Some short-term effects of coumestrol on egg production, hatchability, and reproductive system morphology in chickens

by

Ann Leslie Schwink

A Thesis Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

MASTER OF SCIENCE

Department: Veterinary Pathology
Major: Veterinary Pathology
(Veterinary Toxicology)

Iowa State University
Ames, Iowa

1980

TABLE OF CONTENTS

INTRODUCTION

Natural estrogens in plants have been the subject of concern since their connection with infertility in grazing sheep was first demonstrated by Bennetts et al. (1946). Ewes which had grazed estrogenic subterranean clover for prolonged periods developed "clover disease" and became permanently infertile. The infertility was thought to be due primarily to abnormal cervical secretions, which impaired the establishment of spermatozoa in the cervix after mating. In some cases, the grazing of estrogenic pastures was also felt to be associated with dystocia and uterine prolapse in ewes.

Further investigation resulted in the discovery of three general classes of estrogenic compounds, isoflavones, resorcyclic acid lactones, and coumestans, occurring naturally not only in subterranean clover, but also in alfalfa, Ladino clover, soybeans, carrots, cabbage, peas, hops, wheat bran, wheat germ, rice polish, vegetable oils, and pomegranate seeds (Pieterse and Andrews, 1956). Of the various compounds produced by these plants, coumestrol (3,9-dihydroxy-6H-benzofuro [3,2-c] [1] benzopyran-6-one) was found to have the highest estrogenic activity in animals, as measured by bioassay methods (Bickhoff et al., 1958), comparing ability to cause increased uterine weight in mice or ewes. Coumestrol was reported to be approximately 1/3 as potent as diethylstilbestrol, and 7 times as potent as estrone (Bickhoff et al., 1958).

Coumestrol has been found in most alfalfa samples, frequently at levels of 3-5 ppm, but occasionally as high as 350 ppm (Pieterse and

Andrews, 1956). Since the inception of the use of diethylstilbestrol as a growth promotant, researchers have looked for other estrogenic compounds with similar growth-promotant activity. Many studies in the middle and late 1960s produced conflicting evidence about the growth promotant and feed efficiency-increasing activities of coumestrol, but the general conclusion was that this action was insignificant (Adler and Trainin, 1961; Elam, 1961; Fox and Oldfield, 1962; Matsushima, 1961; Matsushima et al., 1962, Oldfield, 1961; Oldfield and Fox, 1962, 1963; Oldfield et al., 1960; Oldfield et al., 1966; Story et al., 1957).

Dehydrated alfalfa meal is incorporated in most poultry rations used in the United States at a level of 2%. The meal is used mainly to improve skin color and egg yolk color, rather than as a major protein source because higher levels have been found to decrease palatability of the ration.[1] Coumestrol has been found to decrease egg production and delay the onset of sexual maturation in hens exposed continuously from their hatching date (Adams, Casida, and McGibbon, 1951). However, in most commercial poultry operations, rations are formulated on a least-cost basis. Feed is mixed in batches, 1-3 weeks before being used. Therefore, the source of feed ingredients varies from batch to batch, and any two batches may not be expected to contain the same levels of coumestrol, since the coumestrol content in alfalfa varies with variety, climatic conditions, and senescence of the plant (Loper, 1968b; Loper and Hanson, 1964).

---

[1]Dr. Jerry Sell, personal communication, September 1979. Department of Animal Science, Iowa State University, Ames, IA.

The purpose of this study was to investigate the effects of short-term coumestrol exposure in laying hens on egg production and hatchability. Histologic sections of the reproductive tracts and livers were also examined, in the hope of elucidating an anatomic correlation with the physiologic or functional changes noted.

REVIEW OF LITERATURE

Coumestrol in Plants

Coumestrol is the dominant estrogen in alfalfa (Frances and Millington, 1965). It has also been detected in several species of clover and many plant products consumed by man (Pieterse and Andrews, 1956). The coumestrol content of plants appears to be influenced by varietal and genetic differences and the conditions and stage of growth. Another factor that influences the synthesis of coumestrol in plants is the invasion of insects or foliar disease.

Bickhoff et al. (1961) demonstrated that infection with Pseudopeziza medicaginis, the common alfalfa leafspot organism, resulted in a sharp increase in coumestrol content in two major varieties of alfalfa. They also demonstrated a 4-5 fold increase in the levels of coumestrol, when the plants were sampled at 14 days after infection and compared with plants sampled 7 days after infection. Coumestrol content was roughly related to infection score, and indicated that coumestrol content depended on degree of disease development. Accumulation of coumestrol occurred in the immediate vicinity of the lesion caused by the common leafspot organism. The biosynthetic pathway of disease-induced coumestrol production was unknown. The highest concentrations of coumestrol were found in varieties most susceptible to infection by foliar pathogens. Loper (1968b) found levels as high as 2362 ppm in the leaves of mature medic plants exhibiting severe physiogenic leaf spotting.

Pieterse and Andrews (1956) found that the estrogenic activity of

alfalfa increased with maturation, although this varied with the cutting. Bickhoff et al. (1958, 1960) found that estrogenic activity of alfalfa increased with maturation, and that it usually reached the highest level at the full bloom stage. However, when alfalfa was allowed to mature to the seed pod stage, dehydrated alfalfa meals made from these plants contained 340-560 ppm coumestrol. The USDA (1969) reported that the usual levels of coumestrol in commercially available alfalfa meals was less than 100 ppm. Frances and Millington (1965) found that while in green plants, coumestrol was concentrated in the leaves, in the dried standing plants, it was concentrated primarily in the stems and seed pods.

Loper and Hanson (1964) reported that under controlled environmental conditions, similar strains of alfalfa did not contain different levels of coumestrol when grown under different temperature regimes. Climates which produced stress were associated with an increase in coumestrol content. This increase was probably a result of a reduction of plant resistance to disease.

Loper (1968a) found that infestation by aphids caused the buildup of coumestrol in the aphid-damaged portions of the leaves. The more aphid-resistant varieties accumulated less coumestrol than did the susceptible varieties.

Bickhoff and Booth (1960) compared coumestrol with Captan[1] as a germination inhibitor and with PCNB[2] and Dixon[3] as a mycelial growth

---

[1] Captan^{R}, Chevron Chemical Co., San Francisco, CA.

[2] PCNB^{R}, Olin Corp., Little Rock, AR.

[3] Dixon^{R}, Chevron Chemical Co., San Francisco, CA.

inhibitors for four species of fungi. Although the reference compounds were active at 10 ppm, coumestrol was inactive even at 1000 ppm. The USDA (1969) also reported two other studies of the fungitoxic activity of coumestrol in which no effect could be found. The reason for accumulation of coumestrol in response to disease was not known.

Coumestrol in Mammals

Trace amounts of coumestrol have been detected in the depot fat and plasma of ewes grazing estrogenic clover (Lindner, 1967), and in cows fed a ration containing estrogenic alfalfa meal (Adler et al., 1970). Lindner (1967) published a chromatographic method for isolation of coumestrol in microgram quantities from fat or plasma.

In mammals, coumestrol is metabolized primarily into simple acids and phenols (Lindner, 1967). These metabolites have not been specifically identified, but Adler (1961) was unable to find any unchanged coumestrol in the urine of mice fed the compound. However, Cayen and Common (1965) injected hens intramuscularly with tritiated coumestrol and recovered both radiolabelled phenolic products and intact coumestrol in the urine.

Most of the work comparing the relative estrogenic potencies of phytoestrogenic compounds has been done by measuring the differences in weights of uteri of ovariectomized mice fed the compounds being examined for a specific time period. Hoelscher (1979) published a table comparing the relative estrogenic potencies of several phytoestrogens with diethylstilbestrol. He assigned diethylstilbestrol an arbitrary value of 100.0, and relative to this, found coumestrol to have an estrogenic potency in

mice of 35, while for purposes of comparison, estrone was rated at 6.9. The other common phytoestrogens were all rated 1.0 or less.

The clinical effects of estrogenic pastures were first noticed in sheep in the middle 1940s. Lambing rates in certain areas of Western Australia were consistently below the national average, due to low numbers of twins, and the failure of many ewes to produce lambs yearly. Adams (1975) found that statistical evidence suggested that lamb marking rates were decreased in pasture areas characterized by subterranean cultivars of clover, such as the Dwalganup and Yarloop varieties. Bennetts, Underwood, and Shier (1946) reported that severe infertility was also associated with ewes on these pastures. Schinkel (1948) found that the infertility frequently became permanent.

Adams (1977) characterized the infertility associated with "clover disease" as permanent or temporary. Temporary infertility was seen in animals ingesting phytoestrogens during the mating period, and was associated with changes in the reproductive tract, primarily a chemical change in the character of the cervical mucus, and a metaplasia of the glands of the cervix to a more uterine appearance. The permanent type of infertility was associated with a degranulated and hyperactive histologic appearance of the gamma basophils in the pituitary gland, an increase in neurophysin storage in the hypothalamus, some shrunken, hyperchromatic neurons in the hypothalamus, and an increase in adrenal and thyroid gland weights. These changes could be observed within three weeks after exposure.

In another study, Adams (1979) found a mean increase of 3-4 mm in the teat length of wethers grazing estrogenic pastures for 10 days. He

also described masculinization of the external genitalia of ewes exposed to phytoestrogens over a prolonged period of time. The changes were partial fusion of the labia of the vulva and hypertrophy of the clitoris. These changes were found to be permanent, and to correlate with the amount of uterine metaplasia in the cervix.

Story et al. (1957) reported that extracts of estrogenic forages stimulated growth in lambs. With further work, he was unable to substantiate this finding, however. Oldfield (1961), Oldfield and Fox (1962, 1963), and Oldfield et al. (1960, 1966) conducted a series of feeding trials with lambs to investigate the possible growth promoting effects of coumestrol. While organoleptic tests consistently demonstrated improved tenderness and juiciness scores for lamb roasts from animals on high coumestrol diets, no growth-promotant effect could be demonstrated.

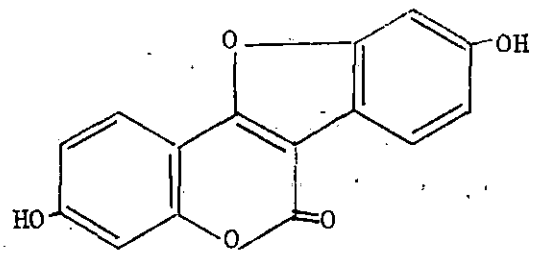
In beef cattle, Matsushima (1961) and Matsushima et al. (1962) reported no significant differences in gain or feed efficiency among steers fed levels of coumestrol from 0-250 ppm. Elam (1961) found no significant difference in digestibility and nitrogen utilization in steers fed levels of coumestrol from 0-209 ppm.

Adler and Trainin (1960a, 1960b, 1961) reported a hyperestrogenic syndrome in dairy cattle characterized by irregular estrus cycles, cystic ovaries, and low fertility, as well as precocious mammary and genital development in heifer calves. It was found that the onset of these signs coincided with the availability of fresh alfalfa. After alfalfa was excluded from the ration of the herd, in two separate cases, there were subsequent increases in conception rates and a decrease in frequency of

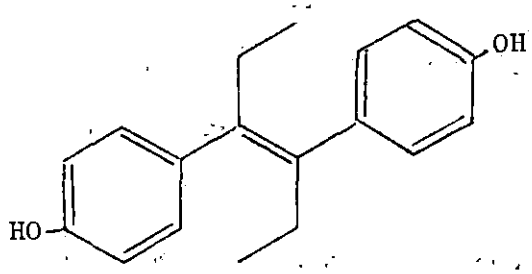
cystic ovaries. Ochi et al. (1964) found that cows fed Ladino clover hay seemed to have an earlier postparturient estrus, and a slightly higher incidence of reproductive disorders, as well as a lower conception rate than cows not fed the clover hay. Similar findings were reported by Foltin (1959).

Chemical Characteristics of Coumestrol

Coumestrol was first synthesized in the laboratory by Emerson and Bickhoff (1958). Its structure was found to be strikingly similar to diethylstilbestrol.



coumestrol



diethylstilbestrol

Figure 1. Structural formulas of coumestrol and diethylstilbestrol

A rapid quantitative paper chromatographic procedure for detecting small amounts of coumestrol in plant materials has been described by Livingston et al. (1961). In addition, a method for alfalfa leaf protein concentrates, which contain high levels of chlorophyll, has been described by Knuckles, Miller, and Bickhoff (1975). The major change was

the employment of a chloroform extraction of basic aqueous solution (pH = 10) to remove the chlorophyll. (Greater than 99% of the coumestrol was in the aqueous phase.) This method proved to be superior to phasic distribution of the extract with hexane for removal of chlorophyll pigments. Sensitivity was approximately 2 ppm with a maximum error of 5%.

Lookhart, Jones, and Finney (1978) have published a quantitative procedure for coumestrol using high performance liquid chromatography. They also reported a thin layer chromatographic method which gave a sensitivity limit of 10 ppm within 25 minutes, or a sensitivity limit of 50 ppm within 2 minutes.

A sensitive quantitative paper chromatographic procedure, using a fluorometer, was developed by Bailey (1960), which allowed detection of quantities of coumestrol in the range of 0.2-1.0 micrograms, with a standard deviation of 7%.

Ultraviolet absorption data for coumestrol have been characterized by Emerson and Bickhoff (1958). High intensity absorption is generally seen in the 340-355 millimicron region, in the 230-250 millimicron region, and in the 200-215 millimicron region, along with low intensity absorption in the 300-320 millimicron region.

Barnes and Occolowitz (1964) reported the mass spectral measurements of a number of coumestan compounds. The characteristic fragmentation under electron impact is the ready loss of CO from the pyrone ring to form an ion having the benzofuran structure, followed by a further loss of the remaining oxygen atoms, again as CO. Coumestrol in particular had a molecular ion that was so stable that the loss of CO was the only

significant fragmentation.

Coumestrol in Chickens

Alfalfa meal was at one time considered for inclusion as a major proportion of poultry diets, since it provided from 30-40% as much total digestible nutrients per unit of weight as the common cereal grains, and was normally available in large quantities at prices often below that of grains and other accepted ingredients. Cooney, Butts, and Bacon (1948) conducted a study of growing broiler chicks, feeding diets containing 0%, 5%, 10%, 15%, 20%, and 25% alfalfa meal. They found that growth was equal to, but not better than, the control in the group fed only 5% alfalfa meal. As the percentage of alfalfa meal in the diet increased, there was an associated depression of growth. Although previous reports had not always supported this data, they felt that the wide variation in alfalfa meals in regard to harvesting, varieties, processing, and storage, might account for conflicting results.

Lepkovsky et al. (1950) repeated the experiment of Cooney, Butts, and Bacon, and obtained similar results. In addition, they found that sulfuring and blanching, storage of alfalfa meal at different temperatures, autoclaving at various pHs, or adding B complex vitamins to the ration, did not alter the growth-depressing effect. However, the growth-depressant effect could be removed by repeated extraction with hot water, and when the extract was fed to chicks, the depression of growth was replicated.

Heywang (1950) reported that alfalfa meals varied considerably in

their growth-depressing effect. He also replicated the growth-depressant effect in broiler chicks. In laying pullets, he did not find any depression of feed consumption, but did find a significant depression of egg production. Egg production decreased proportionally as the level of alfalfa meal in the diet was increased. Hatchability of eggs from pullets on the alfalfa meal containing diet was also depressed.

Thayer, Jaap, and Penquite (1945) examined the possibilities of fattening chickens by feeding estrogens, diethylstilbestrol or dianisylhexene. The rationale was that normally, when a pullet began to lay, body fat deposition and lipemia increased in correlation with increased levels of estrogens. They found an increase in fat grade only in males, probably because of suppression of maleness, but superior carcass quality when distribution of fat and skin quality were considered. They also found decreased feed consumption, without decreased gain, in pullets receiving oral estrogen treatment. When overdosed with estrogen, the birds developed osteoporosis and paralysis, associated with marked hypercalcemia.

Adams, McGibbon, and Casida (1950) reported on the effects of orally administered estrogen (dianisylhexene) in laying single-comb White Leghorn pullets. These scientists found that ovulation was severely depressed by estrogen when it was incorporated in the diet at .04%. They did not find a significant change in feed consumption after adjustment for differences in ovulation rate. They found no apparent effects on body weight. More recently, Mohsin and Pal (1975) compared the effects of coumestrol and diethylstilbestrol in the feed of chicks from 0-60 days of age.

Coumestrol was supplied via lucerne (Medicago sativa L., closely related to alfalfa) meal at levels of 2.8 ppm and 10.66 ppm. Mohsin and Pal found no significant difference in weekly body weights, however, the consumption of feed was significantly lower in the group of birds receiving the higher level of coumestrol. Similar results were obtained with diethylstilbestrol. They concluded that incorporation of these estrogenic compounds in the feed resulted in improved feed efficiency in young chicks, without affecting growth.

Continuing their investigations, Mohsin and Pal (1977) studied coumestrol in lucerne as a factor in egg production and quality in White Leghorn pullets. Birds receiving coumestrol were provided with 15% lucerne meal in their diets, which supplied 2.8 and 10.6 ppm in their feeds respectively. Sexual maturity was delayed by 21 days, egg production was lowered by 7 eggs during a period of 100 days, and the eggs were found to be lighter in weight by approximately 2 grams in the high coumestrol treated birds in comparison to control birds. This is interesting in light of the report discussed earlier by Heywang, of lowered egg production by birds kept on sun-cured alfalfa meal at 10% of the diet. Mohsin and Pal also found in this experiment, that the absolute values of white weight and values in percent egg weight suggested an increase in the white of egg from high coumestrol-treated birds when compared with nontreated. The specific gravity and shell weight, as well as shell thickness were significantly reduced in coumestrol-treated birds.

In light of the evidence available, coumestrol must be considered to be of probable economic significance to poultry producers.

MATERIALS AND METHODS

Experimental Animals

Experiment 1

One hundred-sixty fertilized single-comb White Leghorn eggs were collected from the same day's lay. These eggs were randomly assigned to treatment groups, inoculated, and incubated in a bulk incubator. Incubation was at a temperature of 100 F and 88% relative humidity for the first 3 days, then the humidity was decreased to 86%. At 14 days, the temperature was decreased to 99 F. One day before hatching, the humidity was increased to 88%.

Experiment 2

Eighty single-comb White Leghorn pullets 24 weeks of age were caged individually and placed in a laying house. Birds were randomly assigned to cages, with the restriction that since there were two rows of cages, one facing a wall, and one facing other birds, half of the birds of each experimental group were placed in each row. Eggs were collected and birds observed until the birds were well-established in the housing, and laying had stabilized for a period of 5 days. At this time, all pullets were inseminated, using a commingled semen sample from approximately twenty single-comb White Leghorn roosters. Pullets were inseminated a second time that week, and once weekly thereafter, with collection of eggs for incubation beginning one week after the first insemination.

Experimental Ration

During the acclimation period and the entire experimental period, the hens were fed from a single premixed batch of a proven basal ration for layers.[1] Because the ration contained soybean meal, it was analyzed by thin layer chromatography and found to contain less than 0.5 ppm coumestrol. Since this level was low in comparison to the levels being used for treatment, and the same ration was fed to all birds, one community feeder per 4 cages, it was decided that this ration was acceptable for the trial.

Table 1. Layer ration formula

Ingredient	Amount
ground corn	335.25 lb.
soybean meal (approx. 48% protein)	100.00 lb.
animal fat	10.00 lb.
meat and bone meal	10.00 lb.
dicalcium phosphate	7.50 lb.
layer vitamin premix	2.50 lb.
layer mineral premix	1.50 lb.
d-l methionine	0.25 lb.
ground limestone	33.00 lb.
TOTAL	500.00 lb.

[1] Dr. Jerry Sell, personal communication, October 1979. Department of Animal Science, Iowa State University, Ames, IA.

Table 2. Layer vitamin premix formula

Ingredient	Amount
vitamin A (30,000 IU/g.)	800 g.
vitamin D_3 (3,000 IU/g.)	2,400 g.
vitamin B_{12} (60 mg./lb.)	114 g.
riboflavin (20 g./lb.)	450 g.
pantothenate (160 g./lb.)	56 g.
niacin	56 g.
choline chloride (50%)	3,520 g.
Santoquin[a] (66.6% pure)	330 g.
soy oil	300 g.
wheat middlings	6,964 g.
TOTAL	15,000 g.

[a]Santoquin[R] (ethoxyquin), Monsanto Chemical Corp., St. Louis, MO.

Table 3. Layer mineral premix formula

Ingredient	Amount
manganese sulfate·HOH (32.5% Mn)	20.5 g.
iodized salt	9,979.5 g.
TOTAL	10,000.0 g.

Experimental Coumestrol

Crystalline coumestrol was obtained from a commercial source.[1] By comparison with analytical standard coumestrol, which was approximately 99% pure,[2] the coumestrol used for the experiment was 50% pure compound.

Coumestrol was administered in clear #5 gelatin capsules.[3]

Coumestrol was weighed into the capsules using a microbalance.[4]

Egg Collection

Eggs were collected once daily at 2:00 p.m., labelled, recorded, and incubated immediately. Eggs laid after 2:00 p.m. were collected with the next day's eggs. Whenever possible, soft-shelled or otherwise abnormal eggs were recorded. However, eggs with extremely soft shells or no shells would simply fall through the wire mesh of the cage floors and be lost in the lagoon flush system.

Bird Handling

Throughout the pullets' stay in the laying house, they were fed by the same individual. During the period of treatment, the handler removed each bird individually from its cage and administered an oral gelatin-capsule, beginning at 4:00 p.m., so that each individual was treated at

---

[1] Eastman Chemical Products, Inc., Kingsport, TN.

[2] E. M. Bickhoff, Western Regional Research Laboratory, USDA-ARS, Albany, CA.

[3] Elanco Products Co., Indianapolis, IN.

[4] Cahn electrobalance^{R}, Cahn Div., Ventron Instrument Corp., Paramount, CA.

the same time each day. Treatment of the entire flock took approximately 25 minutes each day.

Necropsy and Collection of Tissues

No hens died during the course of the experiment.

On the appointed day of necropsy, birds were killed by holding the feet in the right hand, the head between the thumb and forefinger of the left hand, and pulling to separate the cervical vertebrae and sever the spinal cord. The limbs were examined for gross abnormalities. The ischiatic nerves were also checked grossly in each bird. The abdominal viscera were also examined.

Tissues for histopathologic examination were collected and immersed in 10% neutral buffered formalin, at a volume approximately 10 times the volume of the tissue sample. Tissues routinely collected were the left lobe of the liver, ovary, and three sections of reproductive tract, including infundibulum, magnum, and uterus. In birds which were sexually immature, ovary, and a small section of the tubular genitalia were collected. A few birds also had cystic abnormalities of the tubular genitalia, and these were also collected for histopathologic examination. Gross abnormalities noted in any other organ systems were also collected.

In addition, the liver tissue not collected for histopathologic examination was individually wrapped and frozen for possible later chemical analysis.

Histologic Procedures[1]

Tissues were fixed in 10% neutral buffered formalin. Processing then involved dehydration by serial passage through 70% alcohol, 95% alcohol, and absolute alcohol. Tissues were passed through xylene to remove the alcohol, and paraffin for infiltration prior to embedding in blocks. After embedding, sections were cut at 6 micron thickness. After sections were placed on slides, they were passed through xylene to remove the paraffin, absolute alcohol to remove the xylene, 95% alcohol, 70% alcohol and distilled water to accomplish rehydration. Sections were then stained with hematoxylin for nuclear detail. Acid alcohol was used to remove hematoxylin from the cytoplasm. Lithium carbonate was used to "blue" the hematoxylin. Slides were rinsed with distilled water, then stained with eosin. Sections were then dehydrated by passing back through 70%, 95%, and absolute alcohol, and xylene to clear the section.

Slides were examined by A. L. Schwink.

Chemical Analysis[2]

The method of chemical analysis used for the liver coumestrol levels determination was developed to overcome the difficulties presented by the fatty livers.

---

[1] K. L. Pierce, personal communication, November 1979. Department of Veterinary Pathology, Iowa State University, Ames, IA.

[2] Dr. H. M. Stahr, personal communication, January 1980. Iowa Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA.

A liver sample of 20 grams was blended with 100 milliliters of acetonitrile (90%, in water) until a slurry was obtained. This slurry was centrifuged to remove the fluid layer. The fluid layer (acetonitrile/water) was extracted four times with 100 milliliters of petroleum ether. KCl in water (5%, in water, 100 milliliters) and chloroform (100 milliliters) were added to the acetonitrile/water layer in a 500 milliliter separatory funnel. The funnel was shaken and the layers allowed to separate. A cloudy chloroform layer resulted because of the suspended aqueous phase. The chloroform layer was passed through anhydrous sodium sulfite to break the emulsion (remove water), and the chloroform layer was reserved. A second chloroform extraction was done in a similar manner, and the 200 milliliter chloroform solution was treated with 0.8 grams Darco^{R} G-60 carbon.[1] The carbon was removed by filtration and the solvent was evaporated to dryness. A control composite liver sample, a composite sample from the high treatment group livers, and an unrelated liver sample were analyzed by this technique.

An aliquot equal to 2 grams of each sample was spotted on Merck[2] silica gel thin layer chromatography plates (0.2 millimeters thick), along with standard coumestrol for comparison. The development solvent was 3 parts ethane, 2 parts ethyl acetal acetate, and 1 part acetone. The plate was observed under long wave ultraviolet light for a coumestrol band. The remaining extract (18 grams) was spotted on a silica gel thin

[1] J. T. Baker Chemical Co., Phillipsburg, NJ.

[2] Merck and Co., Inc., Rahway, NJ.

layer chromatography plate (0.5 millimeters thick),[1] with spike and standard, and developed in a similar manner.

The feed analysis was made the Iowa Veterinary Diagnostic Laboratory method, as reported by Stahr (1975, 1976).

Statistical Evaluation

Experiment 1

Eggs were derived from single-comb White Leghorn hens of the same strain, and inseminated from a single, commingled semen sample from single-comb White Leghorn roosters of the same strain. They were assigned to treatment groups using a table of random numbers from Snedecor and Cochran (1967).

Experiment 2

The pullets were hatched from eggs derived similarly to the eggs used in the first trial, and hatched together. Since they were caged in two rows, one which faced a wall, and one which faced other birds, half of the number of cages in each treatment group was randomly assigned to a position in each row. Each bird was then assigned a number from a table of random numbers from Snedecor and Cochran (1967), and paired with a cage. This resulted in random distribution of the birds within each row, with an equal number of birds from each group on each row. This was hoped to negate any psychological differences which might result from differences due to facing either the wall, or other birds.

---

[1] Dr. H. M. Stahr, personal communication, February 1980. Iowa Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA.

Histologic changes in livers were assigned scores from 1 to 5, and together with egg production and hatchability data, were examined by the techniques of analysis of variance. F-values were determined from computed sums of squares and mean squares for numbers of eggs laid, percent eggs hatched, length and numbers of glands in the magnum and uterus, gross length of the tubular genital tract, and histologic changes in the liver. Statistical significance was assigned to F-values according to Snedecor and Cochran (1967).

Experimental Design

Experiment 1

Commercial coumestrol of varying strengths dissolved in 95% ethanol (in water) were inoculated into the allantoic cavity of eggs. Each egg received 10 microliters of inoculum. The dosages of coumestrol used were 0.003 micrograms, 0.03 micrograms, 0.3 micrograms, and 3.0 micrograms per egg. The solution of 0.3 micrograms/microliter which was to provide the dose of 3.0 micrograms per egg was too viscous to draw into the syringe with ease, and only three eggs were inoculated with this dose. Twenty eggs were inoculated with each of the other dosages. In addition, four control groups of twenty eggs each were treated. The first group was not opened, but merely incubated along with the rest to give an indication of the basal hatchability of the eggs. The shells of eggs in the second group were drilled as if to be inoculated, then sealed with paraffin. The third control group was inoculated with 10 microliters of 95% ethanol. The fourth control group was inoculated with 100 nanograms

per egg of diacetoxyscirpenol, the LD_{50} of this mycotoxin for eggs,[1] to verify that the various inocula being used were in fact being deposited properly.

Table 4. Experimental treatments

Group	Treatment
A	control, no treatment
B	control, drilled and resealed
C	control, 10 microliters of 95% ethanol
D	0.003 micrograms coumestrol
E	0.03 micrograms coumestrol
F	0.3 micrograms coumestrol
G	3.0 micrograms coumestrol
H	control, 100 nanograms of diacetoxyscirpenol

Experiment 2

Eighty laying pullets were assigned at random to four different oral daily doses of coumestrol.

The trial was divided into three time periods. To establish baseline data for hatchability, fertile eggs were collected and incubated

[1]Dr. H. M. Stahr, personal communication, July 1979. Iowa Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA.

from all birds for 10 days before any treatment was begun. For the following 10 days, the hens were treated orally as appropriate for their respective dosage levels. For the following 10 days, treatment and handling ceased, but eggs continued to be collected and incubated for a comparison of carryover effects and recovery. Histologic sections of the livers, ovaries, and tubular genitalia of all birds were examined, coded, and examined for statistically significant differences.

The effects of coumestrol on layability, hatchability, and histology of the liver, ovary, and tubular genital tract were compared between treated birds (groups 2, 3, and 4) and untreated birds (group 1) immediately following treatment, and after a brief period of withdrawal of treatment.

The level of 0.1 mg. per day was chosen to correspond to that which might commonly be found in commercially available feeds. The level of 1.0 mg. per day was chosen to correspond to the level of exposure which might be expected in a bird receiving a high, but reported level of coumestrol in the ration (Bickhoff, 1959). The third level, 10.0 mg. per day, was chosen to show any effect which might be seen at extremely high levels.

Table 5. Experimental treatments

		Time 1 (days 1-10) daily dose coumestrol	Time 2 (days 11-20) daily dose coumestrol	Time 3 (days 21-30) daily dose coumestrol
Group 1	subgroup 1	0.0 mg.	0.0 mg.	0.0 mg.
	subgroup 2	0.0 mg.	0.0 mg.	--
Group 2	subgroup 1	0.0 mg.	0.1 mg.	0.0 mg.
	subgroup 2	0.0 mg.	0.1 mg.	--
Group 3	subgroup 1	0.0 mg.	1.0 mg.	0.0 mg.
	subgroup 2	0.0 mg.	1.0 mg.	--
Group 4	subgroup 1	0.0 mg.	10.0 mg.	0.0 mg.
	subgroup 2	0.0 mg.	10.0 mg.	--

RESULTS

Experiment 1

No significant differences were found in relation to treatment with coumestrol.

Table 6. Experimental results

Group	A	B	C	D	E	F	G	H
number inoculated	20	20	20	20	20	20	3	20
number hatched	15	15	16	14	18	13	2	8

Experiment 2

Egg production

There were no significant differences in egg production between groups within time 1, or within time 2. There was a significant difference in production between groups in time 3, however, examination of the data showed this to be due to 2 individuals in group 2, which never reached sexual maturity.

In all cases, when the individual group was examined over all three time periods, there was a significant ($P < .05$), and in most cases, a highly significant ($P < .01$) increase in egg production with time. However, there could not be demonstrated any group by time interaction.

Table 7. Mean egg production

Day	Group 1	Group 2	Group 3	Group 4
1	0.55	0.55	0.60	0.50
2	0.40	0.75	0.45	0.55
3	0.60	0.55	0.65	0.55
4	0.65	0.75	0.70	0.70
5	0.60	0.75	0.70	0.70
6	0.60	0.75	0.60	0.70
7	0.55	0.75	0.65	0.65
8	0.55	0.50	0.55	0.55
9	0.60	0.80	0.60	0.70
10	0.75	0.75	0.60	0.70
11	0.65	0.75	0.70	0.70
12	0.65	0.70	0.65	0.70
13	0.70	0.80	0.65	0.70
14	0.75	0.80	0.75	0.75
15	0.85	0.60	0.60	0.75
16	0.80	0.80	0.70	0.75
17	0.80	0.85	0.60	0.80
18	0.75	0.75	0.75	0.80
19	0.95	0.75	0.75	0.80
20	0.65	0.75	0.60	0.70
21	0.80	1.00	0.60	0.90
22	0.70	0.80	0.70	0.80
23	0.80	0.60	0.80	0.70
24	0.90	0.90	0.30	0.90
25	0.90	0.90	0.90	0.80
26	0.70	0.80	0.70	0.70
27	0.70	1.00	0.70	1.00
28	0.80	0.60	0.80	0.70
29	0.80	1.00	0.60	0.90
30	0.70	0.70	0.70	0.90

Table 8. Mean egg hatchability

Day	Group 1	Group 2	Group 3	Group 4
1	0.73	0.73	0.91	1.00
2	0.88	0.93	1.00	0.82
3	0.82	0.91	1.00	0.91
4	0.82	0.79	0.83	0.91
5	0.75	0.80	0.71	0.93
6	0.83	0.67	0.58	0.64
7	1.00	0.60	0.93	0.93
8	1.00	1.00	0.91	0.82
9	0.83	0.75	0.92	0.86
10	0.63	0.80	0.92	0.93
11	0.85	0.87	0.86	0.93
12	0.92	0.93	0.77	0.71
13	0.86	0.80	0.80	0.83
14	0.93	0.88	0.80	0.87
15	0.77	0.83	0.83	1.00
16	0.94	0.73	0.85	1.00
17	1.00	0.94	1.00	0.88
18	0.93	1.00	0.87	0.93
19	0.78	0.80	0.80	0.69
20	0.87	0.93	0.83	0.57
21	0.81	1.00	0.73	0.94
22	0.86	1.00	0.86	0.75
23	1.00	1.00	0.75	0.71
24	0.89	0.70	1.00	0.89
25	0.78	0.44	0.89	1.00
26	0.86	0.75	1.00	0.71
27	0.71	0.78	0.86	0.80
28	0.88	0.86	1.00	1.00
29	0.88	0.90	0.83	1.00
30	1.00	0.75	1.00	0.89

Egg hatchability

No significant differences were found in egg hatchability either over time, or between treatment groups. These results are consistent with the results of experiment 1.

Reproductive system morphology

There were no visible differences in the ovaries.

The data obtained from the infundibulae were not amenable to statistical analysis, because the wide variation observed in gland numbers and lengths was probably due to the difficulty of collecting the same anatomic level of tract in each case. However, no lesions were seen, and except for those birds which were physiologically immature, this portion of the tubular genital tract appeared to be normal in all cases.

There were no significant differences in gross length of the tubular genital tracts, either between groups, or between subgroup 1, which was killed and necropsied at the termination of the experiment, on day 31, and subgroup 2, which was killed and necropsied after the last day of treatment, on day 21.

Table 9. Mean gross length of the tubular genital tract

	Group 1	Group 2	Group 3	Group 4
Subgroup 1	48.9 mm.	49.3 mm.	44.9 mm.	53.8 mm.
Subgroup 2	51.9 mm.	52.5 mm.	55.2 mm.	48.1 mm.

There was a highly significant difference ($P < .01$) in the number of glands in the magnum between subgroups, and also a significant difference ($P < .05$) between groups, however, no group by subgroup interaction. On further breakdown of the data, it was found that although there were no significant differences in gland numbers between groups in the birds killed earlier, by 10 days later, when the last birds were killed, there was a significant change in magnum gland numbers, seen primarily as a decrease, in the control group (group 1), and an increase in the low coumestrol dose group (group 2). The decrease in the intermediate dose group (group 3) approached significance. However, these changes did not appear to be dose-related.

Table 10. Mean magnum gland numbers (circumferential section)

	Group 1	Group 2	Group 3	Group 4
Subgroup 1	26.9	22.2	27.3	29.0
Subgroup 2	33.0	33.1	32.7	30.6

No significant differences were found in lengths of glands in the magnum between groups or subgroups. Glands overall were nonsignificantly shorter in the older birds (subgroup 1).

In the uterus, or shell gland, the number of glands was significantly higher ($P < .05$) in each group receiving a higher level of coumestrol, in both subgroups. Additionally, there was a significant decrease

Table 11. Mean magnum gland length

	Group 1	Group 2	Group 3	Group 4
Subgroup 1	3.17 mm.	3.37 mm.	3.06 mm.	3.49 mm.
Subgroup 2	3.60 mm.	2.69 mm.	3.51 mm.	3.04 mm.

($P < .05$) in number of glands between the two necropsy dates in groups 1 and 3, and approaching significance ($P = .06$) in group 2, there was a significant increase in uterine gland numbers in group 4, the high coumestrol dose group.

Table 12. Mean uterine gland numbers (circumferential section)

	Group 1	Group 2	Group 3	Group 4
Subgroup 1	19.1	32.2	37.1	40.8
Subgroup 2	40.0	33.9	40.2	32.0

There was a highly significant difference ($P < .01$) in gland length in the uterus, between groups in those birds killed at day 21, but not in those killed 10 days later. The length of uterine glands increased with increasing coumestrol dosage, except in the high coumestrol dose group, where the mean gland length was less than that of the control group. After withdrawal of coumestrol treatment, a decrease was seen in the length of uterine glands in all groups except group 4, where there

was a large increase in gland length.

Table 13. Mean uterine gland length

	Group 1	Group 2	Group 3	Group 4
Subgroup 1	2.21 mm.	2.41 mm.	1.88 mm.	2.36 mm.
Subgroup 2	2.36 mm.	2.50 mm.	2.66 mm.	2.08 mm.

Liver

A commingled sample of liver tissue from the birds in group 4, subgroup 2, was found to contain 1 part per billion coumestrol, by thin layer chromatography (100% spike recovery).[1]

Histologically, the livers of all birds showed varying degrees of fatty change. A significant increase ($P < .05$) in fattiness was seen over time. There was no significant difference between groups, and no group by subgroup interaction.

Table 14. Mean liver fattiness scores (scale of 0-5, 95% repeatable)

	Group 1	Group 2	Group 3	Group 4
Subgroup 1	2.2	2.7	2.0	2.1
Subgroup 2	1.4	1.1	1.8	1.4

[1]Dr. H. M. Stahr, personal communication, November 1979. Iowa Veterinary Diagnostic Laboratory, Iowa State University, Ames, IA.

DISCUSSION

Experiment 1

In the negative, physical treatment, and solvent control groups, respectively, 75%, 75%, and 80% of the eggs hatched. In the diacetoxyscirpenol control group, only 40% of the eggs hatched, indicating that the method of inoculation was in fact delivering compound into the eggs. There were no significant differences in hatchability in the eggs receiving 0.003, 0.03, and 0.3 micrograms of coumestrol. There also did not appear to be any effect in the eggs receiving 3.0 micrograms of coumestrol, but the number of eggs receiving this dose was too small for statistical analysis. The results of this experiment indicated that coumestrol had no effect directly on the hatchability of chicken eggs.

Experiment 2

There was a significant to highly significant increase in egg production in each group during the time period of the experiment. However, there did not appear to be any differences between groups at any given time point. Production was lower in group 3, and this lower production reached statistical significance in the third time block. However, this was due to the lower number of units in this time block, therefore the overall higher contribution to the mean of 2 birds in group 3 which failed to mature sexually.

The increase in production over time was more interesting. The pullets were 30 weeks old when the experiment began. At that time, they

had been laying for approximately 4 weeks. It was felt that the increase in production with time was an effect of increasing maturity of the pullets, rather than of treatment. The fact that no statistically significant differences were found between groups except as discussed earlier, as well as the fact that no significant group by time interaction could be demonstrated, supported this conclusion. Egg production by chickens is a cyclic biologic phenomenon, continually increasing or decreasing throughout the productive life of the bird. Therefore, any drug effect would have to be very pronounced to show up over this natural flux in productivity.

No significant differences in egg hatchability were found either between groups, over time, or as an interaction. Since other researchers have found only small variations in egg composition when the hens were treated with coumestrol, this was a somewhat expected conclusion. Coumestrol has been demonstrated to cause hypercalcemia and resultant soft-shelled eggs in chickens (Mohsin and Pal, 1977), and while shell-less eggs could not be expected to hatch, these could not be counted under the caging conditions of this experiment, where eggs without shells dropped through the wire floors of the cages and were lost in the flush-through lagoon system.

The ovaries and infundibulae appeared to be normal in all individuals. In 2 birds, sexual maturity had not occurred at the time of necropsy, and the ovaries and tubular genitalia of these birds were small and hypoplastic, but otherwise normal.

Individuals killed at the termination of treatment and 10 days later

had no significant differences in the gross lengths of their tubular genitalia, either between groups, or over time.

Length of glands in the magnum was not affected by treatment. However, numbers of glands increased significantly after treatment withdrawal in the control and low coumestrol dose group, and increased at a level approaching significance in the intermediate coumestrol dose group. The increase seen in the intermediate group would probably have been significant, if it had not been for the 2 birds mentioned previously, which did not reach sexual maturity, and thus lowered the mean value of the gland numbers for this group. Since increased gland development occurred in the control group also after cessation of treatment, these results probably reflect the effects of handling, as well as the drug. However, since the amount of increase was highest in the control group, and was factorially less in each succeedingly higher dose group, the decreased rate of magnum hyperplasia following withdrawal of the stress of handling, must be considered to be an effect of coumestrol.

The effects of coumestrol administration were most marked on the uterus. The number of uterine glands was significantly higher in each successively higher dose group, in each time period. Additionally, uterine gland numbers increased during the 10-day post treatment period in groups 1, 2, and 3, and decreased in group 4. Group by time interaction was very highly significant, suggesting that as well as treatment and period of time post treatment, maturity might also be a factor in the uterine development observed. However, uterine hyperplasia is a reported effect of coumestrol in sheep, cattle, mice, and rats, and these

findings would include chickens in this group, despite the somewhat different development of the avian reproductive tract.

Length of uterine glands also appeared to be affected by coumestrol treatment. At the immediate post treatment necropsy date, there were significant differences in the mean length of uterine glands between treatment groups, with the low and intermediate dose groups somewhat successively longer than the control group, and the high dose group substantially shorter than the control group. However, by 10 days later, at the final necropsy date, the difference between the groups had disappeared due to a decrease in mean length in groups 2 and 3, and an increase in group 4. These findings are in agreement with the data on the effects on uterine gland numbers, with the low and intermediate doses appearing to stimulate uterine hyperplasia, while the high dose appeared to decrease uterine development, while maturational changes were occurring concurrently, as indicated by the control group.

Coumestrol was highly insoluble, and the recovery of only 1 part per billion from the livers of birds which had received 10.0 mg. of 50% coumestrol per day for the 10 days preceding, the last dose only 24 hours previously, probably indicated poor absorption from the gastrointestinal tract. There was an increase in fattiness of the livers of birds over time. This change was not found to be related to group, or to have any interaction with group. This change was probably related to other physiologic processes within the pullets, and not due to coumestrol treatment.

SUMMARY

Pertinent literature on naturally occurring phytoestrogens and clinical syndromes resulting from exposure thereto in domestic animals was reviewed. Coumestrol was reported as having the strongest estrogenic activity of these compounds, causing hyperestrogenic clinical syndromes in many species of food-producing and laboratory animals.

Coumestrol was inoculated into the allantoic cavity of chicken eggs to determine its direct effect on the hatchability of eggs, since its partitioning in the live hen was not known. It was not found to affect the hatchability of these eggs.

Coumestrol was then administered to 80 single-comb White Leghorn pullets, orally in capsules, at doses of 0.0, 0.1, 1.0, and 10.0 mg. of 50% pure compound per day for 10 days. Half the birds were killed and necropsied after 10 days of treatment. The other birds were kept, but not treated, for another 10 days and then killed and necropsied. Throughout the experimental period, eggs were collected and incubated, to collect data on egg production and egg hatchability. When the birds were necropsied, the ovaries, infundibulae, magnums, uteri, and livers were collected and preserved in 10% neutral buffered formalin for later histological examination. Fresh liver was also collected for chemical analysis for coumestrol, in the event that a significant amount was found to be partitioned there. Gross length of the tubular genital tract was also measured.

Coumestrol was found to cause uterine hyperplasia, as shown by

increased numbers and length of uterine glands, which receded following withdrawal of treatment. However, the 10.0 mg. per day treatment, approximately 100 times a daily dose likely to ever be found in nature, appeared to retard uterine development, although it did not affect egg production or hatchability. Coumestrol also appeared to increase the number of glands found in the magnum, at the 0.1 and 1.0 mg. per day dosage, but not at the 10.0 mg. per day dosage.

This study demonstrated that the major effects of coumestrol in laying hens, at doses likely to be found in nature, are subclinical, and regress following withdrawal of the compound. Hyperplasia of uterine glands was the major morphologic effect of coumestrol reported in sheep, cattle, rats, and mice, and these studies confirmed that this was also true in chickens.

REFERENCES

Adams, J. L., Casida, L. E., and McGibbon, W. H. 1951. Some characteristics of eggs from estrogen-fed pullets. Poultry Science 30: 694-700.

Adams, J. L., McGibbon, W. H., and Casida, L. E. 1950. The effect of orally administered synthetic estrogens on single-comb White Leghorn pullets. Poultry Science 29: 666-671.

Adams, N. R. 1975. A pathological and bacteriological abbattoir survey of the reproductive tracts of Merino ewes in Western Australia. Australian Veterinary Journal 51: 351-354.

Adams, N. R. 1976. Pathological changes in the tissues of infertile ewes with clover disease. Journal of Comparative Pathology 86: 29-35.

Adams, N. R. 1977. Morphological changes in the organs of ewes grazing oestrogenic subterranean clover. Research in Veterinary Science 22: 216-221.

Adams, N. R. 1979. Masculinization of the external genitalia in ewes with clover disease. Australian Veterinary Journal 55: 22-24.

Adler, J. H. 1961. Antioestrogenic activity in alfalfa. Bulletin of the Research Council of Israel 9 E: 167.

Adler, J. H., and Trainin, D. 1960a. A hyperestrogenic syndrome in cattle. Refuah Veterinariah 17: 108-115.

Adler, J. H., and Trainin, D. 1960b. Diet and bovine infertility. Veterinary Record 72: 1171-1172.

Adler, J. H., and Trainin, D. 1961. The apparent effect of alfalfa on the reproductive performance of dairy cattle. Proceedings of the Fourth International Congress on Animal Reproduction: 451-456.

Adler, J. H., Weitzkin, G., and Marinov, U. 1970. Determination of free and conjugated coumestrol in bovine blood. Journal of Animal Science 30: 451-453.

Bailey, G. F. 1960. An instrument for the measurement of fluorescence of paper chromatographic spots. Analytical Chemistry 32: 1726-1727.

Barnes, C. S., and Occolowitz, J. L. 1964. The mass spectra of some naturally occurring oxygen heterocycles and related compounds. Australian Journal of Chemistry 17: 975-986.

Bennetts, H. W., Underwood, E. J., and Shier, F. L. 1946. A specific breeding problem of sheep on subterranean clover pastures in Western Australia. Australian Veterinary Journal 22: 2-12.

Bickhoff, E. M. 1959. The chemistry and distribution of estrogens in forages. Proceedings of the Sixth Technical Alfalfa Conference: 47-51. USDA-ARS number 74-15.

Bickhoff, E. M., and Booth, A. N. 1959. Estrogenic factor. U.S. Patent number 2,890,116. U.S. Patent Office, Office Gazette 743: 418.

Bickhoff, E. M., and Booth, A. N. 1960. Preparation of coumestrol esters and use thereof. U.S. Patent number 2,929,713. U.S. Patent Office, Office Gazette 752: 989.

Bickhoff, E. M., Booth, A. N., and Hendrickson, A. P. 1961. Estrogen-like substances in plants. Proceedings of the 22nd Biological Colloquim on the Physiology of Reproduction: 93-118.

Bickhoff, E. M., Booth, A. N., Livingston, A. L., and Hendrickson, A. P. 1960. Observations on the effect of drying on estrogenic activity of alfalfa samples of varying maturity. Journal of Animal Science 19: 745-753.

Bickhoff, E. M., Booth, A. N., and Lyman, R. L. 1958. Isolation of a new estrogen from Ladino clover. Agricultural and Food Chemistry 6: 536-539.

Bickhoff, E. M., Loper, G. M., and Hanson, C. H. 1967. Effect of common leafspot on coumestans and flavones in alfalfa. Crop Science 7: 259-261.

Cayen, M. N., and Common, R. H. 1965. An investigation of urinary conversion products of coumestrol in the fowl. Biochimica et Biophysica Acta 100: 567-573.

Cooney, W. T., Butts, F. S., and Bacon, L. E. 1948. Alfalfa meal in chick rations. Poultry Science 27: 828-830.

Elam, C. J. 1961. Digestibility and nitrogen balance studies with low- and high-coumestrol alfalfa. Proceedings of the Seventh Technical Alfalfa Conference 7: 53-55.

Emerson, O. H., and Bickhoff, E. M. 1958. Synthesis of coumestrol, 3,9-dihydroxy-6-benzofuro (3,2-c) (1) benzopyran-6-one. Journal of the American Chemical Society 80: 4381-4383.

Foltin, E. 1959. Observations on bovine infertility in the Jordan Valley. Refuah Veterinariah 16: 193.

Fox, C. W., and Oldfield, J. E. 1962. Growth response of lambs fed high coumestrol alfalfa. Proceedings of the Western Section of the American Society of Animal Science 13(XLVII): 1-6.

Frances, C. M., and Millington, A. J. 1965. Wether bioassay of annual pasture legumes IV: The estrogenic activity of annual medic pastures. Australian Journal of Agricultural Research 16: 927-935.

Heydon, R. A., and Adams, N. R. 1977. Histochemical studies on cervical glands in ewes with clover disease. Journal of Comparative Pathology 87: 353-361.

Heywang, B. W. 1950. High levels of alfalfa meal in diets for chickens. Poultry Science 29: 804-811.

Hoelscher, M. 1979. Exposure to phytoestrogens may surpass DES residues. Feedstuffs 15-1943: 67-69.

Knuckles, B. E., Miller, R. E., and Bickhoff, E. M. 1975. Quantitative determination of coumestrol in dried alfalfa and alfalfa leaf protein concentrates containing chlorophyll. Journal of the American Organization of Analytical Chemists 58: 983-986.

Lepkovsky, S., Shaeleff, W., Peterson, D., and Perry, R. 1950. Alfalfa inhibitor in chick rations. Poultry Science 29: 208-213.

Lindner, H. R. 1967. Study of the fate of phytoestrogens in the sheep by determination of isoflavones and coumestrol in the plasma and adipose tissue. Australian Journal of Agricultural Research 18: 305-333.

Livingston, A. L., Bickhoff, E. M., Guggolz, J., and Thompson, C. R. 1961. Quantitative determination of coumestrol in fresh and dried alfalfa. Journal of Agricultural and Food Chemistry 9: 135-137.

Lookhart, G. L., Jones, B. L., and Finney, K. F. 1978. Determination of coumestrol in soybeans by high performance liquid and thin-layer chromatography. Cereal Chemistry 55: 967-972.

Loper, G. M. 1968a. Effect of aphid infestation on the coumestrol content of alfalfa varieties differing in aphid resistance. Crop Science 8: 104-106.

Loper, G. M. 1968b. Accumulation of coumestrol in barrel medic (Medicago littoralis). Crop Science 8: 317-319.

Loper, G. M., and Hanson, C. H. 1964. Influence of controlled environmental factors and two foliar pathogens on coumestrol in alfalfa. Crop Science 4: 480-482.

Matsushima, J. K. 1961. Estrogens and dehydrated alfalfa in beef cattle feeding: Review of five years' work. Proceedings of the Seventh Technical Alfalfa Conference 7: 50-52. USDA-ARS number 74-25.

Matsushima, J. K., McCullough, M. W., and Clanton, D. C. 1962. Plant estrogens in cattle rations. 50th Annual Feeders' Day Report. Nebraska Agricultural Experiment Station, Lincoln, Nebraska.

Mohsin, M., and Pal, A. K. 1975. Phytoestrogen in relation to physiology of birds I: Effect of plant and synthetic estrogen on feed utilization and growth response in chickens from day old to 60 days of age. Indian Journal of Poultry Science 45: 93-98.

Mohsin, M., and Pal, A. K. 1977. Coumestrol in lucerne (Medicago sativa L.) as a factor in egg production and its quality in White Leghorn pullets. Indian Journal of Experimental Biology 15: 76-77.

Ochi, I., Takahuru, S., Kunifoshi, I., and Shinnosuke, D. 1964. Reproductive conditions of dairy cows fed Ladino clover. Bulletin of the National Institute on Animal Health 48: 22-28.

Oldfield, J. E. 1961. A review of Oregon research on estrogens and forages in sheep feeding. Proceedings of the Seventh Technical Alfalfa Conference 7: 56-61.

Oldfield, J. E., and Fox, C. W. 1962. Growth responses of lambs fed high-coumestrol alfalfa. Journal of Animal Science 21: 667. (Abstract)

Oldfield, J. E., and Fox, C. W. 1963. Growth factors for sheep, with particular reference to estrogenic materials. Proceedings of the Eighth Technical Alfalfa Conference 8: 24-27. USDA-ARS number 74-26.

Oldfield, J. E., Fox, C. W., and Bickhoff, E. M. 1960. Effects of estrogenic activity in alfalfa on growing lambs. Journal of Animal Science 19: 1281. (Abstract)

Oldfield, J. E., Fox, C. W., and Bahn, A. V. 1966. Coumestrol in alfalfa as a factor in growth and carcass quality in lambs. Journal of Animal Science 25: 167-174.

Pieterse, P. J. S., and Andrews, F. N. 1956. The estrogenic activity of alfalfa and other feedstuffs. Journal of Animal Science 15: 25-36.

Schinkel, P. G. 1948. Infertility in ewes grazing subterranean clover pastures: Observations on breeding behavior following transfer to "sound" country. Australian Veterinary Journal 24: 289-294.

Snedecor, G. W., and Cochran, W. G. 1967. Statistical Methods. Iowa State University Press, Ames, Iowa.

Stahr, H. M., Ed. 1975. Analytical Toxicology Methods Manual. Iowa State University Press, Ames, Iowa.

Stahr, H. M., Ed. 1976. Analytical Toxicology Methods Manual. 2nd ed. Iowa State University Press, Ames, Iowa.

Story, C. D., et al. 1957. The effects of low levels of diethylstilbestrol and plant estrogens upon performance of fattening lambs. Proceedings of the Iowa Academy of Science 64: 259-266.

Thayer, R. H., Jaap, R. G., and Penquite, R. 1945. Fattening chickens by feeding estrogens. Poultry Science 24: 483-495.

United States Department of Agriculture. 1969. Studies on the chemical and biological properties of coumestrol and related compounds. USDA-ARS number 1408. 94 pp.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. W. Eugene Lloyd, chairman of my graduate committee, for his invaluable guidance and support throughout my graduate studies.

I wish to thank the other members of my graduate committee; Dr. John P. Kluge, chairman of the Department of Veterinary Pathology; Dr. David Cox; and my special thanks to Dr. Michael Stahr, for his advice and assistance with this research project.

I would like to thank Dr. Vaughn A. Seaton, head of the Iowa Veterinary Diagnostic Laboratory, for his support and encouragement with this project and my studies.

I also wish to thank Mr. William Cunningham for his contributions to the protocol and execution of this study.

I extend my appreciation to the staff of the Iowa State University Poultry Science Center, especially Mr. Les Williams and Mr. Wayne Miller, whose patience and conscientious animal care, and sound advice were of immeasurable help to me in the completion of this project.