (Applied Biosystems/MDS SCIEX API 4000 Foster City, California; HPLC-MS/MS) operating in positive Atmospheric Pressure Chemical Ionization (APCI) mode. All of the mass spectrometer settings can be found in Havens et al., 2010. The instrument was calibrated by generating a relative response ratio between each analyte at 1, 2, 5, 10, 25, 50, 100, 250 and 500 ng/mL and their respective ISTDs (Table 2) added to each calibration point at a concentration of 50 ng/mL for the runoff and GFF sample extracts. Calibration curves were based on linear or quadratic regressions with 1/*x* weighting with all calibration coefficients always exceeding 0.990. The target analyte concentration in the sample extracts was calculated by interpolating the relative response ratio in the sample extracts to those in the calibration curve. The instrument detection limits for the extracted samples were 1.0 ng/L.

Bioassay Analyses

The water samples intended for the bioassays were extracted separately from those for LC-MS/MS analyses due to interferences in the bioassays using extracts generated with that method. Instead, water samples were extracted with the Empore™ SDB-RPS Extraction Disks (3M, St. Paul, MN). The disks were swelled by soaking in 3 mL of acetone and 3 mL of isopropryl alcohol for 3 minutes each. The disks were then sequentially rinsed with 10 mL of dichlormethane-ethyl actetate (1:1, v:v), 10 mL methanol and 20 mL ultra-pure water. The filtered water samples (~700-1000 mL) were then drawn through the preconditioned extraction disks and the hormones were subsequently eluted with 5 mL of ethyl acetate, 5 mL dichloromethane-ethyl acetate (1:1, v:v) and 5 mL dichloromethane. The extracts were concentrated with a gentle stream of nitrogen gas and reconstituted with methanol to a final volume of 0.5 or 1.0 mL, depending on the volume of sample extracted. The extracts were stored in a freezer until they were analyzed.

E-screen and A-screen Bioassays

For steroid hormones, activity is based on a chemical binding to the hormone receptor and eliciting the response caused by the hormone binding. The E-screen uses MCF-7 breast cancer cells that proliferate in response to estrogenic compounds. The A-screen uses MCF7-AR1 cells, which are stable transfectants of MCF-7 cells that express the wild-type human androgen receptor (Szelei et al., 1997) and respond to androgens by decreasing their proliferation response to 17β -estradiol. The MCF-7 and MCF7-AR1 cells were a gift from Drs. Sonnenschein and Soto at Tufts University (Boston, MA). The methods for the E-screen and A-screen are based on those described in Soto et al., 2004 and were conducted as described in Havens et al., 2010. Briefly, the MCF-7 or MCF7-AR1 cells were seeded into 24-well plates. Twenty-four hours after seeding, the media was replaced with experimental media containing sample extracts (<1%) or standards. Standard curves were generated with 15 concentrations of 17β-estradiol for measuring estrogenic activity and 15 concentrations of 5α-androstan-17β-ol-3-one (DHT) ranging for measuring androgenic activity. After five days of incubation, the cell proliferation was measured using the sulphorhodamine B dye (SRB; Sigma-Aldrich, St. Louis, MO) protein assay, which evaluates the total cell numbers by measuring the total protein content. The standard curves were fit with a four-parameter logistic equation with Softmax PRO v 2.6 (Molecular Devices, Sunnyvale, CA). The 17β–estradiol equivalents (EEqs) and DHT equivalents (AEqs) of the samples were determined by interpolating the results from the standard curves and correcting for the dilution and concentration of the samples. The detection limits for these bioassays vary depending on the run as well as the specific concentration and dilution steps used for the particular sample. Extracts containing components that interfere in the assay response (e.g. toxicity) are identified by spiking 17β–estradiol at each dilution in the E-screen assay and determining which dilution the interference is no longer occurring. This dilution is also assumed to be interference-free in the A-screen bioassay. To determine the estrogenic or androgenic potency of each respective target analytes in these bioassays, dose-response curves were conducted using at least 8 different concentrations. The concentration causing 50% of the maximum cell proliferation (EC_{50}) was calculated using a four-parameter logistic equation with Softmax PRO v 2.6 (Molecular Devices, Sunnyvale, CA). The potency of each target analyte was then determined relative to the EC_{50} of 17β-estradiol and DHT for estrogenic and androgenic activity, respectively (Table 3).

Table 2. Target Analytes with Corresponding Unlabeled Chemical Abstracts Service (CAS) Number, ISTD Analog used in Analyses, Molecular Weights, Partition Coefficients and Ranges in Published Octanol-Water Partitioning Coefficients.

Biological Effect	Origin	Target Analyte [CAS #]	ISTD used	Molecular Wt (g/mol)	LogP[1]	Log K_{ow}[2]
Estrogen	Natural	17β-estradiol [50-28-2]	17β-estradiol-*d5*	272.38	4.13 ± 0.25	2.24 – 4.01
		17α-estradiol [57-91-0]	17β-estradiol-*d5*	272.28	4.13 ± 0.25	
		Estrone [53-16-7]	17β-estradiol-*d5*	270.36	3.69 ± 0.30	2.71 – 3.44
		Estriol [50-27-1]	estriol-*d3*	288.37	2.94 ± 0.28	2.45 – 2.69
	Synthetic / Fungal	α-zearalenol [36455-72-8]	α-zearalenol-*d4*	320.38	4.17 ± 0.79	
		Zearalenone [17924-92-4]	α-zearalenol-*d4*	318.36	3.83 ± 0.80	
		Zearalanone [5975-78-0]	α-zearalenol-*d4*	320.38	3.45 ± 0.55	
Androgen	Natural	Testosterone [58-22-0]	testosterone-*d5*	288.42	3.48 ± 0.28	1.84 – 3.32
		5α-androstan-17β-ol-3-one (DHT) [521-18-6]	testosterone-*d5*	290.44	2.75 ± 0.33	3.40 – 3.90
		Androsterone [53-41-8]	testosterone-*d5*	290.44	3.75 ± 0.33	3.69
		5α-androstane-3,17-dione [846-46-8]	testosterone-*d5*	288.42	3.17 ± 0.38	3.60
		4-androstene-3,17-dione [63-05-8]	testosterone-*d5*	286.41	2.90 ± 0.34	2.68 – 2.75
	Synthetic	17β-trenbolone [10161-33-8]	17β-trenbolone-*d3*	270.37	2.27 ± 0.36	
		17α-trenbolone [80657-17-6]	17β-trenbolone-*d3*	270.37	2.27 ± 0.36	
Progestogen	Natural	Progesterone [57-83-0]	progesterone-*d9*	314.46	4.04 ± 0.28	3.19 – 3.99
		17,20-dihydroxyprogesterone [1662-06-2]	progesterone-*d9*	332.48	2.88 ± 0.40	
	Synthetic	Melengestrol acetate [2919-66-6]	melengestrol acetate-*d3*	396.52	4.21 ± 0.43	
		Melengestrol [5633-18-1]	melengestrol-*d3*	354.48	3.42 ± 0.41	

[1] Calculated using ACD/ChemSketch software.

[2] Obtained from LOGKOW© databank (http://logkow.cisti.nrc.ca/logkow/) accessed January 2011.

RESULTS

Potency of Hormones

When comparing the E-screen activity of the other endogenous estrogenic compounds to 17β-estradiol, the endogenous hormone estriol was the closest in potency (26% of the activity of 17β-estradiol) followed by estrone (12%), and 17α-estradiol (3.5%) (Table 3). The zearalenol compounds were in a similar range to the endogenous estrogens, with α-zearalenol, zearalanon and zearalenone at 29, 6.6 and 1.9%, respectively. With the A-screen, the synthetic androgenic compound 17β-Trenbolone was 10% more potent than the most potent endogenous androgen DHT. 17α-Trenbolone was much less potent at only 4.6% of the activity of DHT. Testosterone was 25% of the activity of DHT and androsterone, 5α-androstane-3,17-dione and 4-androstene-3,17-dione were less than 1% (Table 3).

Table 3. Estrogenic and Androgenic Activities and Potencies Relative to 17β-Estradiol and DHT, Respectively.

Target Analyte (Bioassay Used)	Concentration Response	Potency
Estrogens (E-Screen)	**EC_{50} ng/L**	**Relative to 17β-Estradiol**
17β-estradiol	2.1	1.0×10^{0}
17α-estradiol	61	3.5×10^{-2}
Estrone	18	1.2×10^{-1}
Estriol	8.2	2.6×10^{-1}
α-Zearalenol	7.2	2.9×10^{-1}
Zearalenone	110	1.9×10^{-2}
Zearalanone	32	6.6×10^{-2}

Androgens (A-Screen)	IC_{50} ng/L	Relative to DHT
5α-Androstan-17β-ol-3-one (DHT)	16	1.0×10^{0}
Testosterone	65	2.5×10^{-1}
Androsterone	20000	8.0×10^{-4}
5α-Androstane-3,17-dione	4000	4.0×10^{-3}
4-Androstene-3,17-dione	3500	4.6×10^{-3}
17β-Trenbolone	15	1.1×10^{0}
17α-Trenbolone	350	4.6×10^{-2}

QA Results

Most lab and field blanks indicated that results were below detection limits. However, when blanks indicated concentrations above detection limits, the concentration seen in the blank samples was subtracted from the concentration found in the samples for the corresponding batch of samples. The relative percent difference (RPD) for the duplicate samples at the surface water site indicated good reproducibility with RPDs ranging from 4 to 34% (Table 4). Duplicate sample results also were consistent in the results that were below detection limits. The matrix spike from the piezometer samples ranged in percent recoveries between 81 and 180% for the chemical analyses (data not shown). A matrix spike for the bioassays was apparently mis-spiked as no E-screen activity was detected. After this was noticed, a different sample was spiked and results indicated 96% recovery for the E-screen and 136% recovery for the A-screen. In general, the A-screen assay is more erratic than is the E-screen, with more variability in the cell responses from run to run.

Surface and Tile Site Results

Hormones were detected in snow melt and storm water runoff from fields. Of the 18 hormones analyzed for, only six hormones were detected in these samples (Table 4). The highest concentrations of hormones and hormone activity were found at Farm B, the grazing diary. Concentrations of hormones were usually lower in the tile sites. For one sampling event at Farm A and the only sampling event at Farm C, no hormones were detected by the chemical analyses in the tile samples. In the other sampling event at Farm A and both sampling events at Farm B, hormones were detected in both the surface and tile sites, although the number of hormones, the concentrations and the activity were generally lower in the tile sites than the surface water sites.

The only estrogen detected by chemical analyses in these samples was 17α-Estradiol (Table 4). This hormone was also only found at one site, Farm B. E-screen activities for the samples from Farm B were also the highest of the samples collected. Differences between the E-screen and chemical analyses were evident. E-screen activity was detectable in all but one of the samples. In samples with low E-screen activity (e.g. < 2.4 ng/L), much of the discrepancy could be due to differences in the detection limits. E-screen can reliably detect concentrations as low as 0.03 ng/L of 17β-estradiol, whereas LC-MS/MS instrument detection limit is closer to 1 ng/L, but interferences in the matrix can increase the detection limit somewhat in a sample and chemical specific basis. Because 17α-estradiol is not nearly as active as 17β-estradiol (only 3.5% as active), the results also indicate a poor quantitative match of chemical and biological analyses. For example, the sample with 89 ng/L of 17α-estradiol would be expected to contain approximately 3 ng/L of 17β-EEq, but the actual E-screen results were 28 ng/L EEq. Other than errors

associated with analysis (e.g. variability in the cellular responses not corrected for by the standard curve), explanations for the mismatch between bioassay and chemical analysis results can be due to differences in extraction recoveries, different sets of interferences between the two analytical approaches, or biologically active compounds not measured for by chemical analyses.

Table 4. Hormones and Hormone Activity Found in Water Samples from Three Farms in ng/L.

Farm ID Sample type	Sample Date	17α-Estradiol	4-Androstene-3,17-dione	Testosterone	17β-Trenbolone	Progesterone	17,20-Dihydroxy Progesterone	E-screen Eeq[1]	A-screen DHT Eq[2]
Farm A									
Tile Site1	3/14/08	ND	ND	ND	ND	ND	ND	0.34	<0.6
Tile Site 2	3/14/08	ND	ND	ND	ND	ND	ND	0.038	<0.6
Surface Site 3	6/5/08	ND	17.4	2.7	ND	16.5	ND	2.4	<6
Tile Site 2	6/5/08	ND	6.8	0.8	ND	4.1	ND	0.9	<3
Farm B									
Surface Site 5	3/14/08	89	56	ND	ND	358	109	28	10
Tile Site 6	3/14/08	59	67	ND	ND	232	ND	4.3	1.5
Surface Site 5-1[3]	1/24/10	293	97	ND	214	250	68	30	28
(RPD)		(27)	(34)		(12)	(6)	(13)	(7)	(4)
Surface Site 5-2	1/24/10	155	107	ND	140	164	33	0.63	<9
Tile Site 6	1/25/10	13	ND	ND	ND	9.1	ND	2.9	<3
Farm C									
Tile Site 1	3/27/09	ND	ND	ND	ND	ND	ND	0.09	<1
Tile Site 2	3/27/09	ND	ND	ND	ND	ND	ND	<0.05	<1

1. E-screen measures estrogen agonist activity in 17β-estradiol equivalents (Eeq).
2. A-screen measures androgen activity in DHT equivalents.
3. Duplicate sample. Mean result reported with relative percent difference (RPD) in parenthesis on the line below.

Three androgens were identified in these samples. The most commonly found androgen was 4-androstene-3,17-dione, which was found at two farms (A and B). Testosterone was only found at Farm A in both the surface water and tile site, and 17β-Trenbolone was found at farm B in the January surface site samples. Farm B was the only site that had active A-screen samples, with active samples at both sampling events. Comparing the bioassay results with the chemical analyses indicated that they were not well related. Some samples indicated higher A-screen activity than would be expected based on chemicals analysis. For example, the only androgen found in the 3-14-08 Farm B samples was 4-androstene-3,17-dione at 89 ng/L. This compound has less than 1% the potency of DHT in the A-screen. Thus, detectable A-screen activity would not be expected, yet the A-screen indicated 10 ng/L of activity. On the other hand, the samples with 17β-trenbolone would have been expected to have 110% the DHT activity (e.g. the sample with 214 ng/L 17β-trenbolone would be expected to have 235 ng/L DHT equivalents) but in the assay it

only had 28 ng/L DHT Eq. The subsequent surface water sample was also high in 17β-trenbolone (140 ng/L), but there was no detection in the A-screen. It is hard to know what accounts for these discrepancies, but the explanations outlined for the E-screen certainly apply to the A-screen samples as well.

Progesterone was the most commonly found hormone, detected in all of the samples except the four tile samples with no hormones detected. Progesterone was also found at the highest concentration: 358 ng/L in the spring snowmelt sample at Farm B. The progesterone metabolite, 17,20-dihydroxyprogesterone, was also found although always at lower concentrations and in fewer samples.

Piezometer Results

Piezometers were used to sample groundwater from depths ranging between 22 and 101 feet at UW-Platteville's Pioneer Farms. Nitrogen and chloride contamination was evident in many of the groundwater samples. Near-surfaces sources were determined to be the primary source of the contamination (Macholl and Kraft, 2010). No hormones or bioassay activity were measured above limits of detection in any of the samples collected (Table 5, Figure 1).

Table 5. UW-Platteville (Farm D) Piezometer site results (Nov.-Dec. 2009)

Well Number	Depth (ft)	NO_2+NO_3 (N)[1](mg/L)	Chloride[2] (mg/L)	Conductivity[3] (mg/L)	Hormones[4]	Bioassay[5]
LF462-3	50	0.7	16.9	650	ND	ND
LF462-4	39	20.3	21.5	806	ND	ND
LF463-2	49	20.2	19.9	914	ND	ND
LF467-1	40	0.4	2.7	553	ND	ND
LF467-2	30	<0.1	1.9	559	ND	ND
LF467-3	22	<0.1	2.2	566	ND	ND
LF469-1	52	0.2	5.5	580	ND	ND
LF469-2	35	14.2	15.8	814	ND	ND
LF470-1	101	1.0	24.3	781	ND	ND
LF470-2	85	14.8	32.4	868	ND	ND
LF470-3	64	10.2	10.2	764	ND	ND
LF471-1	42	10.0	31.5	817	ND	ND
LF471-2	28	9.7	30.6	803	ND	ND

1. Nitrogen (NO_2+NO_3) was measured at UW-Stevens Point with method 4500 NO3 F.
2. Chloride was measured at UW-Stevens Point with method 4500 CL E.
3. Conductivity was measured at UW-Stevens Point with method 2510 B.
4. Target hormones were analyzed by HPLC-MS/MS, LOD ~1 ng/L.
5. For bioassays, E-screen measures estrogen agonist activity in 17β-estradiol equivalents (Eeq) LOD~0.3 ng/L. A-screen measures androgen activity in DHT equivalents LOD~4ng/L.

DISCUSSION

Due to the very limited numbers of samples collected, it is inappropriate to draw many conclusions regarding the patterns in hormone occurrence and distribution from this work alone. In conjunction with other recent publications it becomes evident that the profile of hormones present at livestock operations depends on the type and reproductive status of animals in the livestock operation, whether implants are used, how manure is managed, and environmental factors such as weather, temperature and soil type and structure, as well as changes that occur over time due to chemical and microbiological reactions. To put our results into context with other studies, the hormone profiles reported from research at other dairies, and reported from studies on implanted cattle will be described in this discussion. This will be followed by a summary of research on the fate and transport of hormones from livestock operations and a discussion of studies addressing likely effects of steroid hormones on fish reproductive success.

Hormone Profiles Found at Dairies

Kolodziej et al., (2004) analyzed dairy lagoon samples, groundwater monitoring wells and tile drain samples for six hormones. Not surprisingly, the number and concentration of hormones was highest in lagoon samples. The highest hormone concentration found was for estrone, at 650 ng/L. The lagoon was sampled twice, in May and September, with different profiles and concentrations each time. Overall, estrone was the most consistently found hormone, followed by testosterone. Samples from monitoring wells downstream from the lagoon also contained hormones, but fewer and in much lower concentrations. Hormones were sporadically found under fields, but not in up gradient control wells, the deep aquifer well or sample from the tile drain system.

Zheng et al., (2008) studied hormones from one 2000 head dairy (1000 milking). They followed dairy effluent separately from the manure solids. Sample collections included fresh effluent, three sites along the sewage lane and three sequential lagoons. Although they analyzed for both, neither progesterone nor testosterone were found. Total estrogens (consisting of 17α, β-estradiol and estrone) decreased two orders of magnitude from fresh dairy effluent to the second lagoon (from about 2700 ng/L to less than 10 ng/L). The composition also changed from 17α-estradiol being responsible for ~70% of the total estrogens to estrone contributing the largest component prior to entry into the lagoons. In this study, concentrations of estrone increased throughout the flow, due either to oxidation (estrone can be converted to 17α- or 17β-estradiol) or deconjugation of the glucuronide or sulfate metabolites. A similar pattern was found for the solid manure, with 17α-estradiol at highest concentrations initially (1400 ug/kg) and estrone concentrations increasing with time.

Gadd et al., (2010) measured dairy farm effluents from 18 farms in New Zealand. The estrogen at the highest concentration was 17α-estradiol (110-1100 ng/L) followed by estrone (10-580 ng/L) and 17β-estradiol (1-310 ng/L). E-screen results ranged from 1.3-670 ng/L.

Shappell et al., (2010a) tested dairy farm effluent, tile drains and creeks from the drainage region of a >2000 head dairy using best management practices. E-screen analyses indicated that EEq were over 1000 ng/L in the diary farm effluent. Chemical analyses revealed that the effluent mainly consisted of estrone and 17α-estradiol. The estrogen

concentrations in tile samples were always less than 0.3 ng/L, with no significant differences found between upstream and downstream sites.

Lagoon samples were collected from an 800 head dairy in New York in April (prior to land application) and analyzed for the four major estrogens. The lagoon samples contained 475, 98 and 104 ng/L of estrone, 17α-estradiol and 17β-estradiol, respectively (Zhao et al., 2010). Streams near the dairy were analyzed for the four major estrogens over an entire year. In the stream samples, only 17α- and β-estradiol were found and the maximum concentration of 17β-estradiol found was < 0.4 ng/L. Seasonally, snowmelt lead to detections of hormones in the streams (e.g. during a January thaw) as did a rain event in early spring. However during May-June, detections were considered due to groundwater inputs because no precipitation had occurred in the days preceding the sampling.

To summarize these results, the predominant estrogen does vary, with estrone and 17α-estradiol often being found at the highest frequencies and concentrations. 17α-estradiol is excreted by cattle at a higher proportion of total estrogens than humans or other livestock (e.g. poultry or swine) so it can be considered a marker of cattle manure. However, transformation between 17α, β-estradiol and estrone can occur under various conditions lessening the value as a biomarker. That we did not find estrone in any of our samples was inconsistent with most other reported research, especially in the samples where high levels of other hormones were found (e.g. Farm B). It is unclear if this is due to analytical difficulties or particularities with the samples analyzed in our study. Fewer studies measured androgens or progestogens. When they were analyzed for, testosterone, progesterone and 4-androstene-3,17-dione were sometimes detected, although not always.

In our current study, hormone concentrations were highest at Farm B, the grazing dairy, likely because the field is receiving inputs of excrement continuously, rather than the occasional applications that occur at other field sites. A somewhat analogous situation was studied by Kolodziej et al., (2007). They collected water samples from rangeland creeks impacted by grazing cattle in central California. In the surface water samples, 78% of the samples contained steroids, with estrone being reported with the highest frequency. The steroid found at the highest concentration was 4-androstene-3,17-dione (44 ng/L) but 17α and β-estradiol, testosterone, progesterone and medroxy-progesterone were also found. Highest hormone numbers and concentrations were found after a heavy rainfall and in areas where cattle had unimpeded access to the streams. They did not find a significant relationship between nitrate, E.coli or coliform and steroid concentrations, indicating differences in fate and transport of these chemical classes.

Effect of Hormone Implants on Hormone Concentrations

Lagoon samples from implanted cattle was analyzed for the four major estrogens and trenbolone acetate metabolites (17α- and β- trenbolone and trendione) over nine weeks following implantation (Kahn and Lee, 2012). The highest concentration of an estrogen measured was estrone, which was found ranging between 118-1810 ng/L. Estriol was also found in high concentrations ranging between 1-960 ng/L. 17α-estradiol levels were high, ranging from 9-480 ng/L. 17β-estradiol was found at concentrations between 4.5- 90 ng/L. For the trenbolones, the metabolite found at the highest concentration was 17α-trenbolone ranging between 22-1720 ng/L, which was

approximately 20 times higher than 17β-trenbolone or trendione. The highest concentration of 17β-trenbolone measured was 110 ng/L. The peak concentrations varied over time for each hormone. In the lagoon water used for irrigation, 17α-trenbolone and estrone were detected at the highest concentrations, both at concentrations over 1000 ng/L.

A study, conducted over two years collecting natural rain runoff events from feed lots holding either control or implanted cattle revealed that overall endogenous hormone profiles were fairly similar between implanted and unimplanted cattle (Bartelt-Hunt et al., 2012). 4-Androstene-3,17-dione and progesterone were among the most frequently found hormones in the runoff, with concentrations averaging 139 and 59 ng/L, respectively. Contrary to many other studies, 17β-estradiol was found more often and at higher concentrations than 17α-estradiol. This occurred in both implanted and control animals. Of the estrogens, estrone was found at highest concentration, with an average concentration of 269 ng/L. As expected, synthetic forms of the hormones (17α-trenbolone, 17β-trenbolone and melengesterol acetate) were found in the implanted cattle manure samples. However, 17α-trenbolone and 17β-trenbolone took 10 days after implantation to be detected in fresh manure and 17α-trenbolone was found at concentrations approximately 100 times that of 17β-trenbolone. Although found briefly in manure, trenbolones were only rarely found in the runoff (i.e. only 17β-trenbolone was found, once, but at a high concentration of 270 ng/L). Zearalenols were found in both treated and control animals. No samples in our study had detectable levels of the zearalenol compounds.

A different study, reported in two separate papers (Mansell et al., 2011; Webster et al., 2012), measured runoff (using artificial rain) from steer feedlot cattle using steroid implants. This study was unique in that it followed changes in hormone profile in the pen over time from fresh manure, to surficial soils (defined as manure that accumulated over the 14 days the cattle were in the pen), to a simulated rain event, followed by an additional seven day aging period (without cattle) and a final simulated rain event. Interestingly, in fresh manure, 17α-estradiol was found in the highest concentration (15 ng/g) and testosterone was also detected (2 ng/g). But over the 14 days, the surficial soil samples composition changed: 4-Androstene-3,17-dione and progesterone went from close to no detect in the fresh manure to 50-60 ng/g and 17α-estradiol and testosterone decreased. Concurrently, 17β-estradiol and estrone were found in higher concentrations than in the fresh manure (Mansell et al., 2011). The authors conclude that about 25% of 17α-estradiol is converted to estrone and 17β-estradiol. If looked at as total concentration of estrogens combined, there was very little change over the 3 weeks. Testosterone concentrations were reduced by about 70% over the experiment. Changes due to rainfall were hormone dependent. Estrogens concentrations increased, even though some estrogens were found in the runoff. The increases were suggested to be due to deconjugation of the conjugated forms of the hormones. Testosterone concentrations were slightly reduced following rain and concentrations of progesterone and 4-androstene-3,17-dione were greatly reduced after the rain. Very little of the loss of mass that occurred for the androgens or progesterone in response to the rain was accounted for by concentrations detected in the runoff. The loss was likely due to microbial degradation.

Analyzing for trenbolones from the same experiment, Webster et al., (2012) reported technical difficulties with recoveries of deuterated internal standards and matrix spikes in runoff samples, so concentrations reported are not considered robust. 17β-trenbolone (3 ng/g dry weight) was found at 15% of the concentration of 17α-trenbolone (21 ng/g dw) and no trendione (a metabolite) was found in the manure. The rain facilitated the conversion of 17α-trenbolone to 17β-trenbolone and trendione. In addition to the analytical challenges reported in this study, other problems including heterogeneity in results were reported. For example in one trial, in duplicate pens, no 17β-trenbolone was detected at any time point in one pen, whereas 17β-trenbolone was detected in every sample collected from the other pen. Overall, the range of trenbolone actetate metabolite concentrations found in CAFO runoff was 1-390 ng/L. Our finding of high concentrations of 17β-trenbolone only was fairly unusual in that 17α-trenbolone is usually found in much higher concentrations than is 17β-trenbolone. However, given the variability found in these studies, there may be localized microclimates favoring certain metabolite transformations to account for this variation.

As outlined above, it is clear that livestock operations can generate high concentrations of hormones. However, there are many instances where streams, tile drains and groundwater wells within livestock operations indicate little to no contamination by hormones (e.g. Kolodziej et al., 2004; Shappell et al., 2010a; Zhao et al., 2010). In this current study, the groundwater collected at UW-Platteville Pioneer Farms indicated no measurable hormones, even though groundwater had evidence of contamination from nitrite/nitrates and chloride. Additionally, samples at other farms included two sets of tile drain samples that also had no detectable hormone concentrations. The two major factors explaining the depletion of hormones are microbial degradation and sorption to soil.

Factors Influencing Hormone Loss: Degradation and Sorption

Microbial Degradation

Microbial activity is clearly important in the removal of steroids from the environment. As mentioned earlier, this became apparent as internal standards were degraded in field runoff that was not preserved (Havens et al., 2010). In laboratory studies, steroid hormones (e.g. testosterone and 17β-estradiol, which have been studied most frequently) are rapidly degraded with half-lives typically ranging from a few hours to a few days. The rates of degradation depend on factors such as temperature and moisture (Jacobsen et al., 2005 and references therein). Increasing temperatures will lead to greater rates of degradation. Additionally, it is posited that organic contaminants must be dissolved in the aqueous phase to be available for microbial degradation (Fan et al., 2007a) therefore soil moisture is necessary for microbial degradation to occur. Microbes that are contained in the manure transform testosterone into 4-androstene-3,17-dione and estradiol into estrone (Jacobsen et al., 2005). However, this is complicated by the interaction between the manure components and the soil microorganisms, as manure can be toxic to the soil microbial community or reduce bioavailability of the hormones due to increased sorption to the organic matter (Jacobsen et al., 2005). Fan et al., (2007a) studied the degradation of 17β-estradiol and testosterone in agricultural soils comparing aerobic and anaerobic conditions as well as autoclaved compared to native soil. With respect to 17β-estradiol, only 6% and 0.9% was mineralized to CO_2 for aerobic and anaerobic soil, respectively, after 5 days. Much more testosterone was mineralized at 63% (aerobic soil) and 46% (anaerobic soil). If the soil was autoclaved

first, then no mineralization occurred. Even though 17β-estradiol was not mineralized, the parent molecule was largely degraded under non-sterile conditions. Thus, biological processes account for most of the degradation and transformation of 17β-estradiol and testosterone. Degradation is slow in anoxic environments, thus high concentrations remain high in non-aerated lagoons/piles and in the water table (Schuh et al., 2011).

Hormones are excreted in urine mainly as a conjugated (i.e. glucuronide or sulfate) form. The polar conjugate aids in excretion by increasing solubility in water. Once in the environment, deconjugation appears to be mediated by microorganisms and is especially rapid for glucuronides. Therefore, glucuronide conjugates are not likely to be especially relevant in most environmental samples. In the few studies where conjugates have been studied, 17α-estradiol-3-sulfate, 17β-estradiol-3-sulfate and estrone-3-sulfate occur in the highest concentrations in lagoon samples (e.g. Hutchins et al., 2007; Gadd et al., 2010). Scherr et al (2008) modeled the degradation of estrone-3-sulfate in agricultural soils and found degradation to occur via microorganisms (i.e. not in sterile soil), rates were temperature dependent and occurred on the order of hours to days. Although estrone was increased from the deconjugation of estrone-3-sulfate, estrone was also further mineralized under the same conditions. The conjugated forms are not biologically active. Thus, in fresh manure samples, biological activity can increase as deconjugation occurs, releasing the active form of the hormone, but will decrease as microbial degradation removes the active hormones.

Interestingly, progesterone and 4-androstene-3,17-dione were the most frequently found hormones in our study. Mansell et al., (2011) noted that these two compounds have been found in environmental samples in much higher concentrations than expected based on concentrations excreted, or in the case of 4-androstene-3,17-dione, due to the conversion of testosterone by microorganisms. They provide a possible explanation: that progesterone and 4-androstene-3,17-dione are produced with microbial transformation from fecal steroids (i.e. cholesterol and stigmasterol). They cite studies indicating that cholesterol concentrations are very high in cow manure and soil bacteria can convert cholesterol into 4-androstene-3,17-dione. Additionally, progesterone has been shown to be converted to 4-androstene-3,17-dione by soil bacteria. Further research is necessary to explore this possibility.

A review of research on hormones in both manure and wastewater treatment plant systems by Combalbert and Hernandez-Raquet (2010) concluded that microbial degradation is central in hormone loss in manure, rather than removal due to sorption on solids. However, fate and transport of hormones is determined by biological, chemical and physical properties that vary widely across the environment (Fan et al., 2008). Overall, soprtion plays a key role in the distribution of hormones in the environment.

Sorption

The high octanol-water partition coefficients (Table 2) indicate hormones should strongly associate with soils. A study by Caron et al., (2010) determined that soil organic carbon (SOC) was the most important factor in the sorption of estrogens in soil. The soils with the lowest SOC resulted in the least sorption. Sorption coefficients (K_d) values varied by about an order of magnitude across soil types and much of this variation (50-75% depending on which estrogen) was explained by soil organic carbon. However, it is important to consider that hormones are

excreted in a dissolved organic carbon (DOC)-rich matrix (i.e. urine and feces) and sorption to soil is more complicated because of this. Stumpe and Marschner (2010) explored whether organic DOC from manure affects sorption of estrogens to soil. They found that manure-associated organic carbon decreased sorption of estrogen to soil, such that transport would be enhanced and biotransformation would be reduced. Similar work was also conducted by Jacobsen et al., 2005. In terms of CAFO runoff samples, Mansell et al., (2011) reported that the reason such high concentrations of steroids were found in the filtered fraction (i.e. after centrifugation and filtration at 1 μm) was because the steroids were not actually dissolved, but bound to colloidal organic matter. They proposed that this may explain why settling of suspended particles in not an effective treatment at removing steroids.

Although in the spectrum of chemicals, steroids seem structurally fairly similar, differences in the chemical structure among hormones result in differences in their degradation and sorption properties. Fan et al., (2007a) found that 73% of the C^{14} from labeled 17β-estradiol mixed with soil remained associated with humic substances in the soil after 5 days. Further research clarified that < 2% of the sorbed C^{14} was the parent 17β-estradiol. The remaining C^{14} was associated with estrone (20%) and another unidentified metabolite (55%) (Fan et al., 2008). In contrast, only 19% of the C^{14} from testosterone was bound to humic substances, with the majority of C^{14} (64%) being mineralized to CO_2 from aerobic soil that was not autoclaved (Fan et al., 2007a). An explanation for these results is that the aromatic structure of 17β-estradiol is more stable than the cyclohexane ring of testosterone, and is therefore more resistant to degradation (Fan et al., 2007a). Differences in the functional groups (e.g. ketones or hydroxyl groups) of the hormones will alter their sorption to DOC (Neale et al., 2009) and this will vary depending on pH and type of DOC. Qiao et al (2011) found that isomers (α, β-estradiol and α, β-trenbolone) differed slightly in binding to DOC, with β-isomers sorbing more strongly. For example, with Leonardite humic acid, log K_{DOC} was 3.5 and 3.8 for 17α-estradiol and 17β-estradiol, respectively and 3.1 and 3.6 for 17α–trenbolone and 17β-trenbolone, respectively. Thus, any DOC-enhanced transport would be greater for β-isomers. K_d for testosterone (0.5 ml/g) is lower than K_d for estradiol (10 mL/g) therefore will expect greater penetration depths (Arnon et al., 2008).

Factors Influencing Mobility: Macropore Flow and Colloid Facilitated Transport

Given strong sorption affinity of hormones, vertical mobility is unlikely under packed soil conditions. However in soils where macropore flow is high, leaching of hormones is more likely (Sarmah et al., 2008). This may explain the results presented from our study in which tile samples did contain measurable concentrations of hormones in conjunction with fairly high surface sample concentrations (e.g. tile samples at Farm A and Farm B—Table 4). Similarly, Kjaer et al., 2007 studied fields amended with pig manure slurry and found tile drainage water collected during a storm event to have concentrations of 17β-estradiol as high as 2.5 ng/L and 68 ng/L of estrone. The rapid response of the drainage water to the precipitation events indicated that the transport was due to macropore flow between root zone and tile drains, which allows water to bypass the soil matrix.

Using soil lysimeters, Steiner et al., (2010) found that macropore flow can account for rapid leaching of hormones from a dairy farm effluent as the bromide tracer and the hormone concentrations peaked shortly after application.

(This was done with dairy effluent that was spiked with additional hormones). The highest recovery was only 13% (for estrone). Thus, even with macropore flow, much of the hormone was lost due to sorption and degradation (samples were accumulated over 2 days so degradation in the collection vessel was also possible). However, when the lysimeter had no evidence of macropore flow, estrogen concentrations peaked prior to the bromide tracer peak. This indicates enhanced transport of hormones assumed to be due to associations with colloids. Compounds associated with colloids may have enhanced transport compared with chemical tracers because their size limits their movement through the intergranular pore spaces and they move mainly through channels and secondary pore structures (McCarthy and Zahara 1989). Other than Steiner et al., (2010), additional researchers have suggested that colloid associated transport is likely important in the distribution of hormones (e.g. Arnon et al., 2008; Sarmah et al., 2008; Thompson et al., 2009; Stumpe and Marschner 2010; Mansell et al., 2011).

Overall, Fan et al., (2007, 2007b, 2008) were able to model fate and transport of 17β-estradiol and testosterone under lab conditions by including simultaneous transformation and sorption properties and their distribution among the aqueous and reversible and irreversible sorbed phases. However, they caution that it is difficult to adjust these lab determined rates and processes across the wide range of environmental characteristics that occur across space and time. Given that measurable concentrations of hormones are found in the environment, there appears to be an incomplete understanding of the processes involved in their fate and distribution. For example, research done on a swine farm in North Dakota using lysimeters (Thompson et al., 2009) and evaluating soil core water (Schuh et al., 2011) found widespread 17β-estradiol occurrence, although it was variable over space and time and not necessarily related to manure application. The finding of 17β-estradiol in control lysimeters and at depths where it was not expected lead to the speculation that 17β-estradiol could be distributed via the water table. The low temperatures and anoxic conditions can increase its persistence and result in enhanced lateral mobility of estrogens. The implications for health of those exposed to environmental hormones are still being evaluated.

Effects of Hormones on Fish Populations

In trying to put the results from occurrence studies into the context of implications for aquatic life such as fish, it is important to realize that concentrations in runoff do not equal in-stream concentrations. The surface water samples in our study were collected at the edges of fields, not as discharges into surface waters. Therefore, this study does not indicate the likelihood of hormones being found in surface waters near livestock operations. Factors described above, such as sorption and degradation will decrease hormone concentrations as will factors such as dilution that occur as water enters streams. Although the in vitro bioassays such as the A-screen and E-screen are used to assess steps of the hormone signal transduction pathway (binding of compounds to hormone receptors and eliciting the transcriptional response), whether hormones are active to organisms in the environment is more complex. Factors such as bioavailability (e.g. are hormones associated with sediments or colloids more or less available to the organism?), complexities in the uptake and internal distribution of the hormones by the organisms and other metabolic or elimination differences that can occur result in differences between in vivo and in vitro activity. Fish toxicity studies with a reproductive endpoint are much more time and resource consuming than are the in vitro tests.

Difficulties achieving appropriate dosing are frequently reported. Additionally, variability in the reproductive endpoints in controls is often quite high, making it difficult to achieve statistical significance. However, conducting in vivo tests with fish is essential to determine which hormones (and at what concentrations) populations of fish may be affected.

Estrogens

Because estrogens have been the most frequently studied of the hormones in fish, Caldwell et al., (2012) was able to review literature to derive predicted-no-effect concentrations (pNECs) for the three endogenous estrogen hormones and the birth control pill estrogen (ethynyl estradiol—EE2). The pNECS for EE2 and 17β-estradiol were based on reproductive endpoints using studies from several different fish species and were determined to be 0.1 and 2 ng/L for EE2 and 17β-estradiol, respectively. Fewer studies with reproductive endpoints have been conducted using estrone and estriol, so pNECs for these compounds were based on the induction of vitellogenin and were calculated (based on an estimation of the relationhip of vitellogenin induction to fecundity endpoints) to be 6 and 60 ng/L for estrone and estriol, respectively. 17α-estradiol has been predicted to be 8-17 times less potent than 17β-estradiol in fish studies (Huang et al., 2010; Shappell et al., 2010b). Because the only estrogen we found in our samples was 17α-estradiol, at concentrations as high as 293 ng/L, it is possible that this hormone could affect fish reproduction, although this possibility is based on very limited data on 17α-estradiol effects in fish. It is interesting to note that the rank of potencies is not a perfect match between the E-screen bioassay and the fish pNECS: 1) EE2 is much more potent relative to 17β-estradiol in the in vivo fish than in the in vitro E-screen; 2) Estriol is more potent than estrone in the E-screen whereas the order is reversed for fish; 3) 17α-estradiol is 100x less potent in the E-screen than is 17β-estradiol, but likely only 10x less potent in the fish system.

Androgens

Fewer studies have been reported exploring in vivo effects of androgens on fish reproduction than of estrogens. In contrast to mammals for which DHT is the primary active form of androgen, 11-ketotestosterone is the primary androgen used in teleost fishes. Testosterone mainly serves as a "prohormone", i.e. a precursor to estrogen and 11-ketotesosteorne. Androgen receptor binding assays indicate fairly similar affinities of a wide variety of androgens for rainbow trout, fathead minnow and human androgen receptors (Wilson et al., 2007). However, effects of specific androgens within an in vivo test system have been less frequently studied. Testosterone, which was present at one site (Farm A) for one sample collection (Table 4) found at concentrations below 3 ng/L, has not been reported to have been tested in fish reproduction assays. In the A-screen, testosterone is about 25% the potency of DHT (Table 2). 17β-trenbolone is one of the most frequently studied androgens with respect to reproductive effects in fish. In the A-screen, potency of 17β-trenbolone is very similar to DHT. In fathead minnows, fecundity was significantly reduced during a 21-day exposure of 17β-trenbolone at concentrations above 27 ng/L (Ankley et al., 2003). With Zebrafish, exposure to 17β-trenbolone from hatch to sixty days at concentrations above 15 ng/L resulted in a population consisting of 100% males (Morthorst et al., 2010). Thus, concentrations found in our study (greater than 100 ng/L, Table 4), if present in fish habitats, would likely pose serious risks to fish populations. The androgen that is generally the most frequently found and also often found at the highest concentration is 4-

androstene-3,17-dione. The activity of this compound in the A-screen bioassay is less than 0.5% of the activity of DHT (Table 2). The effects of 4-androstene-3,17-dione on fish have previously been studied as a potential candidate to explain masculinization of mosquitofish (*Gasterosteus aculeatus*) in a pulp and paper mill effluent (Stanko and Angus, 2007), as an additive to fish food to enhance biomass production, and for its role as a fish pheromone (Sorensen et al., 2005). A laboratory exposure assessing reproductive endpoints using a 21-day exposure was conducted and although no significant reduction in fecundity was found even at the highest nominal concentration tested (1000 ng/L), there was a trend towards reduced reproduction at 100 and 1000 ng/L and the gondaosomatic index was significantly reduced at those two concentrations (DeQuattro et al., 2011). More studies addressing the effects of environmental androgens on fish would be useful.

Progestogens

In mammals, progesterone is the primary progestogen. The major progesterone used by teleost fish is 17,20-dihydroxyprogesterone, which is responsible for both egg and sperm maturation (Chen et al., 2010 and references therein). In fact, 17,20-dihydroxyprogesterone is also known as maturation-inducing hormone (MIH) due to its role in ooctye maturation in fish. Additionally, 17,20-dihydroxyprogesterone is released by female fish as a pheromone (Sorensen et al., 2005). However, progesterone is synthesized in fish as a precoursor to 17,20-dihydroxyprogesterone and apparently 17,20-dihydroxyprogesterone is produced as a metabolite in livestock, as this hormone is found in samples associated with livestock (Table 2). When both compounds were found, concentrations of progesterone were higher than concentrations of 17,20-dihydroxyprogesterone. In terms of reproductive effects, progesterone was found to significantly reduce fecundity at 100 ng/L and above in a 21-day exposure bioassay using fathead minnows (DeQuattro et al., 2012). Thus, concentrations of progesterone measured in this study could be high enough to affect fish reproduction. Studies on reproductive effects of 17,20-dihydroxyprogestereone have not been published, although males are very sensitive to this pheromone and detect it at concentrations as low as 1.7 ng/L (Sorensen et al., 2005 and references therein). Like androgens, progestogens have been less studied than the estrogens, but given the important roles as MIH and pheromones, more research is warranted.

CONCLUSIONS

With regards to the objectives of our research, to identify and quantify endocrine active compounds from livestock operations, six hormones were quantified, including one estrogen, two progestogens and three androgens. The bioassays indicating estrogen and androgen receptor activation corroborated the finding of hormones, although the quantification did not always agree. The only estrogen identified in our samples was 17α-estradiol, which has lower potency than 17β-estradiol. A review of other published studies indicates that estrone is the most commonly found estrogen associated with livestock operations and it is unclear why estrone was not detected in this study. Two biologically potent androgens were found, testosterone and 17β-trenbolone, but each was found at only one site. The most frequently found androgen, 4-androstene-3,17-dione, has very low androgenic potency, although research on effects in aquatic organisms is limited. Progesterone and 17,20-dihydroxyprogesterone were widely distributed and occasionally found in high concentrations.

Importantly, we found that microbiological degradation of hormones can be rapid. Clearly, livestock do produce large concentrations of hormones, with concentrations reported to exceed 1000 ng/L. The combination of microbial degradation and sorption accounts for the reduction in hormone concentrations from surface samples, to tile drains to the elimination of detectable hormones when groundwater wells were sampled. The modeling of the fate and distribution of hormones in the environment is complicated and rates of degradation and sorption vary depending on a wide number of factors that vary considerably (e.g. microbial communities present, temperature, moisture, structure of hormone, whether conditions are anaerobic or aerobic, soil types, soil structures). Hormones are especially labile under conditions of high soil moisture, warm temperatures and in an aerobic environment. Additionally, hormones sorb most strongly to soil with high carbon content. These factors clearly are protective in many environments. However, some environmental conditions promote stability. For example, there is evidence that hormones that reach groundwater may persist due to cold temperatures and anaerobic environment.

Because the scope of the current study was very limited, it would be important to sample a wider variety of locations to have a better understanding of whether hormones from livestock operations are found in groundwater. Undertaking this type of study requires a team of researchers including hydrogeologists, agriculture experts, chemists and toxicologists. Technical difficulties in all parameters of the study were encountered. Chemical and toxicological studies on environmental samples where compounds are biologically relevant at ng/L levels in complicated matrices are challenging. Future work should include finding sites where the hydrogeology is well understood and build on the sampling and analytical techniques developed during this study.

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Figure 1. Wells Sampled at UW-Platteville's Pioneer Farm. Depth to Water Table, Depths Sampled, Nitrogen (N) (NO_2+NO_3 mg/L) and Hormones (Endocrine Disrupting Compounds [EDC], both Bioassay and Chemical Analyses.). More details can be found in Table 5.