# MINERALIZATION OF THE STEROIDAL HORMONES 17β-ESTRADIOL, ESTRONE, AND TESTOSTERONE IN POULTRY LITTERS

by

SARAH NICOLE JULIA HEMMINGS

(Under the Direction of Peter G. Hartel)

#### ABSTRACT

When poultry litter is landspread, steroidal hormones present in the litter may reach environmental waters, where they may have undesirable biological effects. In a laboratory study, we determined the mineralization of  $[4-^{14}C]$ -labeled 17ß-estradiol, estrone, and testosterone in breeder and broiler litter at three different water potentials (-56, -24, and -12 MPa) and three different temperatures (25, 35, and 45°C). Mineralization was similar in both litters and increased with increasing moisture and decreasing temperature. After 23 weeks at -24 MPa, an average of 27, 11, and <2% of the radiolabeled testosterone applied to breeder litter was mineralized to  $^{14}CO_2$  at 25, 35, and 45°C, respectively. In contrast, mineralization of the radiolabeled estradiol and estrone was <2% after 25 weeks at both water potentials, except after 17 weeks at 25°C and -12 MPa, where up to 5.9% of the estradiol and 7.8% of the estrone was mineralized. The limited mineralization suggests that the litters may still be potential sources of hormones to environmental waters.

INDEX WORDS: Androgens, Breeder litter, Broiler litter, Chicken litter, Endocrine, Estrogens, Temperature, Water potential

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# DEDICATION

For years of encouragement, inspiration, understanding, and late-night proof-reading, I would like to dedicate this work to my family—my mum, Jan, my dad, Ray, and my sister, Laurie— and to Tom, who sat beside me on our favourite rock when I decided to undertake this work and stood beside me the whole way.

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# **CHAPTER I**

## INTRODUCTION

Since the 1990s, poultry production has rapidly increased in the United States, partly in response to demand for low-cholesterol meat. The nation produced more than 8.7 billion broiler chickens (*Gallus gallus domesticus*) in 2004 (Georgia Agricultural Statistics Service, 2005), with a concentration of production the mid-southern states (Moore et al., 1995). Of this total, Georgia grew 1.3 billion birds, ranking first in the nation in broiler production for the 21<sup>st</sup> consecutive year, with a production value of over \$2.8 billion (Georgia Agricultural Statistics Service, 2005).

The poultry industry's increase in production has inevitably increased the generation of poultry litter as well. Assuming a production rate of 1.46 kg of litter per broiler (Perkins et al., 1964), the industry generated 12.7 million Mg of broiler litter in 2004, of which Georgia generated almost 1.9 million Mg. The litter, a mixture of bedding materials, feathers, feed, and feces, is considered a valuable fertilizer because it is relatively dry and high in the macronutrients N, P, and K (Wilkinson, 1979). Over 90% of poultry litter in the United States is landspread (Moore et al., 1995).

Landspread poultry litter can cause environmental problems as a non-point source of excess nutrients and microbial contamination to water bodies. In addition, poultry litter is a source of hormones to the environment because chickens naturally excrete high levels (µg per day) of the steroidal hormones 17β-estradiol (henceforth, estradiol), estrone, and testosterone (Shore et al., 1993; Mathur and Common, 1969). These compounds are primarily responsible for the development of secondary sex characteristics in humans and other vertebrates (e.g., Tapiero et al., 2002), and, as natural hormones, are readily absorbed at low concentrations (ng L<sup>-1</sup>). Researchers believe their presence in the environment may affect vertebrates adversely, particularly in aquatic ecosystems (Lai et al., 2002). A primary mechanism for this effect is runoff from fields amended with animal waste.

Although studies suggest that estradiol and testosterone are sufficiently mobile to be present in surface waters (Finlay–Moore et al., 2000; Nichols et al., 1997, 1998) and even ground waters (e.g., Peterson et al., 2000), these reports seem to contradict other studies where the moderately hydrophobic compounds are rapidly and strongly sorbed to soils and therefore pose little risk of leaching into surface and ground water (e.g., Larsen et al., 2001; Casey et al., 2003). Work describing the degradation of these compounds in soil has shown that estradiol and its main bioactive metabolite, estrone, are rapidly dissipated in soil, but slowly mineralized (Colucci et al., 2001). This research suggests that their bioavailability in soil is low, but persistent.

In contrast, mineralization rates of estradiol and testosterone in biosolids from municipal wastewater treatment systems are greater than in soil, and suggest that microbial adaptation plays an important role in degrading the compounds (Layton et al., 2000). However, studies on the degradation of the steroidal hormones in poultry litter itself have been limited to composting (Hakk et al., 2005) and fermentation (Shore et al., 1993) and have not described mineralization in fresh litter. Therefore, important aspects of the fates of hormones in land-applied poultry litters remain uncharacterized.

My research evaluated the mineralization of the naturally abundant estrogens, estradiol and estrone, and the androgen, testosterone, in three types of poultry litters: fresh breeder (laying hen) litter, fresh broiler (immature chicken) litter, and composted broiler litter. Breeder litter was selected for comparison because it contains higher concentrations of steroidal estrogens and

testosterone than broiler litter (Shore et al., 1993), and we hypothesized that microbial adaptation to these higher levels would affect hormone metabolism. Composted litter was studied because water-soluble concentrations of estradiol and testosterone decline during composting (Hakk et al., 2005). Mineralization studies were conducted with radiolabeled hormones incubated at three different water potentials (-56, -24, and -12 MPa, or 15, 25, and 35% water content) and temperatures (25, 35, 45 °C) typical of conditions in the poultry house.

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# **CHAPTER II**

#### LITERATURE REVIEW

Endocrine disruptors are biologically active compounds in the environment, such as hormone mimics and hormone antagonists that can affect reproductive, neurological, and immune systems in animals. The hypothesis that these synthetic, hormonally active compounds were related to widespread sexual disruption in wildlife and possibly humans was popularized in 1997 by Theo Colborn's Our Stolen Future, a recent analogue of Rachel Carson's Silent Spring. Since the publication of Colborn's book, research has shown that exposing vertebrates to hormonally active substances, particularly during pregnancy and early childhood, may shorten gestation, lower birth weights, reduce IQ and memory, delay neuromuscular development, alter sex ratios, and produce irreversible structural and functional deformities in the reproductive system (e.g., hermaphroditism, rare cancers, and reduced sperm counts; National Research Council, 1999). The current understanding of the full range, magnitude, and variation of effects from hormonally active substances in the environment is limited, but growing. In fact, during the 106<sup>th</sup> Congress in 1999, H.R. 1712 was introduced to "amend the Federal Water Pollution Control Act to authorize an estrogenic substances screening program." Both proponents and skeptics of the endocrine disruptor hypothesis agree that further research into the topic is warranted (Krimsky, 2000).

To date, social pressures have tended to focus research and policy questions on the carcinogenic, toxic, and lethal effects of synthetic compounds. Accordingly, funding and research have been focused on *synthetic* hormonally active chemicals. However, this

concentration has had the unfortunate effect of overshadowing investigation into another potent and pervasive class of hormonally active compounds—steroidal hormones, such as estrogens and androgens, that are naturally excreted by humans and non-human animals. This outcome is unfortunate because natural hormones are more potent and environmentally abundant than synthetic endocrine disruptors. For example, estradiol shows substantially greater estrogenicity in competitive binding, transfection, and uterotropic assays compared to eight other endocrinedisrupting chemicals (Shelby et al., 1996). Natural hormones also deserve special attention because they are harder to control than the synthetic endocrine disruptors: they cannot be replaced or banned, and they come from natural sources that are often non-point sources because animal wastes are applied to land (Lai et al., 2002b). Furthermore, the steroidal hormones are normally excreted together, and there is concern that their environmental effects may be synergistic.

The U.S. Geological Survey (USGS) recently conducted an extensive survey of organic wastewater contaminants in 139 streams across 30 U.S. states (Kolpin et al., 2002). Reproductive hormones were detected in 40% of the streams. Research is needed to better characterize the origins and fate of these molecules in the environment in order to assess risks of exposure.

#### **Natural Steroidal Hormones**

The natural steroidal hormones estradiol and testosterone are primarily responsible for the development of secondary sex characteristics in humans and other vertebrates (e.g., Tapiero et al., 2002; Ogawa et al., 1997). Inside the cell, hormones bind to a protein hormone receptor, activate it, and trigger it to express specific genes. Very low concentrations (e.g., 1 to 2 ng  $L^{-1}$ ) of hormones can induce this effect. Estrogens such as estradiol control reproductive cycles and

pregnancy; they also affect skin, bone, and the cardiovascular and immune systems (Tapiero et al., 2002). Androgens such as testosterone influence the development of the male reproductive system, and sexual and aggressive behaviors in males. However, although estrogens are generally responsible for the development of "female" sex characteristics and androgens are generally responsible for "male" characteristics, both groups of hormones are present and physiologically important in males and females. For example, injections of estrogen contributed to the masculinization of female Zebra finches, leading them to form female-female pairs (Mansukhani et al., 1996). Furthermore, mutant male mice that lack the estrogen receptor (ERKO—Estrogen Receptor Knock-Out mice) are not only infertile, but display reduced male-typical aggressive behaviors and increased female-type open field behaviors, suggesting that both male sexual behaviors and female behaviors are linked to expression of the estrogen receptor. The mechanisms of action and interaction between hormones are complicated; for example, testosterone can be aromatized to estradiol in target tissues such as the brain, and can therefore interact with the estrogen receptor (Ogawa et al., 1997).

Although the natural steroidal hormones are short-lived in the body compared to many synthetic hormones or endocrine disrupting chemicals, they are more potent because they are the actual endogenous molecules "designed" to have the effects that their synthetic counterparts can only mimic or antagonize (Casey et al., 2004). In fact, estradiol and testosterone are carcinogens with neoplastigenic, teratogenic, and tumorigenic effects on animals (Occupational Safety and Health Administration, 2005) because some cancers (e.g., breast and uterine) depend on estrogen for growth, and some (e.g., prostate) depend on androgens (Tapiero et al., 2002). The natural steroidal hormones are readily absorbed through the skin, mucous membranes, and gastrointestinal tract (Occupational Safety and Health Administration, 2005), and this absorption,

coupled with their high potency at low concentrations, makes exogenous hormones a possible hazard to humans and other organisms.

# Physiochemical Properties of Estradiol, Estrone, and Testosterone

Steroidal hormones exist in similar forms in humans and animals; the molecules, which are characterized by a cyclopentan-*o*-perhydrophenanthrene ring, are synthesized from cholesterol (Ying et al., 2002). The natural steroidal estrogens are tetracyclic molecules with an aromatic A ring and a C-13 methyl group (Neef, 1999; Tapiero et al., 2002; Hanselman et al., 2003). The most prominent steroidal estrogen, estradiol, can have two configurations:  $17\alpha$ -estradiol, where the hydroxyl at C-17 points downward, and 17B-estradiol, where the hydroxyl points upward (Fig. 1.1A). The more common epimer in the waste of the non-laying hen is 17B-estradiol (Common et al., 1969). Estradiol is rapidly converted to estrone, its most bioactive metabolite, when the hydroxyl at C-17 is oxidized to a carbonyl group (Fig. 1.1B; Hanselman et al., 2003). Estrone is also excreted by the hen (Mathur and Common, 1969). The androgen testosterone, which is found in the excreta of both male and female birds, differs from the estrogens in that it has a cyclohexene ring rather than an aromatic ring, and a carbonyl group at C-3. Like estradiol, testosterone has a hydroxyl group at C-17 (Fig. 1.1C).

Estradiol, and estrone, and testosterone are poorly soluble in water, with solubilities of 12.96 mg L<sup>-1</sup> for estradiol, 12.42 mg L<sup>-1</sup> for estrone, and 8.7 mg L<sup>-1</sup> for testosterone (Tabak et al., 1981; temperature unknown). Hurwitz and Liu (1977) found a lower value for estrone at approximately 0.8 mg L<sup>-1</sup> at 25 °C, while the Merck Index (Budavari, 1996) reported a much higher aqueous solubility of 30 mg L<sup>-1</sup> at 25 °C for estrone. Yalkowsky and Dannenfelser (1992) reported a lower aqueous solubility for estradiol at 3.6 mg L<sup>-1</sup> at 27 °C and a higher value for testosterone at approximately 23.4 mg L<sup>-1</sup> at 25 °C. As expected, estradiol and estrone are

moderately hydrophobic, and both have a high log octanol-water coefficient ( $K_{ow}$ ) in the range of 3.1 to 4.0 (experimental values are compared to other literature in Lai et al., 2000; Hanselman et al., 2003). Testosterone has a similar log ( $K_{ow}$ ) of 3.3 (Hansch and Leo, 1979). These values indicate that the hormones would tend to be adsorbed onto soils rather than dissolved in water.

Estradiol and estrone are weak acids, with pKa values (ionization constants) of about 10.71 and 10.77, respectively (Lewis and Archer, 1979). According to Hanselman et al. (2003), these values indicate that at normal environmental pH levels, ionized species would not be expected. Furthermore, because both compounds have vapor pressures of about  $3.0 \times 10^{-8}$  Pa (Lai et al., 2000), volatilization should be low and thus gaseous measurements need not be included in the experimental mass balance (Hanselman et al., 2003). Other vapor pressures have been reported: estradiol at about 1.67  $10^{-6}$  Pa, estrone at about  $1.89 \times 10^{-5}$  Pa, and testosterone at about 2.9 x  $10^{-6}$  Pa at 25 °C (Syracuse Research Corporation, 2005).

### **Biological Effects of Steroidal Estrogens**

#### Fish

*In vivo* studies on the estrogenic effects of hormonally active compounds in aquatic systems typically measure two biomarkers in vertebrates: vitellogenin production and relative mass of the gonads. Estrogen stimulates the synthesis of vitellogenin, a protein precursor to egg-yolk that is important to reproduction, in the liver. Male and immature female fish typically carry little-to-no vitellogenin in their blood, though male fish do carry the vitellogenin gene, which will likely be expressed in response to estrogen ("vitellogenesis"). The second biomarker for estrogenicity is the relative mass of the gonads, the development of which is also dependent on hormonal signals (Panter et al., 1998).

The effects of exposure to environmental estrogens on fish vary depending on the reproductive stage of the individual or relative maturation speed of the species, yet concentrations of estradiol as low as 1 ng L<sup>-1</sup> can induce vitellogenin production in male fish (Purdom et al., 1994). Exposure to low concentrations of estradiol (100 ng L<sup>-1</sup>) and estrone (31.8 ng L<sup>-1</sup>) for 3 weeks significantly increased vitellogenin levels in the blood and inhibited testicular growth in male fathead minnows (*Pimephales promelas*; Panter et al., 1998). A group of Japanese Medaka (*Oryzias latipes*) embryos exposed to 1.0  $\mu$ g L<sup>-1</sup> of estradiol contained no males, illustrating how exposure to estrogens affects fish at the population level (Tabata et al., 2001).

Behavioral responses in fish have also been observed in response to environmental hormones. The compounds may interfere with the reproductive behavior and success of fish that use steroidal hormones as pheromonal signals to initiate mating or spawning (Kolodziej et al., 2003). Furthermore, low and high concentrations of estradiol interfered with gender recognition via electrocommunication in adult *Sternopygus*, an electric fish, causing higher discharge of the electric organ, but with a shorter pulse (Dunlap et al., 1997).

# Amphibians

Amphibians may be declining worldwide because of their high sensitivity to poor water quality. One possible reason for this decline may be hormonally active agents. Injection of estradiol caused vitellogenesis in adult male African clawed frogs (*Xenopus laevis*; Palmer and Palmer, 1995). Larval-stage males from the same species were feminized by estradiol injections (Kloas et al., 1999).

# Reptiles

The gonadal sex of many egg-laying reptile embryos depends on the temperature at which eggs are incubated (temperature-dependent sex determination) rather than the organism's genotype (genotypic sex determination). Estrogen may be the "physiologic equivalent of incubation temperature" because it initiates development of the female gonads (Crews et al., 1995). Administering estradiol causes female gonads to develop in turtle eggs incubating at "male" temperatures, and in clutches incubating at intermediate temperatures that produce both males and females, estradiol synergizes with temperature for a greater feminizing effect. Runoff from fields stocked with cattle had an estrogenic effect on painted turtles, *Chrysemys picta*, in farm ponds (Irwin et al., 2001). Low, environmentally relevant concentrations of estradiol induced higher vitellogenin levels in females, but not in males. These results suggest that the reproductive fitness of females with increased vitellogenin levels may be affected, possibly by causing changes in egg size, straining the kidneys, or imposing an excess energy cost. *Birds* 

Reduced breeding success (increased nestling mortality), a female-skewed sex ratio in offspring (significantly more daughters than sons; Williams, 1999), and female-female pairing (masculinization of females) have been observed in Zebra finches exposed to steroidal estrogens via injection. The finches also displayed changes in dancing, singing, and mounting behavior (Mansukhani et al., 1996). Male Japanese quail (*Coturnix japonica*) exposed to the synthetic estrogen ethynylestradiol in the egg developed feminized reproductive tissues (ovary-like tissue in the testis; Berg et al., 1999).

### Invertebrates

Studies are beginning to look at the effects of steroidal hormones on invertebrates. A study of survival and reproductive or developmental effects of natural and synthetic estrogens on the marine copepod, *Tisbe battagliai*, found no observable effects from environmentally relevant concentrations of steroidal estrogens (Pounds et al., 2002). Both low and high concentrations of estradiol significantly reduced the settlement of barnacle (*Balanus amphitrite*) larvae by disrupting the production of cypris major protein, likely affecting barnacle survival adversely (Billinghurst et al., 1998).

### Plants

Environmental estrogens may alter vegetative development, flowering, and the sex determination of flowers (Geuns, 1978). For example, concentrations of estradiol are correlated with sexual expression in the quaking aspen (*Populus tremuloides*), increasing in the spring then peaking during flowering and decreasing as flowers age (Khaleel et al., 2003). Phytoestrogen levels in alfalfa increased after irrigation with sewage water containing estradiol and estrone (Shore et al., 1995b). Since the pathways of estrogen synthesis in animals are similar to those of the phytoestrogens in legumes, estrone may be able to interact with enzymes in both pathways. In turn, elevated phytoestrogen may affect the fertility of grazers, which illustrates how the effects of environmental hormones on individuals my translate into population-level effects through trophic interactions (Lai et al., 2002a).

## Algae

Estrogens were transformed rather than concentrated in the freshwater algae *Chlorella vulgaris* under both dark and light conditions (Lai et al., 2002c). This finding contrasts with the biomagnification of certain persistent organic endocrine disruptors in phytoplankton. The algae

may use biotransformation, which depends on algal cell density and estrogen concentration, to reduce the estrogen's potency or to prepare it for excretion or other metabolic steps. Half of the estradiol applied to the algae was metabolized to an unknown product in the light, and may have become conjugated—a common and rapid detoxification mechanism that deactivates plant hormones and makes them easier to excrete. The algae did not transform the synthetic estrogen ethynylestradiol, a human contraceptive commonly found in wastewater effluent.

## **Biological Effects of Testosterone**

The environmental effects of testosterone have not been as well-studied as the estrogens; however, environmental testosterone may pose health risks because it can be absorbed transdermally (Rolf et al., 2002; Tang et al. 2002). Testosterone affects mouse aggression (Colborn et al., 1997), bird calling (Ball et al., 2002), bird parasite numbers (Saino et al., 1995), snake strikes (King, 2002), the timing of deer antler growth (Suttie et al., 1995), neuron development, fish brooding behavior (Pankhurst and Peter, 2002), and the development of imposex gastropods (Bettin et al., 1996). Medaka (*Oryzias latipes*) exposed to methyltestosterone developed masculine secondary sex characteristics and gonads, and experienced reduced fecundity and fertility (Seki et al., 2004).

There are a number of pathways by which testosterone can reach the external environment. Testosterone is naturally excreted by humans and can be found in municipal biosolids (Lorenzen et al., 2004). It can also exist in high concentrations in animal manures (Lange et al., 2002), including litter from both male and female chickens, and subsequently, testosterone can remain in soils that receive this litter as fertilizer (Finlay–Moore et al., 2000). In the United States, androgens such as trenbolone acetate (TbA) are commonly administered to cattle as growth promoters, and are found in manure (Schiffer et al., 2001). Native female fathead minnows with male characteristics were found downstream from cattle feedlots, and samples of these waters produced androgenic effects in *in vitro* assays. Female minnows exposed to TbA ( $30 \text{ ng L}^{-1}$ ) in the laboratory developed bumps on the head typical of breeding males, and higher concentrations of TbA decreased egg production (Renner, 2002). Finally, androgens fed to fish in aquaculture produce monosex stocks (Chatain et al., 1999). Considering these biological effects and the pathways by which testosterone may exert deleterious effects on wildlife and humans, further research into the behavior and fate of environmental testosterone is warranted.

#### **Population and Community Effects**

Natural steroidal estrogens such as estradiol and estrone affect development and behavior in organisms that express the estrogen receptor, and possibly in those that do not as well (Lai et al., 2002b). In fish, amphibians, and reptiles, estrogenic hormones affect the organ, organism, and population levels of biological organization, including reproductive system development, sex ratios, and reproductive cues (see Lai et al., 2002b, for a review of the pertinent toxicology literature). For example, shifting sex-ratios and the incidence of all-female populations has been linked to estrogen exposure (e.g., Japanese Medaka exposed to estradiol; Tabata et al., 2001). Furthermore, the occurrence of these effects across taxa that commonly co-occur in aquatic habitats suggests that steroidal hormones likely have community effects as well, and researchers are just beginning to extend toxicological data and investigation into community relationships, taking into account trophic interactions and the possibility of biomagnification (e.g., Lai et al., 2002a).

In addition to concerns over the effects of hormone contamination on higher levels of biological organization, the duration of the effects relative to exposure levels and their consequences over longer time scales must be evaluated. For example, one further concern over

the effects of environmental hormones is that short daily movements through plumes of estrogens may have greater effects than expected. Intermittent exposure to estradiol produced vitellogenin levels in rainbow trout almost equal to continuous exposure to the hormone, and recovery in the absence of estradiol was not significant even after 3 weeks (Panter et al., 2000). Furthermore, it is important to consider the evolutionary consequences of continuous or intermittent exposure to steroid hormones beyond the second or third generation, which are commonly the endpoints of toxicological research on hormones. For instance, research should address the effects of environmental hormones on community evolution.

### **Hormones in Sewage Effluent**

Natural steroidal hormones may reach the external environment via sewage effluent. Hormone excretion rates for both human males and females vary between 2 and 259  $\mu$ g of estradiol per person daily, depending on gender and reproductive phase (e.g., pregnant women excrete the highest levels). These amounts are diluted to the ng L<sup>-1</sup> range in sewage (Ying et al., 2002); even so, they remain potent at this low concentration. Natural human hormones such as estradiol, testosterone, and synthetic contraceptives like ethynylestradiol can pass through sewage treatment plants unaffected or as bioactive metabolites (Ternes et al., 1999b; Tabak et al., 1981). For example, estradiol is oxidized to estrone in biosolids, and conjugated estrogens may be cleaved, or de-conjugated, to release free, bioactive estrogens (Ternes et al., 1999a). The concentration and persistence of the hormones is related to their solubility, breakdown into intermediate metabolites, proportion of metabolized and unchanged compounds in the influent, and resistance to hydrolysis and oxidation by microorganisms (Tabak et al., 1981). In addition, because of different methods of wastewater treatment, varying adaptations of microbial

populations in biosolids, and possibly different climatic conditions, facilities differ in their ability to remove hormones (Layton et al., 2000).

Fish and amphibians exposed to effluent from municipal and industrial treatment plants have increased levels of vitellogenin, suggesting the presence of estrogenic compounds in the effluent (Hansen et al., 1998). Effects have been observed at the cellular, organ, organism, and community levels of organization in fish exposed to treated municipal sewage effluent (Porter and Janz, 2003).

#### **Hormones in Poultry Litter**

All animals excrete hormones at rates that depend on species, age, gender, and reproductive stage; thus, steroidal hormones can also reach the external environment via animal wastes (Lange et al., 2002). In particular, concentrated animal operations produce a large amount of waste in a small area, which is frequently land-applied as fertilizer and as a means of disposal (Moore et al., 1995). Because it is relatively dry and high in the macronutrients N, P, and K, broiler litter is considered the most valuable animal waste for fertilizer (Wilkinson, 1979). This material is a mixture of bedding materials, feathers, feed, and feces from immature male and female chickens.

Chickens naturally excrete high levels ( $\mu$ g day<sup>-1</sup>) of steroidal hormones, and hormone concentrations in broiler litter vary. Using a radioimmunoassay, Shore et al. (1993) measured 14 and 65  $\mu$ g kg<sup>-1</sup> dry weight of "estrogen" (likely including both estradiol and estrone) in litter from male and female broilers, respectively. Testosterone concentrations were unaffected by gender; 133  $\mu$ g kg<sup>-1</sup> litter were found from both sexes of broilers. Litter from adult chickens contains higher hormone levels compared to that from immature broilers (Table 1.1). Breeder (laying hen) litter contains nine times more estrogen (533  $\mu$ g kg<sup>-1</sup> dry weight) than immature female broiler litter, and adult rooster litter contains five times more testosterone (670  $\mu$ g kg<sup>-1</sup>

dry weight) than immature male broiler litter. Lorenzen et al. (2004) measured lower values for breeder litter (approximately 70  $\mu$ g estradiol kg<sup>-1</sup> dry weight and 20 to 30  $\mu$ g testosterone kg<sup>-1</sup> dry weight) and broiler litter (approximately 55  $\mu$ g estradiol kg<sup>-1</sup> dry weight and 30  $\mu$ g testosterone kg<sup>-1</sup> dry weight).

In 2004, the more than 8.7 billion broilers produced in the United States generated 12.7 billion kg of litter, assuming a litter production rate of 1.46 kg per broiler (Perkins et al., 1964). Georgia ranked first in the nation for broiler production for the 21<sup>st</sup> consecutive year, and the state's 1.3 billion broilers generated almost 1.9 billion kg of litter (Georgia Agricultural Statistics Service, 2005). In some countries, poultry litter is incorporated into livestock feed; however, the practice of feeding hormone-rich litter to other animals has raised concern because estrogens and androgens in the litter can interfere with reproduction, causing premature udder development and lactation, delayed puberty, or enlarged clitoris (summary in Shore et al., 1993). In contrast, over 90% of poultry manure in the United States is land-applied as part of good agronomic practice (Moore et al., 1995).

## Fates of Hormones from Land-Applied Animal Waste

Hormones may contaminate water and soil when animal waste containing high concentrations of steroidal hormones is landspread. Although the physiochemical properties of estrogens and androgens suggest that they should remain tightly sorbed to litter and soil, studies show that estradiol and testosterone are mobile in runoff, soil, and groundwater. Using enzymeimmunoassay, Finlay–Moore et al. (2000) found high edge-of-field losses of both estradiol (up to 2,530 ng L<sup>-1</sup>) and testosterone (up to 1,830 ng L<sup>-1</sup>) from grazed and ungrazed grasslands amended with broiler litter. The increases in steroid concentrations in runoff and soil depended on the rate of application and the time between application and the runoff event. The presence of

grazing cattle did not contribute hormones to the runoff, but testosterone in the soil was significantly higher in plots grazed by cattle compared to hayed plots.

When Nichols et al. (1997) amended fescue plots with broiler litter, first-storm estradiol concentrations in runoff from amended plots increased linearly with application rate, reaching 1.28 µg L<sup>-1</sup> at the highest rate. Some estradiol persisted for at least 7 days in the field, and though second-storm concentrations in runoff were nearly 70% lower, plots with the highest application rate still contributed significant concentrations of estradiol to runoff. Estradiol concentrations in runoff were positively correlated to both the pH and total organic carbon of the water. The addition of alum, commonly added to poultry litter to reduce ammonia volatilization, reduced mean estradiol concentrations in runoff by over 40%. This reduction may have occurred because alum reduced litter pH, thus making organic carbon compounds less soluble. A subsequent study suggested that estradiol concentrations in runoff from broiler litter-treated plots decreased as the length of grass buffer strips bordering the plots increased (Nichols et al., 1998). Buffer strips of 3.1, 6.2, and 18.3 m in length reduced runoff concentrations of estradiol by 58%, 81%, and 94%, respectively.

Bushée et al. (1998) observed increased estradiol concentrations in runoff from fescue plots amended with municipal sludge and horse bedding. When rainfall was simulated within 1 hour of application, flow-weighted mean estradiol concentrations in runoff from the horse bedding were comparable to rates reported by Nichols et al. (1997) for poultry litter. Estradiol concentrations in runoff were highest within 2 minutes of rainfall and were significantly different from final concentrations after 30 min of runoff. However, the addition of 10% alum did not significantly reduce estradiol concentrations in runoff, presumably because of insufficient mixing or reaction time.

In addition to runoff, estradiol and testosterone are also mobile in soil and groundwater, but the hormones may move through the environment by different routes. Estradiol from poultry and cattle wastes was transported through mantled karst aquifers in Arkansas, with concentrations of estradiol ranging from 6 to 66 ng L<sup>-1</sup> (Peterson et al., 2000). Following landapplication of chicken manure, Shore et al. (1995a) found testosterone, but not estrogen, in subsurface soil samples and well water. Estrogens were leached out in runoff, suggesting that testosterone is leached through soil more readily than estrogens, and that estrogens mainly move through surface runoff.

### **Sorption and Transport**

Sorption studies show that estradiol, and to a lesser extent, testosterone, sorb tightly to soil unless preferential flow occurs. Freundlich sorption coefficients ( $K_d$ ) for estradiol and testosterone are high (Lai et al., 2000; Casey et al., 2003, 2004) and sorption affinity relates to mineral particle size, organic matter content, soil surface area, iron-oxide content, and cation-exchange capacity. In intact soil columns flushed with solutions containing estradiol and testosterone, approximately 50% of both hormones that remained in the columns after flushing was sorbed to the top 0 to 10 cm of soil (Sangsupan, 2005). Thus, long distance transport of the hormones through soil would seem unlikely. In packed columns, testosterone and estradiol sorbed rapidly to soil and did not appear in the effluent when the column was flushed (Larsen et al., 2001). Similarly, Casey et al. (2003) found that only 0.9% of the estradiol flushed through packed soil columns appeared in the column effluent, and that estradiol was transformed to estrone on the sorbed phase.

However, other research suggests that testosterone leaching is much higher than estradiol; 21% of the amount applied to the surface of a packed column was recovered in the effluent

(Casey et al., 2004). In fact, when soil macropores and other pathways for preferential flow are preserved, more testosterone and estradiol are found in column effluent. Averages of 27% of the initial estradiol and 42% of the initial testosterone were eluted from intact soil columns (Sangsupan, 2005). In addition, more estradiol appeared in effluent from intact columns of conventionally tilled soil than from columns of no-till soil. Therefore, despite estradiol and testosterone's high sorption affinities, the hormones may be transported through the soil, particularly under preferential flow. These reports partially parallel the observations of Shore et al. (1995a) that estrogens are primarily transported by surface runoff, whereas testosterone is mobile in surface runoff as well as through the soil into groundwater.

One possible answer to the question of how the moderately hydrophobic steroidal hormones are transported in water may be that they are present in conjugated forms. To facilitate excretion from the body, the steroidal hormones are often sulfated or glucuronidated, making them more water soluble than the free parent forms (Tabak et al., 1981). For example, testosterone can exist in dozens of different conjugates; the most abundant conjugates are sulfated or glucuronidated testosterones. Estrogens are sulfated and glucuronidated in animal urine, and unconjugated in feces (Larsson et al., 1999). These conjugated forms are not as bioactive as the parent forms; however, after excretion, microorganisms can rapidly cleave the conjugates back to the parent forms that exhibit stronger hormonal activity (Ternes et al., 1999a; Larsson et al., 1999). Therefore, in my study, it was appropriate to use the free, parent forms, which are the most bioactive.

#### **Degradation Studies**

Although sorption and transport studies provide important descriptions of what likely occurs when steroidal hormones reach the environment, degradation studies are necessary to give a

complete environmental picture of these compounds. Several studies have monitored the degradation of steroidal hormones in various media. Mineralization of natural hormones has been studied in biosolids (estradiol, estrone, and testosterone, Layton et al., 2000), surface waters (estradiol and estrone, Jürgens et al., 2002), soils (estradiol and estrone, Colucci et al., 2001; Colucci and Topp, 2002; testosterone, Lorenzen et al., 2005), and soils amended with biosolids or swine manure (estradiol and testosterone, Jacobsen et al., 2005) using <sup>14</sup>C or <sup>3</sup>H radiolabeled hormones as tracers. The mineralization studies monitored <sup>14</sup>CO<sub>2</sub> evolution, which denoted cleavage of a carbon ring of the steroid/cholesterol backbone and subsequent inactivation of the hormone. No such studies have been conducted in breeder litter or broiler litter.

Mineralization of <sup>14</sup>C-labeled estradiol and estrone was the same in biosolids from both municipal and industrial water treatment plants, although the facilities differed in their ability to mineralize these hormones (84% mineralized in the municipal compared to 4% in the industrial system; Layton et al., 2000). In 24 hours, municipal biosolids from four plants mineralized 70 to 80% of the total labeled estradiol (rate, k  $\approx$  0.0042), and removed over 90% from the aqueous phase (e.g., through sorption). Loss of estrogenicity in the aqueous phase was verified with the Yeast Estrogenicity Screen (YES) assay. A total of 55 to 65% of the testosterone was mineralized (k  $\approx$  0.0152), and removal of testosterone from the aqueous phase (95%) was approximately twice as fast as for estradiol. In addition, mineralization of <sup>14</sup>C-ethynylestradiol by municipal biosolids was much less than for the other hormones: 40% was mineralized in 24 hours (k  $\approx$  0.0002) with a constant 20% remaining in the aqueous phase. Mineralization in biosolids and removal from the aqueous phase were linked processes with similar rates for each hormone; however, only estradiol's mineralization rate was significantly affected by a 15 °C difference in temperature (between incubation at 5 to 10 °C and 22 to 25 °C). The slower

mineralization of ethynylestradiol was expected because the ethynyl group inhibits degradation, a design feature to ensure that the contraceptive reaches the target organs in the body. The persistence of ethynylestradiol in sewage treatment plant effluent and surface waters has been widely reported (e.g., Ternes et al.,1999a; Jürgens et al., 2002).

River water and anaerobic bed sediments from English rivers rapidly converted estradiol to estrone, which was quickly degraded in aerobic portions of the sediments but tended to accumulate in the anaerobic portions of the sediments; sterile controls showed no significant degradation (Jürgens et al., 2002). Estradiol and estrone had half-lives between a few hours and several days, and accordingly, 99% of estrogenicity declined within 2 weeks, as measured by the YES assay. Mineralization of <sup>14</sup>C-estradiol was consistent with the degradation rates, and about 24 to 45% of the radiolabel was evolved as  ${}^{14}CO_2$  by Day 25, when rates began to slow. The mass balance showed that 10 to 23% of the radiolabel was converted to unknown hydrophilic compounds, and 18 to 32% remained as either estradiol or hydrophobic byproducts. At high (100  $\mu$ g L<sup>-1</sup>) and low (20 ng L<sup>-1</sup>) concentrations in river water, estradiol was degraded at similar rates, which suggested that estradiol will be degraded even when it is present in concentrations too low to stimulate bacterial multiplication. Estradiol also underwent photodegradation (no degradation occurred in dark controls), with a half-life of about 10 days; however, photolysis was slow compared to biodegradation. Estradiol was also quickly biodegraded in aquifer material (sediment and groundwater) under aerobic conditions (half-life of 2 days), but slowly under anaerobic conditions (half-life of 107 days; Ying et al., 2003).

Compared to degradation in biosolids and river water and sediments, mineralization rates of estrogens in agricultural soils are much lower at a range of temperatures and moistures typical of a temperate growing season (Colucci et al., 2001; Colucci and Topp, 2002). Estradiol applied to

three different agricultural soils at a rate of 1 mg kg<sup>-1</sup> substrate was mineralized very slowly: 11.5 to 17.1% of <sup>14</sup>C-estradiol was recovered as <sup>14</sup>CO<sub>2</sub> after a 3-month incubation, with optimal mineralization rates with increasing moisture and incubation at 30 °C and 37 °C (Colucci et al., 2001). Alternatively, the major pathway of hormone removal in these soils was the formation of non-extractable residues rather than CO<sub>2</sub>. Estradiol was converted to estrone in both autoclaved and nonsterile soil, suggesting an abiotic reaction (or incomplete sterilization), whereas estrone was stable in sterile soil, suggesting microbially mediated degradation. Both estrone and estradiol rapidly formed non-extractable soil-bound residues which were slowly mineralized, probably due to low bioavailability. This decrease in extractable <sup>14</sup>C-estradiol and <sup>14</sup>C-estrone concentration, as measured by HPLC, was termed "dissipation." The time to dissipate 50% of the initial estradiol concentration was less than half a day, and after 3 days of incubation at 30 °C, 56 to 90.7% of <sup>14</sup>C-estradiol was non-extractable from agricultural soils. For estradiol, changing the temperature did not significantly affect the rate of dissipation (except at 4 °C, where it was lower) or the loss of total estrogenicity (as measured by the YES assay), but removal of estradiol generally increased with increasing moisture. Replication of this work with environmentally relevant concentrations 1000 times lower (ng L<sup>-1</sup>, using tritiated hormones with a lower detection limit) revealed similar dissipation kinetics and pathways, but did not measure mineralization (Colucci and Topp, 2002).

In contrast, mineralization of testosterone in soils is much higher than the estrogens, with 50% of  $^{14}$ C-testosterone applied to agricultural soils recovered as  $^{14}$ CO<sub>2</sub> in 120 hours (Lorenzen et al., 2005). In addition, amending soils with biosolids and swine manure enhanced the transformation of testosterone and estradiol to less bioactive metabolites, and soil microorganisms were required to mineralize the hormones in the organic amendments (Jacobsen

et al., 2005). The amendments increased the conversion of estradiol to <sup>14</sup>CO<sub>2</sub>, yet transiently inhibited testosterone mineralization, either by slowing microbial activity or reducing bioavailability by increasing sorption.

#### **Fates of Hormones in Poultry Litter**

Because the surface runoff and sorption data for natural steroidal hormones in the environment are somewhat contradictory, the particular fates and behaviors of hormones in poultry litter are not well-understood. The problems with hormone leaching after litter is landspread may be exacerbated as the supply of the material exceeds the availability of agricultural land for application and agronomic rates of landspreading are replaced with disposal rates. Because poultry production tends to be focused in certain regions and transportation of the litter is usually limited to 10 to 20 km, land application may be excessive. For example, as litter application rates are based on N levels, the low N:P ratio of litter (3:1) compared to many crops (8:1) has lead to an excess of P in soils that regularly receive manure (Moore et al., 1995).

Fermentation of poultry litter can affect hormone concentrations, likely due to its effects on the microbial community (Shore et al., 1993). Fermentation at an ambient pH (7) at 30 °C caused estrogen concentrations in male and female broiler litter to decrease, but this effect was not observed when antibiotics were added or litter was incubated at extreme pHs (e.g., pH 1), suggesting that the estrogen decrease was microbially mediated. In contrast, testosterone increased linearly with the length of incubation, and the addition of antibiotics and incubation under anaerobic conditions caused testosterone concentrations in male broiler litter to increase significantly, which was attributed to drastic changes to the microflora. Increases in free testosterone were attributed to the degradation of sulfated or glucuronidated conjugates in the litter. Other studies monitoring the biological effects of different litter processing methods (e.g.,

drying, processing by house fly larvae) have observed similar reductions in estrogenic activity and increases in androgenic activity with short-term aging of the litter, which may have been due to production of testosterone from progesterone by microorganisms (e.g., Calvert et al., 1978). Silaging conditions, the type of processing, and the time of year did not significantly affect testosterone concentrations in poultry litter (Shore and Shemesh, 1993).

Composting reduces hormone concentrations in poultry manure (Hakk et al., 2001). Thermophilic (>40 °C) and mesophilic composting of poultry litter for almost 3 months slowly reduced estradiol concentrations by 56% (from 95 ng g<sup>-1</sup> to 42 ng g<sup>-1</sup>), and reduced testosterone by 87% (from 196 ng g<sup>-1</sup> to 26 ng g<sup>-1</sup>), at a rate three times higher than estradiol. Adding clay to the compost did not increase decomposition rates significantly. Thus, composting reduced, but did not eliminate estradiol and testosterone from poultry litter. The composting process has the added benefit of decomposing pathogens, weed seeds, odors, and dead birds, but it is time consuming and costly, and may not provide more nutrients than fresh litter (Moore et al., 1995).

#### Summary

Chickens excrete high concentrations of hormones; however, we lack important information on the fates of these compounds between their accumulation on the poultry house floor and their appearance in runoff from fields fertilized with litter. While it is standard practice to remove caked litter from the poultry house floor between flocks, the remaining litter can be left in place for one to two years before the house is completely cleaned out (Ritz et al., 2005). It is not clear what happens to hormones that accumulate in this litter that is neither composted, nor fermented. Therefore, this study was conducted to determine the mineralization of estradiol, estrone, and testosterone in fresh breeder and broiler litters at moistures and temperatures typical of the poultry house floor.



Fig. 1.1. Chemical structures of  $[4-^{14}C]$ -labeled estradiol (A), estrone (B), and testosterone (C).
Table 1.1. Concentrations of steroidal hormones in poultry litter. Values are reported by Shore et al. (1993) and <sup>†</sup>Lorenzen et al. (2004). "Estrogen" is likely a combination of both estradiol and estrone (Shore et al., 1993).

Type of litter	"Estrogen"	Estradiol <sup>†</sup>	Testosterone		
	µg kg <sup>-1</sup> dry weight				
Breeder	533	70	254, 23 to $30^{\dagger}$		
Broiler (female)	65	NA	133		
Broiler (male)	14	NA	133		
Broiler (male and female)	NA	55	$30^{\dagger}$		
Rooster	93	NA	670		

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# **CHAPTER III**

# MINERALIZATION OF HORMONES IN POULTRY LITTERS AT DIFFERENT WATER POTENTIALS AND TEMPERATURES<sup>1</sup>

<sup>&</sup>lt;sup>1</sup> Hemmings, S. N. J., and P. G. Hartel. To be submitted to the *Journal of Environmental Quality*.

#### ABSTRACT

When poultry litter is landspread, steroidal hormones present in the litter may reach environmental waters, where they may have undesirable biological effects. In a laboratory study, we determined the mineralization of  $[4-^{14}C]$ -labeled 17ß-estradiol, estrone, and testosterone in breeder and broiler litter at three different water potentials (-56, -24, and -12 MPa) and three different temperatures (25, 35, and 45°C). Mineralization was similar in both litters and increased with increasing moisture and decreasing temperature. After 23 weeks at -24 MPa, an average of 27, 11, and <2% of the radiolabeled testosterone applied to breeder litter was mineralized to  $^{14}CO_2$  at 25, 35, and 45°C, respectively. In contrast, mineralization of the radiolabeled estradiol and estrone was <2% after 25 weeks at both water potentials, except after 17 weeks at 25°C and -12 MPa, where up to 5.9% of the estradiol and 7.8% of the estrone was mineralized. The limited mineralization suggests that the litters may still be potential sources of hormones to environmental waters.

#### **INTRODUCTION**

Poultry litters contain appreciable amounts of the hormones 17ß-estradiol (henceforth, estradiol) and testosterone (Shore et al., 1993; Lorenzen et al., 2004). When the litters are landspread, they can contribute significant amounts of these hormones to environmental waters via runoff, depending on application rate and onset of rainfall (Finlay–Moore et al., 2000), amendment with alum (Nichols et al., 1997), and the length of the buffer strip around the amended grassland (Nichols et al., 1998). Although testosterone can move through soil (Casey et al., 2004), estradiol may (e.g., Peterson et al., 2000) or may not (Casey et al., 2003) move in a similar fashion. Study of these hormones is important because they are responsible for the development of secondary sex characteristics in humans and other vertebrates (e.g., Tapiero et

al., 2002), and they, as well as their metabolites (e.g., estrone, the main bioactive metabolite of estradiol), can have undesirable biological effects in environmental waters (e.g., Lai et al., 2002; Seki et al., 2004). These hormones are also important because of the sheer volume of poultry litter that is applied to land. For example, assuming a litter production rate of 1.46 kg per broiler (Perkins et al., 1964), Georgia's 1.3 billion broilers (Georgia Agricultural Statistics Service, 2005) generated almost 1.9 million Mg of litter in 2004. Most poultry litter is landspread as part of good agronomic practice (Moore et al., 1995).

Concentrations of hormones in poultry litters vary depending on the sex and use of the bird. Broiler litter contains 14 and 65  $\mu$ g of "estrogen" (likely both estradiol and estrone) kg<sup>-1</sup> of litter from male and female broilers, respectively, and 133  $\mu$ g of testosterone kg<sup>-1</sup> of litter from both sexes (Shore et al., 1993). Breeder litter contains almost nine times more estrogen (533  $\mu$ g kg<sup>-1</sup> of litter) and almost twice as much testosterone (254  $\mu$ g kg<sup>-1</sup> of litter) compared to litter from female broilers (Shore et al., 1993). However, lower concentrations of these two hormones have also been observed. Lorenzen et al. (2004) determined that broiler litter contains approximately 55  $\mu$ g of estradiol and 30  $\mu$ g of testosterone kg<sup>-1</sup> from both sexes, and breeder litter contains approximately 70  $\mu$ g of estradiol and 23 to 30  $\mu$ g of testosterone kg<sup>-1</sup> of litter.

The degradation of estradiol and testosterone varies in different media. Aerobic composting of poultry litter reduced concentrations of estradiol (half-life of 69 d) and testosterone (half-life of 46 d; Hakk et al., 2005), whereas fermenting poultry litter reduced estradiol concentrations but increased testosterone concentrations (Shore et al., 1993). In agricultural soils, estradiol is rapidly converted to estrone, and both are slowly mineralized (12 to 17% is recovered as  $^{14}CO_2$ ) after 3 months (Colucci et al., 2001). In contrast, half of  $^{14}C$ -testosterone is recovered as  $^{14}CO_2$  after 120 hours (Lorenzen et al., 2005).

Although it is standard practice to remove caked poultry litter from the poultry house floor between flocks, the remaining litter can be left in place for one to two years before the house is completely cleaned out (Ritz et al., 2005). Mineralization of estradiol, estrone, and testosterone under these conditions has not been described. Therefore, we conducted a study to determine mineralization of these three hormones in fresh breeder and broiler litters. Mineralization (as CO<sub>2</sub>) was determined with [4–<sup>14</sup>C]-labeled hormones. Because poultry litters vary in water potentials and temperatures to which they are exposed, the studies were conducted under three different water potentials (-56, -24, and -12 MPa) and temperatures (25, 35, 45 °C). Fresh broiler litter with and without alum was also tested because alum is often added to poultry litter to control the pH (to reduce ammonia volatilization) and litter moisture (Ritz et al., 2005), and alum significantly reduces estradiol in runoff (Nichols et al., 1997). Composting litter reduces, but does not eliminate estradiol and testosterone (Hakk et al., 2005), therefore composted litter was also included as a treatment.

# **MATERIALS AND METHODS**

#### **Sample Collection and Processing**

Four samples of litter were obtained from commercial poultry houses located in Gordon and Jackson Counties, Georgia: 1) fresh (<2 weeks after bird removal) breeder litter without alum, 2) fresh broiler litter amended with alum, 3) fresh broiler litter without alum, and 4) composted broiler litter. None of the fresh litters had been stacked. Each sample was homogenized by mixing it in a polyethylene bag, and the litter was passed through a 4-mm sieve. The pH and N (total, NH<sub>4</sub>-N, and NO<sub>3</sub>-N), P, and K content of the litters were determined as described by Peters (2003; Table 3.1). Briefly, the pH was determined in a slurry of litter:distilled water (1:2, v/v). Total N was determined by dry combustion (LECO, St. Joseph, MI), and NH<sub>4</sub>-N and NO<sub>3</sub>-

N were determined by distillation and titration. Phosphorus and K were determined by digesting the litter in hot HNO<sub>3</sub> and analyzing the digest with inductively coupled plasma spectroscopy. The water potential of each litter was determined at 25 °C with a dewpoint potentiameter (Model WP4-T, Decagon, Pullman, WA), and the percentage water content of each litter was determined gravimetrically by drying a portion of the litter at 60 °C for 24 hours.

Breeder litter was divided into three equal portions, and the portions adjusted to water potentials of -56, -24, and -12 MPa (equivalent to 15, 25, or 35% water content, respectively) by either air-drying the litter or adding distilled water. The broiler litter amended with alum was treated in a similar fashion except the -56 MPa water potential was not included because of difficulty in maintaining a constant water potential (the treatment absorbed atmospheric moisture). The broiler litter without alum was brought to -12 MPa with distilled water and was split in two portions, one half amended with 20 g of alum kg<sup>-1</sup> (to simulate a commercial application rate), and the other half left unamended. This addition increased the water potential from -12 to -18 MPa. The composted litter was brought only to a water potential of -12 MPa.

#### **Mineralization Studies**

Mineralization studies were conducted with  $[4-{}^{14}C]$ -labeled estradiol, estrone, and testosterone (all approximately 2.00 GBq mmol<sup>-1</sup>; purities >99%; American Radiolabeled Chemicals, St. Louis, MO). For breeder litter, a 200-g portion of each litter was oven-dried at 60 °C, and was passed through a 2-mm sieve. The dried litter was divided into ninety 1.0-g samples, each contained in a 50-mL beaker. A 40-µL portion of radiolabeled estradiol, estrone, or testosterone was each diluted in 9.0 mL ethanol contained in an ice bath (to minimize ethanol evaporation) such that each stock contained approximately 16.44 kBq mL<sup>-1</sup>. A 300-µL aliquot of the ethanolic stock solution was added separately to each of 30 beakers, and the litter was

allowed to air dry at room temperature for 1 h. The radiolabeled litter was thoroughly mixed into 9.0 g of litter (wet weight) with a glass rod, and deionized water was added to bring each sample to 10.0 g at the appropriate water potential. These hormone amendments are similar to amendments used in other studies (e.g., Jacobsen et al., 2005). Each beaker was placed in a wide-mouth Mason jar with a 20-mL glass scintillation vial containing 2 mL of fresh 1 *N* KOH (to trap  $^{14}CO_2$ ). Each jar was tightly sealed and incubated at 25, 35, or 45 °C. In addition, jars containing unlabeled litter for each treatment (negative control) were added to the experiment. Broiler litter was treated in the same way as breeder litter, but the study was only conducted at 25 and 35 °C. The broiler litter with and without alum, as well as the composted litter, was incubated at 25 °C only.

Except for breeder litter, CO<sub>2</sub> traps were removed for counting and were replaced on Days 2, 4, 8, 15, then approximately weekly thereafter for 13 wk. This length of sampling was selected because it is recommended by the USEPA (1996). In the case of breeder litter, the litter was incubated an additional 10 to 12 weeks because estradiol and estrone mineralization was observed fortuitously. Water potential of the litter was maintained by weighing each 50-mL beaker every 2 wk and adding deionized water as needed. At each sampling, 18 mL of ScintVerse cocktail (Fisher Scientific, Fair Lawn, NJ) was added to each scintillation vial, and the radiolabel measured in a liquid scintillation counter (Model LS 6500, Beckman Coulter, Fullerton, CA). Quenching was corrected with an external standard.

## **Litter Oxidation**

To confirm the presence of radiolabeled hormone in each beaker, two 0.1-g dry weight portions of litter from each beaker (for a total of six samples per treatment) were combusted in a biological oxidizer (Model OX500, R. J. Harvey Instrument Corp., Hillsdale, NJ). Beakers were

removed from the Mason jars at the end of each study, placed in a polyethylene bag, and frozen at -18 °C until the analysis. Carbon dioxide was captured in Carbon-14 cocktail (R. J. Harvey), and the radioactivity measured in the liquid scintillation counter. Blank (unlabeled) 0.1-g portions of litter and standards spiked with known amounts of radiolabeled hormone were also oxidized. Finally, one entire 10-g sample of <sup>14</sup>C-hormone-amended broiler litter was divided in 0.1-g portions and was oxidized to determine the distribution of radiolabel in the litter. The inside of the 50-mL beaker was rinsed three times with 2 mL of ethanol, and the rinsate measured in the liquid scintillation counter to recover any radiolabel on the glassware.

#### **Statistical Methods**

Treatments were arranged as randomized blocks, and there were three replicates for each treatment. All data were analyzed with the PROC MIXED procedure by SAS (Cary, NC).

# **RESULTS**

In breeder litter, mineralization of estradiol, estrone, and testosterone generally increased with decreasing temperature and increasing water potential. Testosterone was consistently mineralized more than estradiol and estrone regardless of temperature or water potential. In the case of testosterone, an average of <2% was mineralized at 45 °C at both -24 and -12 MPa (Fig. 3.1B and 3.1C). At 35 °C, testosterone mineralization increased to 10.5% at -24 MPa and 12.6% at -12 MPa (Fig. 3.1E and 3.1F). At 25 °C, testosterone mineralization was highest, with 27% of the [4–<sup>14</sup>C]-testosterone initially applied to the litter converted to <sup>14</sup>CO<sub>2</sub> at -24 MPa, and 21% mineralized at -12 MPa (Fig. 3.1H and 3.1I). In the case of 35 and 25 °C, the lag phase to begin testosterone mineralization ranged from <1 wk (35 °C and -24 MPa) to 3 wk (25 °C and -24 MPa).

Although the experiment was also conducted at -56 MPa, it was difficult to maintain a constant water potential, and the litter absorbed atmospheric moisture until it reached a water potential of approximately -24 MPa by Week 5, where it remained constant for the duration of the study. In this treatment, no mineralization of testosterone was observed at 25, 35, or 45 °C before the 5 wk point (Fig. 3.1G, 3.1D, and 3.1A); however, after this 5-wk lag phase, testosterone mineralization reached 31, 4, and <2% at 25, 35, and 45 °C, respectively.

Mineralization of estradiol and estrone in breeder litter was minimal. Except for samples at 25 °C and -12 MPa, estradiol and estrone mineralization was <1% across all treatments. At 25 °C and -12 MPa, mineralization of estradiol and estrone began at Week 17, and 5.9% of estradiol and 7.8% of estrone were mineralized by Week 25. To a much lesser extent, the same 17-week delay in mineralization was observed at 35 °C and -12 MPa, where 1.4% of estradiol and 1.5% of estrone were mineralized by Week 25. Wherever mineralization of estrone and estradiol was observed, mineralization of estrone was always greater than that of estradiol.

In broiler litter, mineralization of estradiol, estrone, and testosterone was similar to breeder litter at 13 weeks. At 35 °C, 11% of testosterone was mineralized at -24 MPa, and 7.0% of testosterone was mineralized at -12 MPa (Fig. 3.2A and 3.2B). At 25 °C, testosterone mineralization was highest, with 16% mineralized at -24 MPa, and 15% mineralized at -12 MPa (Fig. 3.2C and 3.2D). Compared to breeder litter at 35 °C, testosterone mineralization was 6.0% higher at -24 MPa and 1.0% higher at -12 MPa; at 25 °C, testosterone mineralization was 7.5% lower at -24 MPa and 3.5% lower at -12 MPa. Less than 1% of estradiol and estrone was mineralized in the four broiler litter treatments after 13 weeks regardless of water potential and temperature. Therefore, differences in mineralization between breeder and broiler litter were small.

In broiler litter amended with alum or left unamended, the percentage and rate of mineralization after 13 weeks was similar between the two treatments for estradiol and estrone (total <1%), but the mineralization of testosterone was 2% higher in broiler litter left unamended (Table 3.2 and Fig. 3.3). In composted litter, <0.1% each of estradiol, estrone, and testosterone were mineralized after 13 weeks.

When the litters were oxidized, the recovery of litter-bound <sup>14</sup>C was greater, as expected, in treatments where mineralization was minimal (Table 3.3). When the total radioisotope recovered (litter bound and  $CO_2$ ) was considered, the average percentage recovery was 75% (ranging from 41 to 106%) in breeder litter, 86% (ranging from 51 to 107%) in broiler litter, 76% (ranging from 60 to 95%) in broiler litter with and without alum, and 75% (ranging from 62 to 97%) in composted litter. In the one 10-g broiler litter sample that was divided in one hundred 0.1-g portions, 22 samples contained <8 Bq, 73 samples contained between 8 and 50 Bq, and only 5 samples contained >50 Bq. If the original 10-g sample contained approximately 5 kBq, then each 0.1 g sample should contain approximately 50 Bq. Therefore, considerable heterogeneity in the distribution of the radiolabel among the 0.1-g samples was observed.

## DISCUSSION

Although decreases in water-soluble estradiol and testosterone have been observed in composted poultry manure (Hakk et al., 2005), our study is the first to describe mineralization of hormones in fresh poultry litters under different temperatures (25, 35, 45 °C) and water potentials (-56, -24, and -12 MPa) that might be expected in a poultry house. In all treatments in fresh breeder and broiler litters, mineralization of estradiol, estrone, and testosterone was limited (<30%), but generally increased with decreasing temperature and increasing water potential. More  $[4-1^4C]$ -testosterone was converted to  $^{14}CO_2$  than estradiol or estrone. The greater

mineralization of testosterone compared to estradiol is similar to what has been observed previously in agricultural soils (testosterone, Lorenzen et al., 2005; estradiol and estrone, Colucci et al., 2001), and in soils amended with biosolids or swine manure slurry (Jacobsen et al., 2005). In addition, the rate of testosterone decline is almost two times faster than estradiol decline in composted poultry manure amended with clay (Hakk et al., 2005), and in municipal biosolids (Layton et al., 2000). Furthermore, as expected, estrone and estradiol mineralization was similar in all of the litters tested. Although studies of estrone mineralization are limited, estradiol and estrone are mineralized at similar rates in municipal biosolids (Layton et al., 2000).

Because water potential, temperature, and pH were similar in the breeder and broiler litters, the most likely explanations for greater testosterone mineralization compared to estradiol are the physiochemical characteristics of the hormones. Estradiol (and estrone) contain an aromatic A ring, which is difficult for microorganisms to mineralize (Hakk et al., 2005). Also, estradiol and estrone have higher sorption coefficients ( $K_d$ ), which indicate that these hormones sorb more strongly to soil and are less bioavailable than testosterone (e.g., Casey et al., 2003, 2004). It is unclear why the rate of testosterone mineralization slowed after several months. One explanation may be that testosterone is converted to 1,4-androstadiene-3,17-dione, a compound with a higher K<sub>d</sub> than testosterone, which like estradiol and estrone, would then be less bioavailable (Jacobsen et al., 2005).

Given the high  $K_d$  values of the hormones, the compounds should be tightly sorbed to the organic matter in poultry litters. Indeed, only 52% of estradiol and 62% of testosterone spiked into composted poultry litter is extractable with water after 2 h (Hakk et al., 2005). Most of the sorbed <sup>14</sup>C was observed when the litters were oxidized. However, in some cases, the percentage of <sup>14</sup>C label recovered was low when the litter was oxidized. The most likely explanation was

the variable distribution of the radiolabel in the litter. In the one 10-g sample that was completely oxidized, considerable heterogeneity existed in the distribution of the radiolabel among the 0.1-g subsamples.

The total amount of mineralization observed in the poultry litters was lower than that observed in soil. In agricultural soils, 50% of applied testosterone was mineralized after 120 hours (Lorenzen et al., 2005) and 10 to 17% of applied estradiol was mineralized after 3 months (Colucci et al., 2001). Here, even under conditions of greatest mineralization (25 °C and -12 MPa), the total amount of testosterone mineralization was <30% after 23 weeks, and the total amounts of estradiol and estrone mineralization were <10% after almost double the time monitored in the soil study. There are three likely reasons for the lower percentage of mineralization in poultry litter compared to soil. First, a water potential of -12 MPa is sufficient to limit much microbial activity (Harris, 1981). Ideally, litter should be kept at a water potential less than -12 MPa to limit the proliferation of pathogens (Ritz et al., 2005). Unsurprisingly, the lag phase to mineralize the testosterone was longer (5 weeks) at 25 °C and -56 MPa than at -24 or -12 MPa, and mineralization began in the -56 MPa treatment once the litter had absorbed enough moisture to reach -24 MPa. Therefore, the results from the -56 MPa treatment were not discarded because the water content changed, but rather they can be considered as replicates of the -24 MPa treatment after 5 wk.

Secondly, mineralization may be lower in poultry litter compared to soil because the litter likely does not have the sufficient microbial activity to augment mineralization. Soil enhanced the mineralization of estradiol and testosterone in biosolids and swine manure (Jacobsen et al., 2005). Thirdly, sorption of estradiol and testosterone increases with substrate organic matter content and surface area (e.g., Lai et al., 2000), and the high organic matter content and

considerably large surface area (see Appendices) of the poultry litter likely reduced bioavailability.

Mineralization in broiler litter was similar to breeder litter, with the highest mineralization rates for testosterone at 25 °C, and <1% of the estrogens mineralized in 13 wk. However, it is unlikely that hormone mineralization will be the same in all types of poultry litter, since the feces in some litters may contain antibiotics or the litter may be stacked (Hakk et al., 2005). For example, when antibiotics were added to broiler litter and the litter fermented, estrogen degradation was inhibited, whereas testosterone concentrations increased (Shore et al., 1993). Amending broiler litter with alum had no effect on the mineralization of estradiol or estrone, but reduced testosterone mineralization by 2% of the total. The most likely reason for the reduction was the increase in water potential from -12 to -18 MPa, which, again, would have decreased microbial activity. Therefore, practices to reduce estradiol in runoff by adding alum (Nichols et al., 1997) may reduce hormone mineralization. In addition, although composting of poultry litter reduces water soluble concentrations of estradiol by 84% and testosterone by 90% (Hakk et al., 2005), <0.1% of the estradiol, estrone, and testosterone was mineralized in our composted litter. The most likely reason was the low pH of the composted litter (pH 5.2), which, again, would have reduced microbial activity (Hartel et al., 2000).

Type of litter	pН	Total N	Р	K	water potential
		%	g kg <sup>-1</sup>		MPa
Breeder without alum	8.5	2.19	11.9	18.7	-24
Broiler with alum	8.3	3.82	16.9	26.8	-22

3.82

ND

4.79

19.3

ND

22.8

26.0

ND

32.0

-14

-18

-20

Table 3.1. Initial physical and chemical characteristics of the litters.

8.6

7.3

5.2

Broiler without alum

Composted

Broiler without alum + alum



Fig. 3.1. Mineralization of  $[4-^{14}C]$ -labeled estradiol (closed triangles), estrone (open squares), and testosterone (closed circles) in breeder litter at different temperatures (25, 35, and 45 °C) and water potentials (-56, -24 and -12 MPa). Standard error is shown where the error bar is larger than the symbol. Litter in the -56 MPa treatment absorbed atmospheric moisture and reached -24 MPa after approximately 5 weeks.



# Water Potential, MPa (water content %)

Fig. 3.2. Mineralization of  $[4-^{14}C]$ -labeled estradiol (closed triangles), estrone (open squares), and testosterone (closed circles) in broiler litter at two different incubation temperatures (25 and 35 °C) and water potentials (-24 and -12 MPa).

Table 3.2. Percent of radiolabeled estradiol, estrone, and testosterone mineralized in broiler litter with and without alum amendment, and in composted litter.

	Litter Type				
Hormone	Broiler with alum	Broiler without alum	Composted		
		%			
Estradiol	$0.06 \pm 0.03$	$0.08 \pm 0.01$	$0.10 \pm 0.01$		
Estrone	$0.15 \pm 0.03$	$0.11 \pm 0.01$	$0.10 \pm 0.02$		
Testosterone	$17.2 \pm 2.1$	$19.1 \pm 2.1$	$0.09 \pm 0.03$		



Fig. 3.3. Mineralization of  $[4-^{14}C]$ -labeled estradiol (closed triangles), estrone (open squares), and testosterone with (closed circles) and without (open circles) 20 g of alum kg<sup>-1</sup> of broiler litter. Because estradiol and estrone mineralization were the same in both litters, only one symbol is used to represent the mineralization of each in both treatments.

Table 3.3. Mean percent of initial <sup>14</sup>C recovered as litter-bound residues and mean total percent of initial <sup>14</sup>C recovered (as litter-bound residues and <sup>14</sup>CO<sub>2</sub>). Values are means of six replicates  $\pm$  standard error.

Type of litter	L	Litter-bound <sup>14</sup> C			Total <sup>14</sup> C Recovered			
		Water Potential (MPa)						
	-56	-24	-12	-56	-24	-12		
Breeder								
25 °C								
Estradiol	$91 \pm 35$	$41 \pm 30$	$71 \pm 28$	$91 \pm 35$	$41 \pm 30$	$78 \pm 28$		
Estrone	$105 \pm 25$	$77 \pm 39$	$73 \pm 11$	$105 \pm 25$	$78 \pm 38$	$81 \pm 10$		
Testosterone	$33 \pm 9.8$	$37 \pm 8.2$	$25 \pm 8.0$	$63 \pm 9.7$	$64 \pm 8.7$	$46 \pm 7.7$		
35 °C								
Estradiol	$71 \pm 38$	$89 \pm 36$	$98 \pm 29$	$72 \pm 38$	$90 \pm 36$	$100 \pm 29$		
Estrone	$63 \pm 23$	$98 \pm 22$	$102 \pm 11$	$64 \pm 23$	$99 \pm 22$	$104 \pm 11$		
Testosterone	$66 \pm 27$	$41 \pm 30$	$52 \pm 15$	$70 \pm 27$	$51 \pm 30$	$65 \pm 15$		
45 °C								
Estradiol	$69\pm42$	$66 \pm 37$	$90 \pm 10$	$69\pm42$	$66 \pm 36$	$91 \pm 10$		
Estrone	$71 \pm 23$	$53 \pm 47$	$72 \pm 28$	$72 \pm 23$	$54 \pm 47$	$73 \pm 28$		
Testosterone	74 ± 15	$49\pm47$	$104 \pm 14$	76 ± 15	$51 \pm 47$	$106 \pm 14$		
Broiler with alu	m							
25 °C								
Estradiol	ND	$98 \pm 23$	$91 \pm 40$	ND	$98 \pm 23$	$91 \pm 40$		
Estrone	ND	$106 \pm 57$	$94 \pm 21$	ND	$107 \pm 21$	$94 \pm 52$		
Testosterone	ND	$67 \pm 32$	35 ± 21	ND	$83 \pm 32$	51 ± 23		

	L	Litter-bound <sup>14</sup> C Total <sup>14</sup> C Re			otal <sup>14</sup> C Recov	ered		
		Water Potential (MPa)						
Type of litter	-56	-24	-12	-56	-24	-12		
				%				
Broiler with alum	(Cont'd.)	)						
35 °C								
Estradiol	ND	$82 \pm 32$	$87\pm40$	ND	$82 \pm 32$	$87\pm40$		
Estrone	ND	90 ± 13	$105\pm18$	ND	$90 \pm 13$	$105\pm18$		
Testosterone	ND	$52 \pm 21$	$76 \pm 4.7$	ND	$63 \pm 20$	84 ± 5.5		
Broiler without al	um							
25 °C								
Estradiol	ND	ND	$94 \pm 35$	ND	ND	$94 \pm 35$		
Estrone	ND	ND	$95 \pm 28$	ND	ND	$95 \pm 28$		
Testosterone	ND	ND	$39 \pm 15$	ND	ND	$63 \pm 15$		
Broiler without al	um ameno	ded with alum	1					
25 °C								
Estradiol	ND	ND	$60 \pm 25$	ND	ND	$60 \pm 25$		
Estrone	ND	ND	$76 \pm 43$	ND	ND	$76 \pm 43$		
Testosterone	ND	ND	$45 \pm 25$	ND	ND	$68 \pm 25$		
Composted								
25 °C								
Estradiol	ND	ND	$97 \pm 32$	ND	ND	$97 \pm 32$		
Estrone	ND	ND	$62 \pm 59$	ND	ND	$62 \pm 59$		
Testosterone	ND	ND	$65 \pm 56$	ND	ND	$65 \pm 56$		

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# **CHAPTER IV**

# CONCLUSIONS

This study is the first to describe mineralization of estradiol, estrone, and testosterone in poultry litter under conditions likely to be encountered in a poultry house. Depending on the temperature and water potential conditions, the compounds were either not mineralized, or mineralized to a limited extent. Under optimal conditions, 5.9% of the estradiol and 7.8% of the estrone were mineralized after 25 weeks, and 27% of the testosterone was mineralized after 23 weeks. Adding alum to the litter had little effect on the total amounts of estradiol and estrone mineralized, but reduced the total amount of testosterone mineralization by 2%. The limited mineralization suggests that the litters may still be a potential source of hormones to the environment, where they may have undesirable biological effects.

This research partially fills the gap concerning what occurs between excretion of hormones in the poultry house and their appearance in soil and runoff after poultry litter is landspread. Future studies are needed to monitor the mineralization of these hormones once the litter is applied to soils. Furthermore, in addition to estrone, studies should evaluate the bioavailability and hormonal activity of any metabolites that may form when the parent hormones are partially degraded in poultry litter, but still retain their steroidal structure (i.e. the A ring is not mineralized). In this manner, the ecological risks associated with landspreading poultry litter can be more accurately characterized. Finally, this information will inform the development of engineering- and management-related solutions to reduce the appearance and mobility of these hormones in runoff, or to increase their degradation rates prior to or after landspreading litter.

# APPENDIX. SCANNING ELECTRON MICROSCOPE IMAGES OF SELECTED POULTRY LITTERS

Scanning electron microscope (SEM) images of fresh breeder litter (Fig. A1), fresh broiler litter with alum (Fig. A2), fresh broiler litter without alum (Fig. A3), and composted broiler litter (Fig. A4) were taken at progressively higher magnifications, as shown by the scale bars (mm or µm) on the individual images. Small, representative portions of each litter were vacuum freezedried and fixed to the SEM inspection stubs with a graphite-based adhesive, then rendered conductive by gold sputter coating using an ISI PS-2 unit. The SEM (Model 515, Philips) was equipped with a Link exL energy dispersive X-ray analysis (EDXA) system. Photos are courtesy of Dr. Ray Hemmings, CChem., and Mr. Bruce Cornelius, P.Eng., of Hemmings & Associates, LLC, Atlanta, GA.



Fig. A1. SEM images of fresh breeder litter.


Fig. A2. SEM images of fresh broiler litter with alum.



Fig. A3. SEM images of fresh broiler litter without alum.



Fig. A4. SEM images of composted broiler litter.