Stacking broiler litter to reduce natural hormones

Kate Cassity-Duffey,[*,1] Miguel Cabrera,[*] Mussie Habteselassie,[†,‡] Sayed Hassan,[§] John Rema,[*] and Brian Fairchild[¶]

*Crop and Soil Sciences, University of Georgia, Athens, GA 30602, USA; [†]Crop and Soil Science Department, University of Georgia, Griffin, GA 30223, USA; [‡]Poultry Sciences Department, University of Georgia, Griffin, GA 30223, USA; [§]Lab for Environmental Analysis, University of Georgia, Athens, GA 30602, USA; and [¶]Poultry Science, University of Georgia, Athens, GA 30602, USA

ABSTRACT Estrone, 17β-estradiol, and testosterone are naturally occurring hormones excreted in broiler litter. With some potential for environmental concern from the hormones, understanding management practices effect on hormone concentrations is beneficial for the poultry industry. As the amount of hormones potentially introduced into the environment is directly related to the concentration at the time of land application, the purpose of this study was to investigate hormone dynamics in stacked broiler litter during the storage period before removal from the farm and/or land application. Stack temperatures and hormones concentrations were monitored at 15, 45, 75 cm, and 105 cm (measured from the stack bottom) in 6 different on-farm stack houses over 4 or 8 wk. Significant differences in temperature were determined by height and by stack. Stack temperatures during the first 4 wk ranged from 41.5°C to 54.5°C, and all stacks reached maximum temperature by 7 D. Highest temperatures were observed at the 45-cm or 75-cm height. Average stack temperatures correlated with the ambient temperature. Hormone concentration did not vary with height within each house. In 5 of the 6 stack houses, the concentrations of 17β-estradiol and/or testosterone significantly decreased after stacking for 4 or 8 wk (35 to 64%) with only one house showing a significant decrease in estrone concentration (72% in 4 wk). The percent change of estrone and 17β-estradiol mineralization during the first 4 wk was negatively correlated with the 7-D temperature of the pile (r^2 = 0.80), indicating that the high temperatures observed during stacking may inhibit estrogen mineralization. In this study, hormone degradation decreased with high temperatures. Therefore, stack management favoring at least a period of low temperatures may help promote mineralization of these hormones and reduce any potential for introduction into the surrounding environment.

Key words: stacked, estradiol, testosterone, estrone, poultry litter

2020 Poultry Science 99:1379–1386
https://doi.org/10.1016/j.psj.2019.11.011

INTRODUCTION

Poultry production is a major component of United States agriculture with the production value of broilers, eggs, turkeys, and chickens totaling $46.3 billion in 2018 (USDA, 2018). Broiler production (*Gallus gallus domesticus*) makes up 70% of that total and is the largest sector of poultry production (USDA, 2018). With a yearly production of approximately 9 billion birds (USDA, 2018), an estimated 12 million Mg of broiler litter is generated annually, which must be properly managed, utilized, and disposed. This by-product of the poultry industry is most commonly applied to pastures and crops as an inexpensive/alternative fertilizer. The land application of broiler litter provides plant nutrients nitrogen, phosphorus, potassium, as well as other macronutrients and micronutrients (Stephenson et al., 1990; Kingery et al., 1994). However, broiler litter also has potential as an environmental contaminant as it contains the naturally excreted sex hormones testosterone, estrone, and 17β-estradiol (Shore et al., 1993; Lange et al., 2002).

A wide range of hormone concentrations have been previously reported for broiler litter. Shore et al. (1993) determined an average of 133 ng g^{-1} dry litter of testosterone in broiler litter from a 7-wk growout (male and female), and estrogen concentrations (estrone and 17β-estradiol combined) were 5 times higher in female litter (65 ng g^{-1}) than male (14 ng g^{-1}). Cabrera

Received April 3, 2019.
Accepted November 5, 2019.

[1]Corresponding author: katecass123@gmail.com

et al. (2018) found a range of concentrations for testosterone from 59 to 106 ng g^{-1}, estrone from 13 to 135 ng g^{-1}, and 17β-estradiol from 10 to 64 ng g^{-1} for 3 different broiler houses over time. In contrast, Bevacqua et al. (2011) measured average of 41 ng g^{-1} estrone but no 17β-estradiol or testosterone from 12 different broiler houses. Hormone concentrations in broiler litter can be affected by the number of flocks, age, sex, type, and housing/storage conditions that in turn drive hormone degradation rates (Shore et al., 1993; Bevacqua et al. 2011; Cabrera et al., 2018).

Degradation of hormones, 17β-estradiol, estrone, or testosterone in poultry litter are driven primarily by mineralization, but dissipation because of sorption to soil and organic matter have been previously determined (Bera et al., 2011; Durant et al., 2012). Many studies have indicated that 17β-estradiol is primarily degraded to estrone, but other metabolites have been identified. This microbially mediated process has been shown to occur in both anaerobic and aerobic conditions and is affected by water content and temperature (Adeel et al., 2017). Hemmings and Hartel (2006) determined an increase in mineralization of both testosterone and estradiol in poultry litter as water potential approached 0 MPa and a decrease in mineralization at temperatures greater than 30°C. In poultry litter, high ammonia concentrations may also kill pathogenic bacteria (Kwak et al., 2005), but no studies have been performed determining the microorganisms responsible for hormone degradation in poultry litter.

Even small amounts of sex hormones introduced to the surrounding environment can have detrimental impacts on wildlife and human health (Shore and Shemesh, 2003; Soto and Sonnenschein, 2010). Very low concentrations of estrogen (2–10 ng L^{-1}) have been shown to disrupt reproductive cycles, reduce fish biomass, and affect the organ functions of aquatic wildlife (Thorpe et al., 2003; Adeel et al., 2017), so accurately measuring and managing the concentration of these hormones is important. Hormones in broiler litter can potentially be introduced into surrounding surface waters through runoff after land application or leaching from poultry operations. Studies have shown a wide range of hormones in runoff from fields receiving the application of poultry. Anywhere from 7 to 2,530 ng estradiol L^{-1} and from 3 to 12,830 ng testosterone L^{-1} in runoff from fields receiving broiler litter applications have been reported in the previous literature (Finlay-Moore et al., 2000; Jenkins et al., 2006). Peterson et al. (2000) measured 6 to 66 ng L^{-1} of 17β-estradiol in 5 Arkansas springs, and the hormone concentrations trended with concentrations of fecal coliform and *Escherichia coli* in an area where poultry production was heavily concentrated.

The timing of application and litter amendments, such as alum, can reduce hormone runoff from land-applied litter (DeLaune and Moore, 2013). Cabrera et al. (2018) determined greater estrone, 17β-estradiol, and testosterone concentrations in cake vs. full clean-out broiler litter, with hormone concentrations increasing as the number of flocks grown on the same litter increased. Additionally, the researchers found that water contents nearing 60% water-filled porosity favored hormone mineralization in house, with estrone degradation being the most sensitive to water content. Lu et al. (2014) found that after 4 wk 60 to 90% of estradiol decomposed in stacked litter, with initial concentrations varying with height (719–200 ng g^{-1} litter). However, no important mineralization factors were measured such as litter water content or stack temperature in the study.

When house clean-out does not coincide with appropriate times for land application, excess litter must be stored to reduce the potential for environmental contamination and maximize its fertilizer value (Ritz et al., 2013). Stacking is the most common practice of storage to manage poultry litter prior to land application. This practice requires little labor and can also be used as method of pathogen elimination for broiler litter to be used as livestock feedstuff (Kwak et al., 2005; Bush et al., 2007; Baluch-Gharaei et al., 2015). Deep stacking is done under permanent storage structures. Stacks are recommended to be under 2-m height to avoid fires (Ritz et al., 2013). Under real world conditions, stacks vary in litter composition, temperature, water content, and height (Bush et al., 2007), and therefore vary in potential hormone-mineralizing conditions.

This study was conducted to better understand the dynamics of sex hormones estrone, 17β-estradiol, and testosterone in broiler litter after removal from the house and placed in stacks. The objectives of this study were to (1) determine temperature dynamics of deep-stacks as affected by height (from the floor) and time, (2) determine the concentrations and potential for degradation of sex hormones testosterone, estrone, and 17β-estradiol in deep-stacked broiler litter under real production settings as a function of location in the stack and time, and (3) determine if concentrations of estrogenic hormones measured in stacked poultry litter could be related to the E-Screen bioassay.

MATERIALS AND METHODS

Stack House Sampling

Six stack houses (labeled H1, H2, H1a, H3, H4, and H5) were sampled at the time of litter stacking and approximately 4 wk later, with additional sampling done 8 wk after stacking in Houses 3, 4, and 5. House 1a was performed in the same farm/house as H1, but in a stack placed later in the year. To determine locations for sample collection as well as positioning of temperature probes, the width of each stack was divided by 4 and that distance was measured from the edge of the stack to place a 1.8-m wooden dowel with HOBO (Onset, Bourne, MA) temperature sensors located at 0.15, 0.45, 0.75, and 1.05 m from its lower end (against the stack floor) to record temperature at 15-min intervals. Two other dowels were placed in a line at the same distance from each other. Dependent on stack size, distance

between wooden dowels varied from 1 to 4.6 m. Two additional parallel lines of wooden dowels with sensors were placed to achieve a 3 × 3 arrangement. The distance between parallel lines of dowels ranged from 1 to 2 m dependent on stack size.

At 4 wk (all houses) and at 8 wk (H4, H4, and H5), a litter sample was taken right next to each wooden dowel and divided into 30-cm increments, which led to sampling at 0 to 30 cm, 60 to 60 cm, and 90 to 120 cm measured from the floor. Each sample increment was divided in half longitudinally, with half placed into a separate 500-mL Nalgene bottle to be freeze dried, and the other half placed in moisture cans for soil water content determination (65°C, 48 h). Initial total C and N were determined using dry combustion (Nelson and Sommers, 1982; Table 1). Hormone concentrations were measured using the method described below.

Litter Analysis

Hormones were extracted from the litter as described by Cabrera et al. (2018) in which 1 g freeze-dried litter was wrapped in Nitex-35 μm and transferred into a 40-mL screw-cap vial with 10 mL of hexane. Samples were placed in an ultrasonic bath (Fisher FS110H) at 23°C for 10 min, centrifuged at 252 g for 15 min, and the hexane was discarded. Ten milliliters of dichloromethane (DCM) was subsequently added, and the ultrasonic bath and centrifuge were repeated. The extract was transferred to an evaporation tube, and the DCM extraction was repeated. The second extract was transferred to the same evaporation tube, the whole volume was evaporated to near dryness at 45°C under nitrogen, and 1 mL of acetonitrile was added followed by whole volume evaporation to dryness. The remaining residue was dissolved by cautiously shaking with 3 aliquots of 0.5 mL of DCM that were each transferred to the same 2-mL volumetric. The tube was then placed in an air oven at 55°C until evaporated to dryness. Then, 50 μL of 99:1 N,O-bis(Trimethylsilyl)trifluoroacetamide (**BSTFA**):Trimethylchlorosilane (**TMCS**) was added, and samples were incubated at 55°C for 15 min. An additional 50 μL of BSTFA:TMCS was added, and the incubation was repeated. The sample was subsequently spiked with 50 μL of internal standard solution and brought to volume with acetonitrile. Samples were stored in the dark at room temperature until ready for GC/MS analysis.

All solvents used were HPLC-grade or better. Testosterone, 17β-estradiol, and estrone were obtained from Sigma-Aldrich Corporation (St. Louis, MO). The derivatizing reagent, BSTFA containing 1% of TMCS, was obtained in glass ampoules ready to use from Supelco Analytical.

Gas chromatographic analysis was done using a GC/MS/MS that consisted of a CP-3800 oven, CP-8400 autosampler with CP-8410 auto-injector, and 4000 Ion Trap Mass Spectrometer (Agilent Technologies, Santa Clara, CA). The unit runs both electronic and chemical ionization under MS Workstation, version 6.9 SP1. Separation was done using capillary column VF-5 ms 30 m × 0.25 mm × 0.25 μm from Agilent Technologies, helium mobile phase at a flow rate of 1 mL min^{-1}. Oven temperature started at 90°C and ramped at 12°C min^{-1} to 250°C, followed by a second ramp at 20°C min^{-1} to a final temp of 250°C, which was held for 15 min. Selected ion storage parameters selected for the derivatized hormones were the same as those used by Cabrera et al. (2018). Duplicate samples to monitor analytical performance and spiked samples to evaluate recovery were included every 5 to 10 samples. The limit of quantitation was ∼10 ng g^{-1} dry litter for estrone and 17β-estradiol and 25 ng g^{-1} dry litter for testosterone. Repeatability was 5 ng g^{-1} for estrone and 17β-estradiol and 9 ng g^{-1} for testosterone. The linearity varied from R^2 0.96 to 0.99, and the recovery in spiked samples ranged from 83 to 95%.

E-Screen Test

In samples from 3 stack houses (H1, H2, and H3), the estrogenicity of poultry litter extracts was assessed with the E-screen test as described by Soto et al. (1995) with some modification detailed below. The human breast cancer MCF7-BOS cells used in the assay were obtained from Dr. Ana M. Soto's laboratory in Tufts University School of Medicine (Boston, MA). For routine maintenance, cells were seeded in 25-cm^2 flasks in Dulbecco's modification of Eagle's Medium (DMEM; MP Biomedicals LLC, Solon, OH) with phenol red, L-Glutamine and 4.5 g L^{-1} glucose without sodium bicarbonate supplemented with 5% fetal bovine serum (FBS, Hyclone, Logan, UT) at 37°C under saturating humidity in a Becton Dickinson Vented GasPak 150 Anaerobic System (Sparks, MD) filled with Airgas' compressed biological

Table 1. Initial total carbon, total nitrogen, and the carbon to nitrogen ratio measured for each stackhouse.

Stack house	Total carbon	Total nitrogen	C:N
	g kg^{-1} dry litter		
H1	379.9 (8.6)^{a,1}	51.4 (3.7)^{a}	7.42^{d}
H2	386.7 (8.5)^{a}	46.0 (2.0)^{b,c}	8.42^{b,c}
H1a	386.6 (5.1)^{a}	45.2 (2.4)^{c}	8.57^{a,b,c}
H3	352.2 (11.7)^{b}	38.8 (1.7)^{d}	9.10^{a}
H4	348.3 (33.8)^{b}	39.5 (4.2)^{d}	8.86^{a,b}
H5	393.6 (10.3)^{a}	49.1 (1.9)^{a,b}	8.02^{c,d}

Standard deviations from the mean are presented in parenthesis.
[1]Within each column, means followed by different letters are significantly different at $P < 0.05$.

atmosphere with 6% CO2/94% air mixture (Griffin, Georgia).

To carry out the assay, MCF7-BOS cells were trypsinized and transferred into 12-well plates (Franklin Lakes, NJ) at initial concentrations of 1 × 104 cells per well. Cells were incubated at 37°C with 6% CO2 for 24 h for the cells to attach to the wells before removing the seeding medium (5% FBS in DMEM) and replacing it with the experimental medium (5% charcoal-dextran stripped FBS supplemented to phenol red-free DMEM/F12 with penicillin). To prepare the dose–response curve, a range of E2 concentrations diluted in phenol red-free DME immediately before use were added to the experimental medium in plates and incubated for 5 D at 37°C in 6% CO2. In a separate setup, poultry extracts from the hormone extraction at 1/10 dilution were added to the experimental medium and incubated for same length of time under same conditions. The bioassays were terminated on day 6 (late exponential phase). Samples were processed as per the protocol described by Soto et al. (1995), which quantified DNA with Hoechst 33,258 dye-based assay (Fisher Scientific, Hampton, NH). The fluorescence of the samples was read in the UV mode (excitation wavelength range of 300–400 nm and emission range of 380–650 nm) in a mini-fluorimeter (Model TBS-380; Turner Bio Systems, Sunnyvale, CA). A range of working DNA concentrations (0–20 μg/mL) (calf thymus DNA; Hoefer, Inc., San Francisco, CA) was used to prepare a standard curve that was used to convert the fluorimeter readings to DNA concentration. The test was carried out with 100 μL of extract with 3 analytical replicates in a 96-well plate containing MCF-7 cells and 900 μL of DMEM. The relative estrogenic effect for each sample was calculated as described in Soto et al. (1995).

Statistical Analysis

Significant temperature differences with height in the stacks and ambient air (averages between sampling times) were determined using PROC GLM, MEANS Tukey's Multiple Comparison in SAS (SAS Institute, 2013). Owing to differences in the stack heights and potential in shifting of the upper layers of stacks, temperatures measured near the top of pile were compared with the ambient air temperature. Any sensors that were ±8°C from the air temperate were removed from statistical analysis to avoid sensors that may have been exposed to air because of variations in stack height or any possible shifting. For hormone concentrations, PROC MIXED in SAS (SAS Institute, 2013) was used to evaluate the effect of sampling time, height, and sampling time by height interaction on hormone concentrations in the litter. Compound symmetry was used as the covariance structure, with sampling time and height as fixed effects, and sampling location as a random effect. PROC REG selection = stepwise (SAS Institute, 2013) was used for multiple regression analysis of variables.

RESULTS AND DISCUSSION

Stack Temperature

Average air temperature in the stack houses ranged from 6.6°C to 27°C in the first 4 wk of the 6 studies; these values were statistically significant from one another except for H3 and H4 which averaged 22°C (Table 2). Air temperature fluctuated on a diurnal basis (example Figure 1), with temperature values reflecting those expected for the season. Average temperatures measured within the stacks in the first 4 wk (all heights and locations) ranged from 41.5°C to 54.5°C. Separation of means (Table 2) showed significant differences in the stack temperature during the first 4 wk, with H1a and H3 having the highest average temperatures and H2 the lowest average temperature.

Significant differences in temperature by height were determined in all the houses but H2 (Table 3). The highest temperatures were located in the middle of the stack, at either the 45-cm or 75-cm height. Lowest temperatures were observed at 15 cm for all but H4, where the 75-cm height (near top of the pile) was the coolest. All heights reached thermophilic conditions (>40°C), with the internal heights (45 and/or 75 cm) nearing 60°C at some point during each study. All heights but H5 at the 15 and 45 cm, reached >40°C within a few days after stacking and those heights reached 40°C by 2 wk after stacking. The highest average temperature measured during the first 4 wk was determined in H3 at 75 cm (Figure 1), and those temperatures remained sustained throughout the 8-wk study. All stack houses (average all heights) reached maximum temperature by 7 D, with the rate of temperature increase varying by house (Figure 2).

As would be expected, both the whole stack temperature and the temperature measured nearest the top of

Table 2. Average air temperature and average whole stack temperatures during the first 7 D and during the first 4 wk.

Stack house	Air temp	Avg stack temp	7-D stack temp
		°C	
H1	11.3[d,1]	51.3[a,b]	52.7[a]
H2	14.4[c]	41.5[d]	46.3[c]
H1a	27.0[a]	54.2[a]	52.6[a]
H3	22.9[b]	54.5[a]	50.4[a,b]
H4	20.8[b]	49.3[b]	47.3[b,c]
H5	6.6e	45.6[c]	36.0[d]

[1]Within each column, means followed by different letters are significantly different at $P < 0.05$.

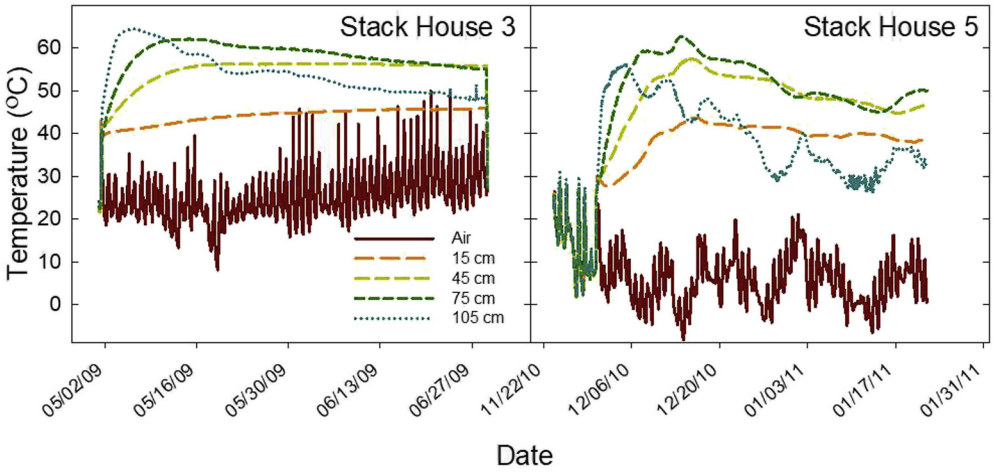

Figure 1. Air temperature and stack temperatures by height (from stack bottom) in stack houses 3 and 5 over 8 wk.

the stack were affected by ambient air temperature (Table 2; Figure 1). Both H3 and H5 reached temperatures of 60°C, but cooler air temperatures in the stack house likely facilitated faster dissipation of heat into the surrounding air, as in the cooling observed in H5. In H5, temperature in the 105-cm height decreased after the first few weeks (Figure 1). Additionally, H5 was much slower to rise in temperature than the other houses. Higher temperatures near the top of the pile (upper 30 cm) have previously been attributed to greater aeration (Kwak et al., 2005), but heat dissipation/accumulation likely play a role as well. Kwak et al. (2005) showed that at ambient temperatures near 30°C, there was a rapid rise in poultry litter stack temperatures (aerated and deep stacked) to 60°C after 3 D. At 10°C ambient temperature, the group observed a slower rise to a maximum of 50°C after 6 or 7 D. Similarly, in a study by Baluch-Gharaei et al. (2015), it took 7 D for stacked litter to reach maximum temperature (50°C–60°C) at 10°C ambient temperature. In a regression of the average stack temperature (average across all heights) during the first 7 D vs. the ambient air temperature during 7 D, the ambient air temperature explained 47% of the variability in stack temperature with a $P = 0.13$. H1 seemed to be an exception with relatively low ambient air temperature and high stack temperature which skewed regression analysis (Table 2). Chaudhry et al. (1998) determined lower temperatures in stacks at 15% moisture content compared with 25 and 35% moisture contents. Water contents measured in this study were greater than those of Chaudhry et al., 1998 (0.4 to 1.16 g H_2O g litter^{-1}; Table 3) and did not correlate with stack temperature. Stepwise multiple regression analysis of average stack temperature vs. ambient temperature, initial water content, and the C:N ratio of the stack litter resulted in no significant relationships for the average stack temperatures during the first 4 wk of the study.

Hormones

Initial hormone concentrations in the stack ranged from 16 to 103 ng g^{-1} for estradiol, from 26 to 232 ng g^{-1} for estrone and from 59 to 202 ng g^{-1} for testosterone (Table 4). In general, no significant differences were found by height for hormones, except for H3 ($P < 0.0001$) in which the 0 to 30 cm height had less estradiol than the 60 to 90 cm height (66 vs. 85 ng g^{-1}) after 8 wk. This is in contrast to findings from Lu et al. (2014) who determined differences in 17β-estradiol concentrations in stacked litter at 0.3-m, 0.6-m, and 0.9-m heights above the floor. The group determined high initial concentrations of 710 μg kg^{-1} 17β-estradiol at the top of the pile, with concentrations decreasing toward the bottom of the pile (311 and 200 μg kg^{-1} 17β-estradiol at 0.6- and 0.3-m heights above the floor, respectively). Furthermore, the group determined that after 4 wk, 90% of the estradiol had degraded in the top height with concentrations of 74, 60, and 57 μg kg^{-1} estradiol at 0.9, 0.6, and 0.3 m, respectively. The researchers stated that higher temperatures may have favored mineralization but did not measure temperature

Table 3. Average temperatures during the first 4 wk at 4 heights (above the floor) in 6 stack houses.

Height (cm)	House 1	House 2	House 1a	House 3	House 4	House 5
15	47.0^{a,1}	40.2^{a}	50.3^{a}	41.9^{a}	49.3^{b}	36.6^{a}
45	55.7^{b}	45.9^{a}	57.6^{b}	53.3^{b}	55.2^{c}	48.1^{b}
75	52.5^{a,b}	36.2^{a}	53.8^{a}	58.6^{b}	37.9^{a}	53.2^{c}
105	49.4^{a,b}	41.0^{a}	NA	57.4^{b}	NA	44.7^{b}

[1]Within each house, means followed by different letters are significantly different at $P < 0.05$.

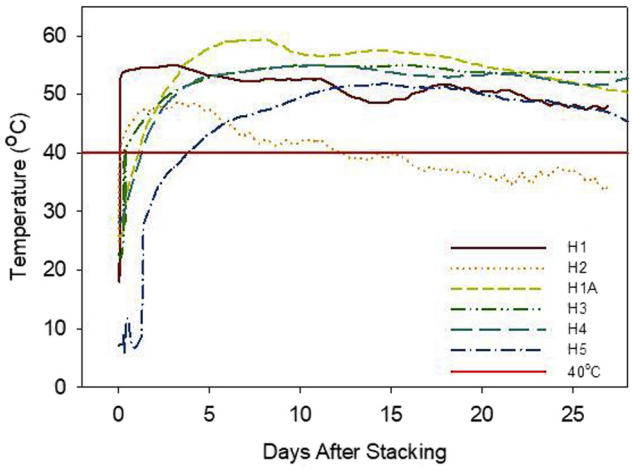

Figure 2. Stack temperature averaged over height in 6 stack houses.

throughout the stack or any other variable such as water content. The lack of effect of height on hormone degradation determined in our study was somewhat surprising because we did determine significant differences in temperature with height. This lack of significant differences may be due in part to the inherent heterogeneity of broiler litter (cake, non-cake, large wood chips), which may have masked actual differences. The lack of significance with height may also be because of the sampling times selected. In stacks where mineralization would occur, there may have been ample time in 4 wk (regardless of the rates occurring at each height) for it to occur at all heights as observed by Lu et al. (2014). A shorter sampling time may have detected differences with height.

In view of the lack of a height effect, all heights and locations were used as replications to identify significant differences in hormone concentrations over time in whole stacks (all sampling heights; Table 4). In 5 of the 6 stack houses, the concentrations of estradiol and/or testosterone significantly decreased after 4 or 8 wk, with no significant differences measured in H1 for estradiol and in H3 for testosterone in H3 (Table 4, Figure 3.). H1a showed the greatest amount of testosterone degradation with 65% of the initial testosterone degraded in 4 wk. H5 showed the greatest amount of 17β-estradiol degradation (52%) of all houses studied. Additionally, only H5 showed a significant decrease (72%) in estrone. Estrone is a metabolite of estradiol degradation, therefore any observed changes in estrone will be because of the degradation of the initial estrone present as well as any produced by estradiol mineralization. This may be one explanation for a lack of observed estrone degradation, although other studies indicate estrone to be more sensitive to environmental factors than testosterone or estradiol (Colucci et al., 2001; Raman et al., 2001; Cabrera et al., 2018). A significant increase in estrone was observed for H1a, from 17 to 30 ng g^{-1}, which may reflect the appearance of estrone from estradiol degradation (Figure 3). Conditions in that house may have been favorable to estradiol mineralization but not estrone. Estrone has been shown to have between 10 and 50% the estrogenicity of 17β-estradiol depending on the assay used (Soto et al., 1995; Colucci et al., 2001), so while its potential for environmental harm is decreased, it is still a contaminant of interest.

For the houses that had significant decreases in hormone concentrations, only H4 showed significant decreases in hormones at week 8, with all other houses showing the majority of degradation occurring in the first 4 wk. None of the houses studied showed a complete

Table 4. Date of sampling, water content, estrone, estradiol, and testosterone concentrations in litter samples taken at stacking time and 4/8 wk later.

	Date	Water	Estrone	Estradiol	Testosterone
		g g^{-1} dry	ng g^{-1} dry		
Stack house 1					
Initial	2/15/08	1.16	26[a]	16[a]	63[a,1]
4 wk	3/13/08	0.83	31[a]	20[a]	23[b]
Stack house 2					
Initial	3/18/08	0.40	85[a]	85[a]	200[a]
4 wk	4/15/08	0.39	77[a]	55[b]	95[b]
Stack house 1a					
Initial	6/22/08	0.81	17[b]	33[a]	59[a]
4 wk	7/23/08	0.75	30[a]	20[b]	21[b]
Stack house 3					
Initial	5/1/09	0.64	211[a]	76[a,b]	99[a]
4 wk	6/1/09	0.61	195[a]	63[b]	87[a]
8 wk	6/30/09	0.55	212[a]	83[a]	97[a]
Stack house 4					
Initial	4/5/10	0.80	61[a]	98[a]	219[a]
4 wk	5/3/10	0.90	50[a]	80[a,b]	204[a]
8 wk	6/2/10	0.90	44[a]	59[b]	157[b]
Stack house 5					
Initial	11/29/10	0.64	232[a]	103[a]	202[a]
4 wk	12/28/10	0.60	64[b]	49[b]	125[b]
8 wk	1/27/11	0.60	59[b]	52[b]	128[b]

[1]Within each hormone, means followed by different letters are significantly different at $P < 0.05$.

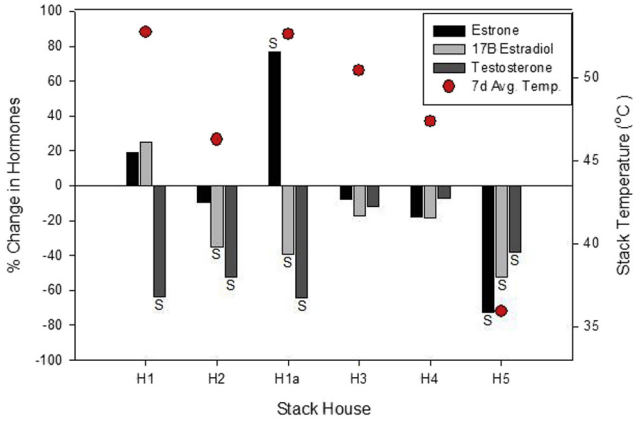

Figure 3. The percent change in hormone concentration after 4 wk and the average temperature during the first 7 D after the study. "S" represents a significant chance from the initial concentrations at $P < 0.05$.

degradation of any of the hormones measured, with considerable concentrations remaining after the 4 or 8 wk, similarly to studies conducted by Hakk et al. (2005) in composted poultry litter.

Previous studies have shown hormone mineralization in poultry litter, manure, and soil to be affected by temperature and water content. Hemmings and Hartel (2006) determined that in broiler litter, a maximum of 27% of testosterone, 6% of 17β-estradiol, and 8% of estrone were mineralized over a 23-wk incubation study, with mineralization increasing with increasing water potentials (−12, −24, and −56 MPa) and decreasing temperatures (25, 35, and 45°C). Durant et al. (2012) found maximal mineralization of 17β-estradiol in soil amended with litter at 30°C, and Raman et al. (2001) determined that estrogen mineralization increased with increasing temperatures from 5°C to 50°C in pressed dairy cake. In addition, Cabrera et al. (2018) hypothesized that mineralization is inhibited at high water contents (greater than >60% water filled porosity) in broiler and breeder houses. While there is potential for abiotic

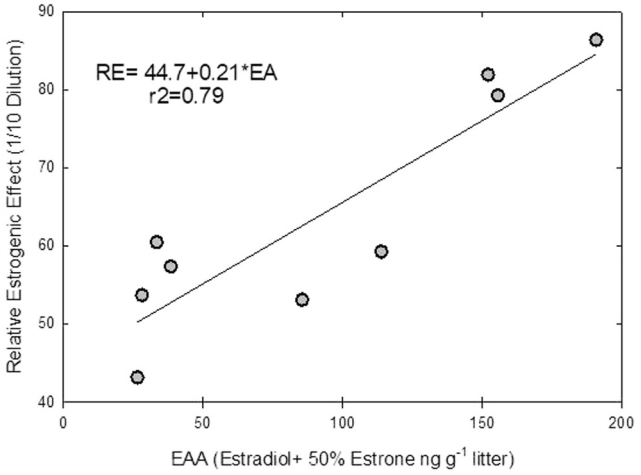

Figure 4. Relationship between relative effect determined by the E screen and the estrogenic activity (EA) determined through GC/MS/MS analyses of broiler litter extracts from 3 houses and sampling times. Estrogenic activity was calculated as the sum of estradiol and 50% of the estrone measured.

degradation (Colucci et al., 2001), significant mineralization is most likely microbially mediated and falls within a "sweet spot" of water contents and temperatures which has yet to be clearly determined. The studies cited above occurred in the lab or in poultry houses; no studies have measured sex hormone mineralization at the high temperatures that occurred during stacking as in our study.

Multiple regression of initial water content, average 4-wk temperature, and carbon: nitrogen ratios did not yield any significant relationships with the percent change of testosterone, estrone, or 17β-estradiol over the first 4 wk. However, when the changes in hormones were regressed against the average temperature during the first 7 D (Table 2), the degradation of estrone and estradiol did show significant relationships with temperature ($r = 0.74$ and 0.40 respectively) with degradation increasing at lower temperatures. A better relationship was obtained between average temperature during the first 7 D and estrogenic activity (ng g^{-1}; **EA**), calculated as the sum of the concentration of 17-β estradiol and 50% of the concentration of estrone (Colucci et al. 2001). The model explained 80% of the variability ($P = 0.016$) and indicated a decrease in EA mineralization with increasing temperature. Under moderate temperatures, hormone degradation has been shown to be rapid (Colucci et al., 2001), which may explain why the 7-D temperature shows a strong relationship with estrogen mineralization. The majority of the mineralization may occur at the beginning of the stacking period, before stack temperatures rise and inhibit degradation. Studies at high temperatures would need to be conducted to verify this potential inhibition. Testosterone degradation appears to be less sensitive to temperature.

E-Screen Test

To determine the effectiveness of the hormone analysis and extraction by GC/MS and to measure estrogenicity, the extracts for the litters underwent the E screen test as outlined by Soto et al. (1995). The relative estrogenic effect determined by the E screen test correlated with the EA (ng g^{-1}) measured (average of all heights for each sampling time) with r^2 of 0.79 (Figure 4). The relative estrogenic effect determined in the E screen test shows the estrogenicity of the poultry litter extracts as compared with the lab-grade estradiol used as positive control in the test (concentration of 1 nmol). It would be expected that higher concentrations of estradiol and estrone measured by GC/MS would correlate with higher estrogenic effects in the E screen test. Further studies should be performed in stacked litter using the E screen test to determine how mineralization, sorption, or abiotic degradation affects the estrogenicity, as well as, the concentration of sex hormones.

CONCLUSIONS

Stacking broiler litter led to high temperatures, with all stacks reaching thermophilic conditions. There was a wide range in sex hormone mineralization in the stacks,

with estrogen degradation being negatively related to temperatures during the first 7 D of stacking. Estrone was the most recalcitrant hormone, only having significant mineralization in one of the 6 houses study. Maintaining a period of low stack temperature immediately after stacking may lead to more hormone degradation and therefore a lower potential of introducing hormones into the environment. The results of this study show that stacking broiler litter may be a viable way of reducing the concentration of sex hormones before land application.

ACKNOWLEDGMENTS

This research was funded by U.S. EPA - Science To Achieve Results (STAR) Program Grant # R833419.

REFERENCES

Adeel, M., X. Song, Y. Wang, D. Francis, and Y. Yang. 2017. Environmental impact of estrogens on human, animal, and plant life: a critical review. Environ. Int. 99:107–119.

Baluch-Gharaei, H., Y. Rouzbehan, H. Fazaeli, and J. Rezaei. 2015. Effect of deep-stacking broiler litter on pathogenic bacteria, intake, digestibility, microbial protein supply and rumen parameters in sheep. Anim. Feed Sci. Tech. 199:73–83.

Bevacqua, C. E., C. P. Rice, A. Torrents, and M. Ramirez. 2011. Steroid hormones in biosolids and poultry litter: a comparison of potential environmental inputs. Sci. Total Environ. 409:2120–2126.

Bera, M., D. Radcliffe, M. L. Cabrera, W. Vencill, A. Thompson, and S. Hassan. 2011. 17β-estradiol and testosterone sorption in soil with and without poultry litter. J. Environ. Qual. 40:1983–1990.

Bush, D. J., M. Poore, G. Rogers, and C. Altier. 2007. Effect of stacking method on salmonella elimination from recycled poultry bedding. Bioresour. Technol. 98:571–578.

Cabrera, M. L., D. E. Kissel, S. Hassan, J. A. Rema, and K. Cassity-Duffey. 2018. Litter type and number of flocks affect sex hormones in broiler litter. J. Environ. Qual. 47:156–161.

Chaudhry, S. M., J. P. Fontenot, and S. Naseer. 1998. Effect of deep stacking and ensiling in broiler litter on chemical composition and pathogenic organisms. Anim. Feed Sci. Tech. 74:155–167.

Colucci, M. S., H. Bork, and E. Topp. 2001. Persistence of estrogenic hormones in agricultural soils: 1. 17β-Estridiol and Estrone. J. Environ. Qual. 30:2070–2076.

DeLaune, P. B., and P.A. Moore, Jr. 2013. 17β-estradiol in runoff as affected by various poultry litter application strategies. Sci. Total Environ. 444:26–31.

Durant, M. B., P. G. Hartel, M. L. Cabrera, and W. K. Vencill. 2012. 17β-estridiol and testosterone mineralization and incorporation into organic matter in broiler litter-amended soils. J. Environ. Qual. 41:1923–1930.

Finlay-Moore, O., P. G. Hartel, and M. L. Cabrera. 2000. 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual. 29:1604–1611.

Hakk, H., P. Millner, and G. Larsen. 2005. Decrease in water-soluble 17β-estridiol and testosterone in composted poultry litter with time. J. Environ. Qual. 34:943–950.

Hemmings, S. J., and P. G. Hartel. 2006. Mineralization of hormones in breeder and broiler litters at different water potentials and temperatures. J. Environ. Qual. 35:701–706.

Jenkins, M. B., D. Endale, H. Schomberg, and R. Sharpe. 2006. Fecal bacteria and sex hormones in soil and runoff from cropped watersheds amended with poultry litter. Sci. Total Environ. 358:164–177.

Kingery, W. L., C. W. Wood, D. P. Delaney, J. C. Williams, and G. L. Mullins. 1994. Impact of long-term land application of broiler litter on environmentally related soil properties. J. Environ. Qual. 23:139–147.

Kwak, W. S., J. Huh, and T. McCaskey. 2005. Effect of processing time on enteric bacteria survival and on temperature and chemical composition of broiler poultry litter processed by two methods. Bioresour. Technol. 96:1529–1536.

Lange, I. G., A. Daxenberger, B. Schiffer, H. Witters, D. Ibarreta, and H. Meyer. 2002. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. Anal. Chim. Acta 473:27–37.

Lu, J., D. Kong, L. Zhao, and Q. Zhou. 2014. Analysis of oestrogenic hormones in chicken litter by HPLC with fluorescence detection. Int. J. Environ. Anal. Chem. 98:783–790.

Nelson, D. W., and L. W. Sommers. 1982. Total carbon, organic carbon, and organic matter. Pages 539–580 in Methods of Soil Analysis Part 2. ASA, SSSA, Madison, WI.

Peterson, E. W., R. K. Davis, and H. A. Ordoff. 2000. 17β-estradiol as an indicator of animal waste contamination in mantled karst aquifers. J. Environ. Qual. 29:826–834.

Raman, D. R., A. C. Layton, L. B. Moody, J. P. Easter, G. S. Sayler, R. T. Burns, and M. D. Mullen. 2001. Degradation of estrogens in dairy waste solids: effects of acidficaiton and temperature. Trans. ASAE 44:1881–1888.

Ritz, C. W., D. L. Cunningham, and W. C. Merka. 2013. Best Management Practices for Storing and Applying Poultry Litter. UGA, Athens, GA. University of Georgia Extension Bulletin 1230. Accessed July 2013. extentison.uga.edu/publications.

SAS Institute. 2013. SAS/STAT 9.4 User's Guide. SAS Inst., Cary, NC.

Shore, L. S., and M. Shemesh. 2003. Naturally produced steroid hormones and their release into the environment. Pure Appl. Chem. 75:1859–1871.

Shore, L. S., E. Harel-Markowitz, M. Gurevich, and M. Shemesh. 1993. Factors affecting the concentration of testosterone in poultry litter. J. Environ. Sci. Health 8:1737–1749.

Stephenson, A. H., T. A. McCaskey, and B. G. Ruffin. 1990. A survey of broiler litter composition and potential value as a nutrient resource. Biol. Wastes 34:1–9.

Soto, A., and C. Sonnenschein. 2010. Environmental causes of cancer: endocrine disruptors as carcinogens. Nat. Rev. Endocrinol. 7:363–370.

Soto, A. M., C. Sonnenschein, K. L. Chung, M. F. Fernandez, N. Olea, and F. Olea Serrano. 1995. The E-SCREEN assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. Environ. Health Perspect. 103:113–122.

Thorpe, K., R. I. Cummings, T. H. Hutchinson, M. Scholze, G. Brighty, J. P. Sumpter, and C. R. Tyler. 2003. Relative potencies and combination effects of steroidal estrogens in fish. Environ. Sci. Technol. 37:1142–1149.

USDA National Agricultural Statistics Services and Census of Agriculture. 2018. Poultry Production and Value 2018 Summary. USDA-NASS, Washington, DC [Online]. Accessed July 2013. http://usda.mannlib.cornell.edu/usda/current/PoulProdVa/PoulProdVa-04-27-2018.pdf/.