AN EXAMINATION OF GOSSYPOL TOXICITY IN BROILERS AND LAYING HENS AND THE DEVELOPMENT OF A NOVEL USE FOR COTTONSEED MEAL IN POULTRY DIETS AND OF A RAPID ASSAY FOR QUANTIFYING GOSSYPOL IN COTTONSEED MEAL

by

MARIA MADALENA DOS SANTOS LORDELO

(Under the Direction of Adam J. Davis)

ABSTRACT

Gossypol is a toxic compound found in cottonseed meal. Experiments were conducted to determine the relative toxicity of the (+) and (-) enantiomers of gossypol in poultry. The individual pure gossypol enantiomers were added to nutritionally complete diets and then fed to broilers, laying hens, and broiler breeder hens. The (-) enantiomer of gossypol was detrimental to feed intake in broilers and broiler breeder hens while the (+) enantiomer of gossypol inhibited feed intake in laying hens. Severe egg yolk discoloration was caused by (+)-gossypol in both laying and broiler breeder hens. Since both (-)- and (+)-gossypol have toxic effects in chickens, the development of a cotton strain containing only one gossypol enantiomer would not be beneficial for the poultry industry.

To determine if cottonseed meal could replace soybean meal in broiler breeder pullet diets, pullets were fed a diet containing either cottonseed meal or soybean meal as the major protein source from 2 to 18 weeks of age. Flock body weight uniformity

during the rearing period was improved in pullets consuming cottonseed meal as the major protein source. Furthermore, subsequent reproductive performance was not affected in broiler breeder pullets fed cottonseed meal during the rearing period. Therefore, cottonseed meal has advantages over soybean meal in broiler breeder pullet diets.

To determine if near infrared reflectance spectroscopy could be successfully utilized to rapidly measure free gossypol concentration in cottonseed meal, a near infrared calibration equation for gossypol was developed. There was a high correlation between the values of gossypol determined by the conventional chemical-based reference method and by the near infrared reflectance spectroscopy method.

The results suggest that there are no apparent advantages for poultry in altering the enantiomeric ratio of the cotton plant, but that cottonseed meal can be successfully utilized by the poultry industry in broiler breeder pullet diets and that the poultry industry could use near infrared reflectance spectroscopy technology to determine the free gossypol content of cottonseed meal.

INDEX WORDS: Broilers, Laying hens, Broiler breeder hens, Cottonseed meal, Gossypol enantiomers, Near infrared reflectance spectroscopy, Toxicity

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DEDICATION

I dedicate this dissertation to my parents, Amélia and Eduardo, and to my brother, Ricardo.

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I acknowledge Dr. Adam Davis, for his advice, guidance and patience at both personal and academic level. I thank Dr. Millard Calhoun, Bobbie Baldwin and Liz Freeman for helping me with my research. I also thank Beatriz Dale and Dr. Nick Dale for being the family when I was far away from home. Special thanks to John Driver, who has been faithfully beside me throughout my entire graduate career.

TABLE OF CONTENTS

CHAPTER 1

LITERATURE REVIEW

1. COTTONSEED MEAL

1.1. Cottonseed processing and products

The United States, China and India account for over 50% of the world's cotton production. Worldwide, there has been a steady increase in consumer demand for cotton textile products. This increase in cotton production has resulted in a greater production of cotton by-products such as cottonseed meal (CSM).

When cotton is harvested, it is composed of both fibers and seeds. The purpose of a modern cotton gin is to dry and clean the freshly picked cotton, separate the fibers from the seed, further clean the fibers, and place the fibers into an acceptable package for commerce (Baker and Griffin, 1984). The fiber portion then enters the textile industries, while the seeds are transported to an oil extraction mill.

At the oil extraction mill, oil, meal, hulls and linters, are the major products that can be obtained from the cottonseed. From the cottonseed processing procedure, the recovery of meal, hulls, oil, linters and losses due to handling and processing are 45, 26, 16, 9, and 4 percent, respectively (Kromer, 1977; Carter et al., 1979).

Lints are the short fibers that still remain attached to the seed after ginning. After being removed from the seed, the collected lint is used for the manufacture of dissolving pulps, fiber pulp, medical grade absorbent cotton and cushions for furniture. After removing most of the lint, the hull of the seed is removed. This is accomplished by a machine employing a series of knives, which cut the hulls and thereby loosens them from the kernel (NCPA, 1990). Then, since the hulls are lighter than the other components of the seed, a vacuum hose is used to remove the hulls. The hulls are used as a feed

ingredient for dairy cattle and as poultry litter. Hulls are not used as a chicken feed ingredient because the bird digests the hull very poorly as it contains approximately 50% crude fiber (Etgen et al., 1987).

Following dehulling, only the meat of the seed remains, which is flaked to optimize oil extraction. There are several methods by which oil extraction can be accomplished. These methods include mechanical, direct solvent, prepress solvent or expander solvent extraction. During mechanical extraction, the cottonseed meats are subjected to high pressure as they are squeezed by a revolving screw-press inside a barrel which forces the oil out of the cottonseed cells. The oil extracted from the meats is then pushed through the small openings of the barrel and collected (NCPA, 1990). The de-oiled residue moves out through the end of the barrel and is then ground into meal.

Cottonseed oil can also be removed from the cottonseed meat by a direct solvent extraction method in which a solvent is poured over the meat flakes. The solvent extracts oil from the meats and then the oil is separated from the solvent. The solvent used currently is a mixture of petroleum fractions that is composed predominantly of hexane. A problem with the direct solvent method is that yields are not optimal since oil remains in intact cottonseed meat cells. To combat this loss of yield, a prepress solvent process of oil extraction was developed. In this procedure, the cottonseed meat flakes are first put through a screw-press under medium pressure and then subjected to direct solvent extraction to remove the remainder of the oil (NCPA, 1990).

Currently, most modern cotton oil mills utilize an expander solvent oil extraction method. In the expander, high temperature and pressure break the membranes of the cottonseed meat cells, which releases the oil that is inside the cells. The flaked

cottonseed meats are then exposed to hexane, which removes the oil. Two fractions are created from this process, one is the extracted oil with solvent called "miscella", and the other is the extracted solid cellular material, which becomes the meal (Gregory et al., 1999). The miscella then passes through a series of evaporators and stills to remove all solvent from the oil. The solvent is then recovered and reused (NCPA, 1990). The crude oil is processed to separate the pure high quality oil from the portion of the oil composed of lower quality lipids such as waxes (Frazier, 2000). The extracted fraction of lower quality lipids is termed soapstock. The remaining solid cellular material goes through a process of desolventizing, toasting, drying and cooling to yield CSM.

Refined cottonseed oil is used by the food industry to fry snack foods and as a component of salad dressing, margarine, and other products. Due to its limited marketability, cottonseed soapstock is usually mixed back with the CSM. Expander solvent CSM is usually sold with a 41% crude protein content. After the oil, CSM is the most valuable product obtained from delinted cottonseed and it usually accounts for about one third of the total product value (NCPA, 1990).

1.2. Concerns when using cottonseed meal in poultry feeds

Cottonseed meal is a widely available plant protein feed ingredient. Nevertheless, as an ingredient for poultry diets there are three major problems associated with the use of CSM. One major problem associated with feeding CSM to poultry is the presence in this feed ingredient of the toxic polyphenolic compound gossypol. Laying hens fed diets containing gossypol produce egg yolks with an olive-brown color (Heywang et al., 1954; Kemmerer et al., 1961; Reid et al., 1984; Panigrahi, 1990; Davis et al., 2002). In

addition, numerous researchers have indicated that high levels of gossypol (greater than 200 mg/kg of diet) in broiler rations are associated with depressed weight gain (Boatner et al., 1948; Lillie and Bird, 1950; Milligan and Bird, 1951; Gamboa et al., 2001) and depressed feed intake and/or feed efficiency (Boatner et al., 1948; Couch et al., 1955; Lipstein and Bornstein, 1964; Waldroup, 1981; Gamboa et al., 2001; Henry et al., 2001).

Another problem associated with the feeding of CSM to poultry is that it contains low levels of available lysine compared to other dietary protein sources (Anderson and Warnick, 1966). This low lysine availability results from the formation of indigestible complexes between lysine and gossypol or carbohydrates during processing (Waldroup, 1981). Feeding diets that are low in available lysine can negatively impact bird growth. However, the amount of available lysine in the diet can be increased to required levels by the addition of synthetic crystalline lysine.

The final problem associated with the use of CSM in poultry diets is the presence of cyclopropenoid fatty acids in this feed ingredient. The presence of more than 0.1% residual cottonseed oil in the laying hen diet will increase the fatty acid content in the egg, such as cyclopropenoid fatty acids that contributes to egg discoloration (Evans et al., 1961; Reid, 1972; Reid et al., 1984). Panigrahi et al. (1989) reported that during cold storage of eggs, cyclopropenoid fatty acids alter the permeability of the yolk viteline membrane such that the alkaline albumen proteins diffuse into the yolk and yolk iron passes into the albumen, which results in pink albumen (Schaible and Bandemer, 1946), and in an enhancement of the brown yolk discoloration caused by gossypol (Kemmerer et al., 1963). The problems associated with cyclopropenoid fatty acids, however, may be avoided by the use of CSM containing low quantities of residual lipid (Panigrahi and

Hammonds, 1990). In cottonseed processing plants that use an expander solvent extraction process to separate the oil from the cottonseed, the cyclopropenoid fatty acids are converted to other fatty acids during the heating process, and almost all of the oil is removed from the seed. Therefore, the concentration of cyclopropenoid fatty acids in CSM is low enough that it is no longer a major concern when feeding CSM.

1.3. Summary

Despite the antinutritional factors and the low levels of available lysine associated with CSM, there has been a continuous interest in utilizing this feed ingredient in poultry and animal feeds due to the continual rise in cotton production around the world. In areas of the world where cotton is grown, CSM is available at a lower price than other protein sources, and therefore, more attractive to the poultry producer.

2. GOSSYPOL

2.1. Occurrence in the cotton plant

Gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3, 3'dimethyl-(2, 2'-binaphthalene)-8, 8'-dicarboxaldehyde] is a yellow pigment first identified and named by Marchlewski in 1899. The structure of gossypol (Figure 1.1.) was subsequently determined by Adams et al. in 1938. The chemical structure of gossypol consists of two naphthalene rings joined by a single bond. Due to the restricted rotation about the bond connecting the rings, gossypol exists in two enantiomeric forms. Thus, gossypol occurs

naturally in the cotton plant as a mixture of (+) and (-) enantiomers (Figure 1.2.). Isolation of the (+) form of gossypol was first reported by King and De Silva in 1968. Later, Dechary and Pradel (1971) reported the isolation of both the (+) gossypol enantiomer and the (-) gossypol enantiomer from cottonseed.

At present, most of the cotton fibers produced worldwide are from the Gossypium hirsutum (Upland cotton) or Gossypium barbadense (Egyptian, Tanguis or Pima cotton) species of cotton. In these cotton strains, the ratio of (+)- and (-)- gossypol is not always 50:50 (Zhou and Lin, 1988; Cass et al., 1991). The Upland cotton variety consistently has a higher percentage of the (+)-gossypol than the (-)-gossypol and in some cultivars the (+)-gossypol accounts for as much as 95% of the total gossypol content (Cass et al., 1991). Interestingly, Pima cotton consistently has a higher level of the (-) enantiomer of gossypol than the (+) enantiomer (Cass et al., 1991).

Gossypol is produced and contained almost exclusively within discrete bodies called pigment glands, which are found in the leaves, stems, roots, and seeds of cotton plants (Boatner, 1948). Work by Fisher et al. (1988) reported, however, that the absence of pigment glands does not ensure complete elimination of gossypol, which suggests that there may be other sites of gossypol production in the cotton plant. The main function of gossypol in the cotton plant is to act as a natural insecticide (Berardi and Goldblatt, 1980).

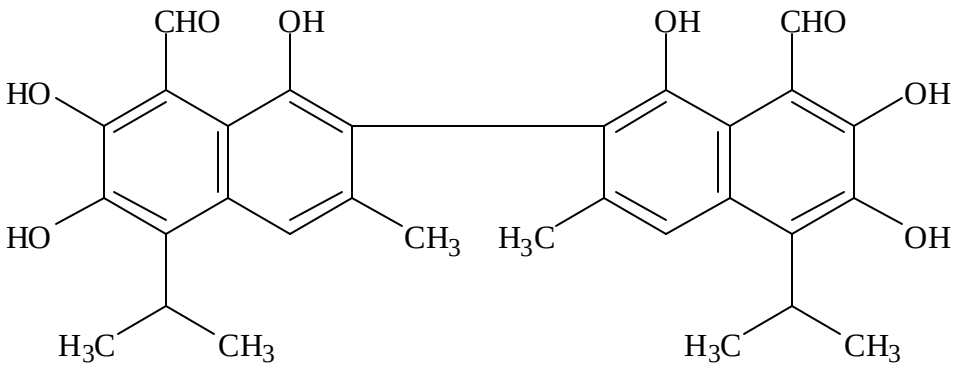
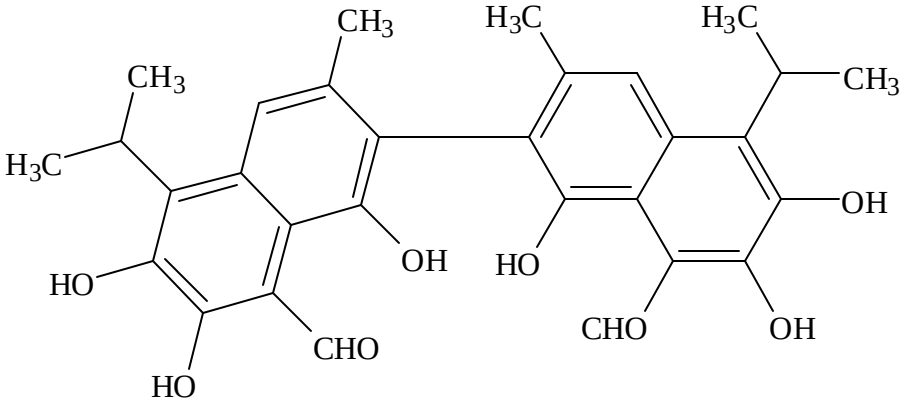
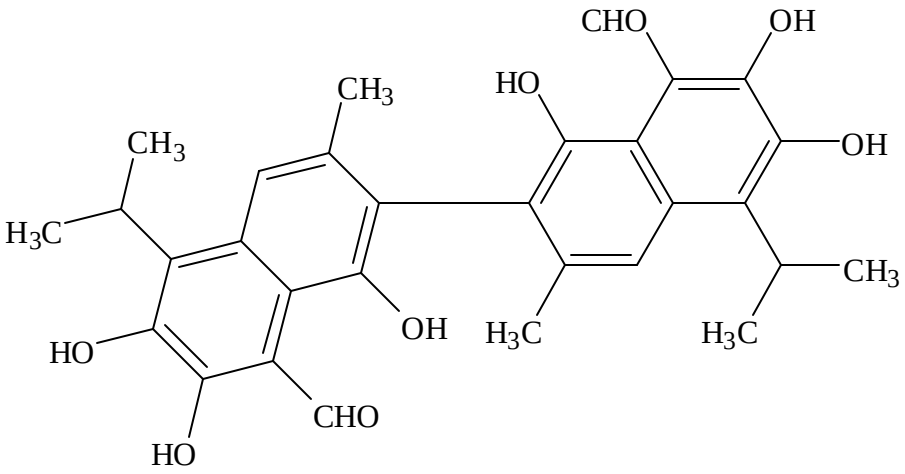


FIGURE 1.1. Structure of gossypol.



(+) Gossypol enantiomer



(-) Gossypol enantiomer

FIGURE 1.2. Structure of the (+) and (-) gossypol enantiomers.

2.2. Total and free gossypol

Free gossypol is officially defined as the "gossypol and gossypol derivatives in cottonseed products that are soluble in aqueous acetone under the conditions of the (AOCS) method" (AOCS, 1985a). Total gossypol is defined as the "gossypol and gossypol derivatives, both free and bound, in cottonseed products that are capable of reacting with 3-amino-1-propanol in dimethylformamide solution to form a diaminopropanol complex, which then reacts with aniline to form dianilinogossypol under the conditions of the (AOCS) method" (AOCS, 1985b).

When cottonseeds are processed, most of the pigment glands in the seeds are ruptured and thus gossypol is released and extracted with the oil. The rest of the gossypol that is not extracted with the oil remains with the CSM in the free and bound state (Heywang et al., 1952). Therefore, the content of gossypol in CSM is affected by the method of oil extraction that was used in the cotton oil mill (Waldroup, 1981). Methods that achieve the highest oil extraction will yield CSM with the lowest gossypol content. Accordingly, free gossypol levels are usually lowest in prepress solvent meals and highest in direct solvent or mechanically extracted meals. Several studies have determined that CSM derived from the expander solvent extraction process has the lowest concentrations of both free and total gossypol (Forster and Calhoun, 1995; Waldroup and Kersey, 2002), thus increasing the feed ingredient quality of the CSM.

Additionally, cottonseed meals to which the soapstock has been added are higher (about twice) in total gossypol content than those with no added soapstock. This is because the soapstock waste from the oil extraction process contains high levels of

gossypol (Dowd, 1996; Davis et al., 2002). In fact, the average content of gossypol in cottonseed oil soapstock is 7.5% (Dowd, 1996).

2.3. Gossypol toxicity

2.3.1. Gossypol toxicity in animals

The toxic effects of gossypol are related to the species of animal, cotton variety, level of consumption and period of consumption. Feeding gossypol is associated with decreased feed intake and body weight gain in broilers (Heywang and Bird, 1955; Lipstein and Bornstein, 1964), cows (Hollon et al., 1958) and swine (Withers and Carruth, 1915).

In the laying hen, gossypol forms a complex with iron in the egg yolk, which is associated with the appearance of a brown yolk discoloration (Swensen et al., 1942; Phelps, 1966; Waldroup, 1981). Heywang et al. (1963) reported, however, that layer pullets could be fed CSM until 20 days prior to the onset of egg laying without producing any adverse effect on subsequent egg color. Heywang et al. (1949) also indicated that the addition of dietary CSM above 10% (120 mg/kg dietary free gossypol) would significantly reduce hatchability. However, when similar levels of glandless CSM were added to the diet, no differences were reported in hatchability (Vohra et al., 1983). Although levels above 120 mg/kg of dietary free gossypol may adversely affect hatchability, these levels are far higher than the 50 mg/kg required to cause egg discoloration (Waldroup, 1981).

Gossypol intake has also been extensively associated with poor male fertility in rats (Winbauer et al., 1982; Wang et al., 1987), hamsters (Hahn et al., 1981; Saksena and

Salmonsen, 1982), humans (National Coordinating Group on Male Antifertility Agents, 1978), monkeys (Shandilya et al., 1982) and bulls (Arshami and Rutle, 1989; Chase et al., 1989). The antifertility effect of gossypol in male birds has been documented in Japanese quail (Lin et al., 1988) and roosters (Mohan et al., 1989). In rodents, this decrease in male fertility is related to lower epididymal sperm motility and sperm counts (Saksena and Salmonsen, 1982; Weinbauer et al., 1982). Additionally, Shandilya et al. (1982) described a sperm tail lesion in gossypol-treated monkeys, which may be responsible for the observed immotility.

2.3.2. Gossypol reactivity

The gossypol molecule contains two aldehyde groups (Figure 1.1.). These two groups have the ability to bind organic compounds of biological importance by a condensation reaction to form a Schiff's base by the elimination of water (Figure 1.3.). Often, the aldehyde groups of gossypol will bind to the free amino groups of amino acids or peptides (Figure 1.3.). In addition, gossypol chelates metal cations such as iron and zinc by trapping or encapsulating them. The exact nature of the cation-gossypol complex formation is still an area of active investigation (Przybylski et al., 2001).

Gossypol can inhibit the activities of several enzymes such as adenylate cyclase and cytochrome P450 (Adams et al., 1960; Wang and Lei, 1987). The inhibition of enzyme activity can be due to gossypol's chelating property, which lowers the available concentration of enzyme cofactors such as Zn^{2+} and Fe^{2+}(Berardi and Goldblatt, 1980).

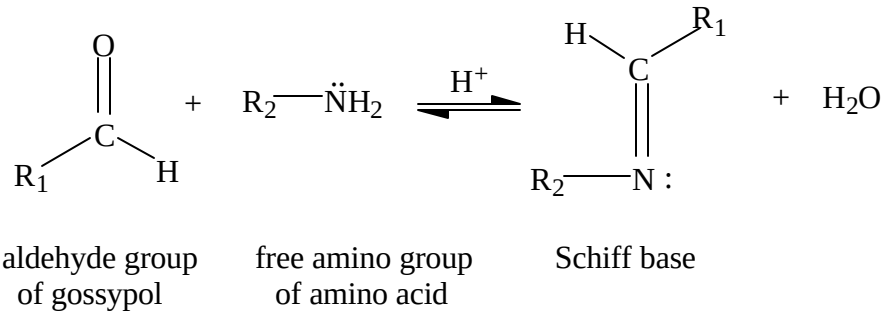


FIGURE 1.3. Condensation reaction between gossypol and amino acids or peptides.

But more importantly, gossypol can inhibit enzyme activity by directly binding to exposed free amino groups within the enzyme structure and thus alter the functionality of the active site of the enzyme (Strom-Hansen et al., 1989).

2.3.3. Gossypol cytotoxicity and therapeutic effects

Gossypol kills cells by inhibiting DNA synthesis (Hu et al., 1993), increasing DNA strand breaks (Quintana et al., 2000) and damaging the mitochondria membrane (Hu et al., 1986). Due to its cytotoxic property, gossypol has been studied as a potential therapeutic agent in many human carcinoma cell lines including breast, ovarian, uterine, prostatic, adrenal, pancreatic and colon (Tuszynski and Cossu, 1984; Band et al., 1989; Wu et al., 1989; Thomas et al., 1991). Recent research also indicates that gossypol may target and antagonize certain antiapoptotic proteins found in cells of cancer patients and thus promote the death of cancer cells (Kitada et al., 2003). In the United States, the National Institutes of Health has already obtained a patent for gossypol as a cancer treatment agent (US patent No. 551353, 1991).

Besides its anticancer properties, gossypol also has antiviral activity against a number of enveloped viruses, including the HIV virus (Polsky et al., 1987). Research suggests that the anti HIV effect of gossypol is caused by inhibition of the enzyme HIV reverse transcriptase by a gossypol derivative (Keller et al., 2003).

2.4. Relative toxicity of gossypol enantiomers

Recently, there has been increased interest in the relative biological responses to the different enantiomers of gossypol. The poor performance of animals that are fed diets

containing gossypol has been associated with the (-) enantiomer in both broilers (Bailey et al., 2000) and lambs (Kim et al., 1996). Similarly, the antifertility effect of gossypol has been almost exclusively associated with (-)-gossypol (Wang et al., 1987). Liu and co-workers (2002) have reported that (-)-gossypol is also the more potent inhibitor of cancerous breast cell growth. Additionally, the activity against the HIV virus has also been attributed to (-)-gossypol (Lin et al., 1989). These results suggest that the utilization of a CSM with a higher (+) to (-) ratio of gossypol enantiomers would be preferable in animal feeds. Additionally, since (-)-gossypol is more active than (+)-gossypol, it would be more effective to use in clinical trials.

2.5. Isolation of gossypol and gossypol enantiomers

For research purposes, there has been an increasing need for pure gossypol and pure gossypol enantiomers. Presently, the general strategy of most of the isolation procedures involves extracting gossypol into an organic solvent, concentrating the resulting solution and precipitating gossypol in this solution by adding acetic acid. The resulting product is a complex containing an equimolar ratio of gossypol and acetic acid (Carruth, 1918). In addition, this isolated complexed gossypol will contain equal amounts of the (+) and (-) enantiomers. The best method for obtaining a good yield of highly purified gossypol acetic acid is to precipitate it from solvent extracted cottonseed soapstock (Dowd and Pelitire, 2001).

Chromatographic separation techniques are currently used for the isolation of pure (+)- and (-)-gossypol (Matlin et al., 1988; Zhou and Lin, 1988). A new approach, however, has been developed to prepare pure gossypol enantiomers by producing large

single crystals of only one gossypol enantiomer from gossypol acetic acid which contains an equal amount of the (+)- and (-)-gossypol (Dowd, 2003). The enantiomeric form of each crystal is then determined by HPLC and the crystals of each form are combined (Dowd, 2003). This new methodology has proven to produce a purer product and if a daily routine is established it yields larger quantities of enantiomeric gossypol than preparative-scale chromatographic procedures.

2.6. Prevention of gossypol toxicity in poultry production

Due to the continued interest of using CSM in poultry diets, several studies have been conducted to determine ways to reduce the negative effects of gossypol in chickens. Reid et al. (1987) concluded that isopropanol extracted CSM had 18 times less free gossypol than CSM extracted with hexane. Thus, this meal could be safely utilized in laying hen diets at levels up to 15% without any detrimental effect on either egg production or yolk color and quality. Panigrahi and Morris (1991) reported that adding ferrous sulphate heptahydrate to CSM at a 4:1 weight ratio of iron to free gossypol also prevented brown yolk discoloration in eggs from laying hens fed diets containing CSM. Additionally, supplementing diets containing CSM with soluble iron also prevented poor growth in broilers (Phelps, 1966).

Since gossypol is contained almost exclusively in the pigment glands of the cotton plant, Waldroup et al. (1968) obtained normal weight gain and feed efficiencies in broilers fed a diet in which a portion of the dietary soybean meal was replaced by glandless CSM. Furthermore, Ryan et al. (1986) indicated that the gossypol content of a CSM produced from a specific glandless cotton strain (Acala C-9) was sufficiently low

that laying hens fed diets containing 20% of this meal had no egg yolk discoloration. In terms of fiber yield, however, the pigment glands are an essential component of the cotton plant since they produce gossypol that acts as an insecticide.

2.7. Quantification of gossypol in cottonseed meal

The gossypol concentration in CSM is variable and is dependent on cotton genetics (Berardi and Goldblatt, 1980), environmental conditions (Berardi and Goldblatt, 1980), and cottonseed oil extraction process (Waldroup, 1981). Due to the problems attributed to the presence of gossypol in animal feeds, the amount of CSM to incorporate in a diet is obviously dependent on the level of gossypol contained within the meal. Therefore, it is important that reliable analytical methods exist to accurately determine free gossypol content.

The American Oil Chemists' Society (AOCS) spectrophotometric-based methods are the adopted official methods for the quantification of total and free gossypol in CSM (AOCS, 1985a, b). Nevertheless, the AOCS methods are laboratory intensive and produce results that have some variation from laboratory to laboratory. Instead of the traditional AOCS procedure, more rapid and reliable methods of quantifying free and total gossypol in CSM have been developed using high-performance liquid chromatography (HPLC) (Hron et al., 1990; Kim et al., 1996; Hron et al., 1999; McMillan, 2000). The existing HPLC methods use reverse phase chromatography for gossypol determination in CSM. The typical sample preparation involves drying and grinding the sample and then adding a gossypol complexing reagent that usually consists of 2-amino-1-propanol and glacial acetic acid. The purified gossypol is then applied to

the HPLC column. The mobile phase typically utilized for the HPLC method is acetonitrile and potassium dihydrogen phosphate buffer (Kim et al., 1996; Hron et al., 1999; McMillan, 2000). The main drawback of the HPLC methods is the expense of the equipment.

2.7.1. Near infrared reflectance spectroscopy

Near infrared reflectance spectroscopy (NIRS) is a technique that allows for the successful determination in feeds and feed ingredients of various parameters such as moisture, protein and fat content. As reviewed by Van Kempen (2001), this technique has also been used to predict metabolizable energy, total amino acid and digestible amino acid content as well as to detect contaminants in feeds. Near infrared reflectance spectroscopy is unusually fast compared to other analytical techniques and is nondestructive, non-polluting and economical.

In NIRS, a light source interacts with the finely ground feed sample and the reflected light is measured with a detector (Figure 1.4.). Because most feedstuffs are opaque, NIRS utilizes the light reflected by the sample. The reflected light from the sample indirectly indicates the amount of light energy absorbed by the sample. Therefore, near infrared reflectance technology is capable of measuring the absorption of infrared radiation by the sample components, such as O-H, N-H, C-H and peptide bonds. Since different molecules have different atom arrangements, each molecule within a feed sample has its own infrared "fingerprint" that can be detected and then quantified.

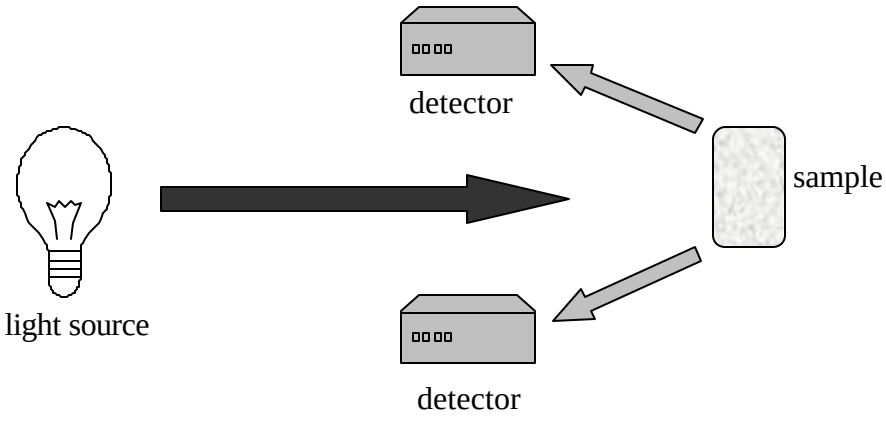


FIGURE 1.4. Near infrared reflectance spectroscopy uses a light source of a defined wavelength or group of wavelengths from the near infrared region, which interacts with a sample. The reflected light is then measured with a detection device.

The drawback of this simple methodology is the amount of preliminary work that must be done to allow the near infrared reflectance instrument to make a reliable prediction of the concentration of the constituent to be analyzed. During the preliminary work, the NIRS system is calibrated and a prediction equation for the component to be analyzed is developed.

For accurate calibration, a wide number of samples must be obtained that reflect the variability in concentration expected for the "real" samples to be tested later. Then, reference analyses must be performed on all of these obtained samples using an accepted reference method. The AOCS method for determination of gossypol concentration in CSM is, for example, an accepted reference method. A spectral analysis is then run for each of the calibration samples in the near infrared reflectance instrument and a prediction equation is formulated. After a prediction equation is obtained, a validation should be performed which correlates the reference data and the spectral data of a set of samples in a regression. A strong correlation (greater than 90%) should be obtained between the two methods if the near infrared methodology is to be used. Near infrared reflectance calibration models can be then transferred from one instrument to another. Transfer of calibrations is necessary since not all NIRS users have the resources to develop their own prediction equations.

Birth and Ramey (1982) reported a good correlation for the concentration of gossypol in cottonseed samples between the near infrared reflectance determination method and the AOCS method. No attempts, however, were made to quantify the amount of gossypol in CSM samples.

2.8. Summary

The toxic compound gossypol has been the subject of intense investigation in various fields of animal science and human medicine. Feeding animals diets containing gossypol is associated with decreased feed intake and decreased growth. Based on current research, the (-) enantiomer of gossypol appears to be more toxic to animals than the (+) enantiomer. Further investigation, however, should be conducted for a better understanding of the relative toxicity of the individual enantiomers of gossypol.

3. FEEDING REGIMENS FOR BROILER BREEDERS

3.1. Feed restriction

In the United States, broiler breeder pullets are fed corn/soybean meal based diets, which are the same type of diets used to feed commercial broilers that reach market size in six weeks. However, to prevent broiler breeder pullets from growing too quickly and becoming too large and/or obese by sexual maturity at 22-24 weeks of age, their dietary intake is severely restricted. Typically, in commercial farms, feed restriction involves giving birds 60 to 80% less feed during the rearing period and 25 to 50% less feed during the laying period than what they would consume ad libitum.

There are conflicting research reports, however, on the optimum timing and duration of feed restriction. Pym and Dillon (1974) indicated that severe feed restriction during the rearing period followed by ad libitum feeding during the breeding period could be the best feeding regimen for broiler breeder hens. McDaniel et al. (1981) and Yu et al.

(1992a,b) suggested that feed restriction should occur during both the rearing and breeding periods for achieving optimum reproductive performance. Robbins et al. (1986, 1988) reported that restricting feed intake during the rearing period followed by ad libitum feeding during part or all of the breeding period increased egg production compared to birds that were feed restricted during both periods. Robinson et al. (1991) indicated, however, that ad libitum feeding during the breeding period resulted in lower egg production. More recently, Bruggeman et al. (1999) reported that ad libitum feeding from 1 to 7 weeks of age followed by feed restriction from 7 to 15 weeks of age followed by ad libitum feeding to first egg, resulted in improved reproductive performance compared to any other combination of ad libitum or restricted feeding during the rearing period.

3.1.1. Advantages of feed restriction

Feed restriction of broiler breeder pullets during the rearing period is important for achieving optimum reproductive performance. Relative to birds fed ad libitum a corn/soybean meal based diet, birds which have been fed restricted amounts of the same diet will produce less over-sized eggs (Fuller et al., 1969). In addition, broiler breeder hens that were provided feed ad libitum during their rearing period develop more large yellow preovulatory follicles at a given time than Leghorn laying hens (Hocking et al., 1987). The increased number of large follicles is often manifested as a double hierarchy with pairs of follicles of similar weights, which is linked to an increased incidence of the production of double-yolked eggs (Whitehead and Hocking, 1988). Full-fed broiler breeder hens also lay fewer eggs (Robbins et al., 1986; Katanbaf et al., 1989; Robinson et

al., 1991; Yu et al., 1992b), have a shorter production period (Fattori et al., 1991), lay more defective eggs and have more multiple ovulations in a single day (Fattori et al., 1991; Yu et al., 1992b) than pullets fed-restricted during both rearing and laying periods. Leeson and Summers (1982) also reported that early attainment of mature body size resulted in early onset of egg production and that birds, which obtained mature body size early, failed to maintain peak egg production. In addition, shell quality is significantly lower and egg weight significantly higher when pullets are not feed restricted (McDaniel, 1983).

3.1.2. Qualitative and quantitative feed restriction

Feed restriction can be achieved quantitatively and/or qualitatively. The main objective of these feeding practices is to achieve slower rates of growth while maintaining flock body weight uniformity. The traditional approach to feed restriction in broiler breeder pullets and hens has been a severe, quantitative feed restriction in which a small volume of a corn/soybean meal based diet is fed every day. However, when the volume of the feed recommended is too small to achieve uniform feed distribution, the feed from two days should be pooled and fed once every other day. Thus, on the days that the birds are fed, there is more feed available and therefore less competition among the birds. But, even with this skip-a-day feeding practice, maintaining body weight uniformity in the flock can be a problem since the aggressive birds tend to consume more of the available feed than weaker and more passive birds.

The severity of feed restriction of broiler breeder pullets can be reduced by feeding qualitatively deficient diets that are diluted with sand (Hogsette et al., 1976) or

with ground fiber sources such as ground oat hulls (Zuidhof et al., 1995). In addition, slower rates of growth can also be achieved by feeding birds diets that are low in sodium chloride, low in protein (Plavnik and Hurwitz, 1990), containing short-chain fatty acids (Pinchasov and Jensen, 1989), or containing high levels of zinc (Bartov, 1996). These low quality diets will reduce appetite, which decreases feed intake and therefore limits growth and improves flock body weight uniformity.

3.2. The importance of flock body weight uniformity

A problem with feed restriction is that the aggressive birds consume more than their share of the food, which leads to poor flock body weight uniformity (Lee et al., 1971; Blair et al., 1976). Flock body weight uniformity is critical for several reasons. A flock with uniform body weights can be managed more effectively since the nutrient requirements of all birds will be similar. More importantly, pullets with similar weights commence producing eggs at roughly the same time and will lay eggs that are more uniform in weight. Egg size is highly correlated with duration of incubation and chick weight at hatching. Eggs collected and incubated from a uniform flock of breeder hens will hatch at the same time and produce chicks of the same size and thus improve hatchery efficiency. Furthermore, broiler farmers that receive chicks of uniform weight are more likely to produce marketable broilers that are of uniform size, which is critical for the automated processing lines, which are adjusted to process birds in a specific size range.

To maximize the uniformity of body weights within a flock, it has been suggested that, when a feed restriction program is employed, adequate feeder space should be

provided (Hubbard Breeder Management Guide) to maximize the opportunity for all birds to have feeder access. Problems with bird weight uniformity can be avoided through very labor-intensive practices. Research has indicated that a flock of broiler breeder females weighed and rearranged in separate feeding groups according to body weight and then fed size-appropriate diets, allows for the use of feed restriction without an associated decrease in uniformity (Pettite et al., 1981). In practice, however, this management technique would be too costly, require too much housing space and be too difficult to efficiently manage.

3.3. Summary

Due to genetic advances, the modern broiler breeder must be feed restricted either qualitatively or quantitatively in order to prevent excessive body weight gain and subsequent reproductive inefficiency. Feed restriction, however, can lead to problems with flock body weight uniformity. Therefore, considerable research has been conducted and continues to be conducted in order to identify the best feed restriction practices to maximize reproductive efficiency and profitability in broiler breeder hen operations.

4. STATEMENT OF PURPOSE

In poultry diets, soybean meal is often used as the main protein source. However, in areas of the world where cotton is produced, CSM is an alternative protein source that is available in large quantities and at a competitive price. Nevertheless, the low levels of

lysine compared to soybean meal and the presence of the toxic compound gossypol have inhibited the widespread use of this feed ingredient in poultry diets. The overall goal of the present research is to enhance the acceptability and utilization of CSM in poultry production.

Bailey et al. (2000) found that broilers fed cottonseed with a higher (+) to (-) gossypol enantiomer ratio grew better than broilers fed cottonseed with a lower (+) to (-) enantiomeric ratio. This result indicated that the (-) enantiomer of gossypol might be the more toxic form of gossypol for broilers. Their work, however, did not conclusively establish that the (+) enantiomer is not toxic to broilers. Thus, one objective of the present research is to determine the exact toxicity of the (+) and the (-) enantiomer by feeding them individually in pure form to broilers, laying hens and broiler breeder hens.

When broiler breeder pullets are reared, they need to be feed restricted because of their propensity to grow at a very rapid rate. If broiler breeder pullets were not feed restricted they would become too large at sexual maturity for efficient reproduction. Cottonseed meal has lower levels of available lysine than soybean meal. Therefore, another objective of this research was to determine if broiler breeder pullet diets based on CSM as the main protein source rather than soybean meal, would naturally restrict pullet growth and allow more feed to be fed to the pullets.

The utilization of CSM in animal diets is often limited because the gossypol content of the meal is unknown. A rapid, on-site gossypol quantification method would be advantageous to feed manufacturers in selecting CSM with the lowest levels of gossypol. Therefore, the final objective of this research is to determine if NIRS can be used to accurately determine the gossypol content of CSM.

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CHAPTER 2

RELATIVE TOXICITY OF GOSSYPOL ENANTIOMERS IN BROILERS[1]

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ABSTRACT

Use of cottonseed meal in poultry diets has been avoided in large part because of the fear of gossypol toxicity. Gossypol exists naturally as a mixture of two enantiomers that are known to exhibit different biological activity. Two experiments were conducted to determine the relative toxicity of these gossypol enantiomers on broilers. In the first experiment, 3-d-old broilers were fed a standard diet containing 0, 100, 200, 300 or 400 mg of gossypol from gossypol acetic acid per kg of diet. This form of gossypol contains both enantiomers in an equimolar ratio. Each dietary treatment had six replicate pens of four birds each. In the second experiment, 3-d-old broilers were divided into 15 pens of four birds each and fed a standard diet supplemented with either no gossypol or one of the gossypol enantiomers at 200 or 400 mg/kg of diet. In both experiments, feed intake and body weight (BW) gain were measured. In addition, several organ and tissue samples were collected at 21 d (experiments 1 and 2) and at 42 d (experiment 1) of age and were analyzed for gossypol. In experiment 1, feed consumption and BW gain were reduced ($P < 0.05$) at 21 and 42 d for the birds fed the highest level of gossypol. The concentration of gossypol in the heart, kidney and plasma were equivalent at 21 and 42 d of age. In experiment 2, total feed consumption was only reduced in the birds consuming (-)-gossypol, but BW gains were lower for birds fed either enantiomer. However, (-)-gossypol was more detrimental to growth than (+)-gossypol. The liver had the highest tissue concentration of both enantiomers and accumulation of (+)-gossypol was higher than (-)-gossypol in all tissues examined. Racemization of the enantiomers did not occur in the tissues analyzed. The results indicate that both gossypol enantiomers are toxic to

broilers, but that (-)-gossypol is more detrimental to efficient broiler production than (+)-gossypol.

Key words: cottonseed meal, gossypol, gossypol enantiomers, broilers

INTRODUCTION

Cottonseed meal (CSM) could be an attractive alternative protein source for poultry diets, but concern over the presence of the potentially toxic agent, gossypol, has limited its utilization. Gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3, 3'-dimethyl-(2, 2'- binaphthalene)-8, 8'-dicarboxaldehyde] is a polyphenolic compound located in the pigment glands that are distributed throughout the cotton plant. Gossypol is composed of two naphthalene rings with restricted rotation around the bond connecting the rings. As a result of this restricted rotation, gossypol occurs naturally as a mixture of two enantiomers [(+)- and (-)-gossypol] that differ in their optical properties (Huang et al., 1987).

Researchers previously reported that feeding broilers diets containing high levels of gossypol resulted in depressed weight gain (Lillie and Bird, 1950; Milligan and Bird, 1951; Phelps, 1966; Waldroup, 1981) and poor feed efficiency (Couch et al., 1955; Heywang and Bird, 1955). Therefore, extensive research has been conducted to establish methods to decrease the negative impacts that gossypol might have on poultry production. Weight gain and feed efficiency were unaffected when broilers were fed a diet in which a portion of the dietary soybean meal was replaced with CSM made from glandless cottonseed, which has a very low concentration of gossypol (Waldroup et al., 1968). Thus, the development of strains of cotton with glandless seeds should alleviate the problems associated with feeding CSM to broilers (Ryan et al., 1986). However, gossypol serves as a natural insecticide in the cotton plant (Bottger et al., 1964) and therefore it is desirable that the plant retains the ability to produce gossypol in its other tissues. At present, all the CSM produced commercially in the United States contains gossypol.

Another avenue of research to lower the impact of gossypol on poultry production has dealt with dietary iron supplementation. The presence of gossypol in poultry diets may be counteracted by the addition of highly soluble iron salts that bind gossypol (Withers and Brewster, 1917; Gallup, 1928; Eagle, 1949). The gossypol-related brown-yolk discoloration of eggs produced by laying hens fed diets containing CSM can be prevented when crystalline ferrous sulphate heptahydrate is added to the diet at a 4:1 weight ratio of iron to free gossypol (Panigrahi, 1990; Panigrahi and Morris, 1991; Panigrahi and Plumb, 1996). However, iron supplementation is costly, contributes to the heavy metal content of the feces and can depress bird performance by reducing the availability of dietary phosphorus (Panigrahi and Plumb, 1996).

Little research attention has been focused on the relative toxicity of the individual gossypol enantiomers in chickens. Poultry experiments conducted to determine the relative toxicity of the enantiomers have only been done with cotton cultivars that produce seeds with different proportions of (+)- and (-)-gossypol. A study conducted with broilers indicated that ground Pima (Gossypium barbadense) cottonseed with a higher (-) to (+) ratio of gossypol was significantly more toxic, based on feed intake and body weight gain than ground Pima cottonseed with a higher (+)-to-(-) ratio of gossypol (Gamboa, 1997). This was later confirmed when broilers fed Moco crushed cottonseed containing a high (+)- to (-)-gossypol enantiomer ratio performed better than those receiving Moco cottonseed with a lower (+)- to (-)-gossypol enantiomer ratio (Bailey et al., 2000). These results suggest that CSM containing a higher proportion of (+)-gossypol relative to (-)-gossypol would be more desirable for broiler production. Nevertheless, for a definitive determination of the relative toxicity of the two enantiomers

in chickens, they would have to be fed individually in pure form. Therefore, in the present study, gossypol acetic acid (GAA), which contains an equal molar ratio of the enantiomers, and the individual gossypol enantiomers were fed to broilers. Because some gossypol amine complexes have been reported to racemize (Si et al., 1990; Fish et al., 1995), the possibility of interconversion of the enantiomers in broiler tissues was also investigated.

MATERIALS AND METHODS

Experiment 1

Two hundred 1-d-old Cobb by Cobb male broiler chicks were fed a standard corn/soybean meal based mash starter diet (Table 2.1) for 2 d. At 3 d of age, the chicks were weighed and those with extreme weights discarded. The remaining birds were assigned to 30 pens of 4 birds each such that the weight profile of each pen was similar. Chicks were wing-banded for individual identification. The 30 pens were randomly split into 5 dietary treatments that consisted of the starter diet supplemented with either 0, 100, 200, 300 or 400 mg/kg of gossypol from GAA. Throughout the experiment, chicks were given free access to water and mash experimental diets, brooded in thermostatically controlled batteries[1] with raised wire floors and reared on a 24-h lighting schedule. The Institutional Animal Care and Use Committee of the University of Georgia approved all animal procedures.

The GAA utilized in this experiment was made as previously described (Dowd and Pelitire, 2001) and contained 89.64% gossypol based on HPLC analysis. This

[1] Petersime Incubator Co., Gettysburg, OH.

percentage was used in calculating the amount of GAA added to the experimental diets. Because GAA contains a 50:50 mixture of (+)- and (-)-gossypol, the concentration of the individual enantiomers was 0, 50, 100, 150 and 200 mg/kg of diet. Each of the diets for the different dietary treatments was mixed on a daily basis to minimize binding of gossypol to other feed components. In order to monitor the stability of gossypol in the feed, feed samples were collected at 0, 4, 8 and 24 h after mixing and frozen at -80 ºC for future determination of free gossypol.

At 21 d of age birds from half of the replicate pens were killed, and the liver, heart, kidney and a portion of the left pectoralis major muscle were collected. The organs were pooled by pen, weighed, and immediately frozen at –80 ºC for future gossypol analysis. Blood was collected from the brachial vein and placed on ice. Heparinized blood samples were then centrifuged for 10 min at 3000 x *g*. Plasma was collected from each sample and frozen at -80 ºC for future gossypol analysis. The remaining birds were transferred to pens in a grower battery and fed daily a standard grower diet (Table 2.1) supplemented with the appropriate levels of gossypol. At 42 d of age, the liver, heart, kidney, testis, a portion of the left pectoralis major muscle, and the blood were removed from the remaining birds. Feed consumption and mortality were recorded daily and BW was determined weekly throughout the experiment.

Experiment 2

The protocol for this experiment was similar to experiment 1, except that 15 pens of 4 birds each were split into 5 dietary treatments that consisted of the starter diet supplemented with either no gossypol or 200 or 400 mg/kg of each gossypol enantiomer.

Tissue samples were collected from the broilers at 21 d of age. The individual gossypol enantiomers were prepared as previously described (Dowd, 2003). The optical purity of the gossypol enantiomers was at least 99.5% based on HPLC analysis. During this experiment, total water consumption for a 24 h period was determined on days 11, 16, and 20 for each pen. In addition, total bile volume and blood packed cell volume were determined for each bird at the end of the experiment. In order to reduce variation in bile amounts related to feed intake, the birds were fasted for 12 h before the contents of the gallbladder of each bird were collected with a needle and syringe. Blood was collected in two heparinized capillary tubes from the brachial vein of each bird. The capillary tubes were immediately centrifuged in a microcapillary centrifuge and percent packed cell volume was determined with a microcapillary reader.[2]

Bile Analysis

Bile dry matter was determined (AOAC, 1970) for each bird from duplicate samples of 100 µL each. To detect differences in bile pigment concentration, 20 µL of bile from each bird was diluted with 3,980 µL of deiodinized water and the absorbance at 625 nm was determined with a DU-530 spectrophotometer.[3] The remaining bile collected from each bird was frozen at –80 ºC for future gossypol determination.

[2] International Equipment Company, Needham Heights, MA.
[3] Beckmann Coulter, Fullerton, CA.

Gossypol Determination

Feed samples were assessed for free gossypol content by the official method of the American Oil Chemists' Society (AOCS, 1985). Tissues were freeze-dried for 48 h (which was sufficient to reach a constant dry weight). Total gossypol and (+)- and (-)-gossypol concentrations in tissues and feed samples were determined by HPLC as previously described (McMillan, 2000).

Statistical Analyses

Data from each experiment was subjected to ANOVA according to the General Linear Model procedure. Tukey's multiple-comparison procedure (SAS Institute, 2001) was used to detect significant differences among diets (experiments 1 and 2) and bird age (experiment 1). For experiment 1, regression analyses were conducted to test for linear and quadratic effects in broiler performance and tissue gossypol concentrations. The resulting regression models were then reduced using a stepwise statistical procedure to eliminate non-significant effects in order to determine appropriate linear and quadratic prediction equations (SAS Institute, 2001). All statistical procedures were done with SAS® statistical software package (SAS Institute, 2001), and differences were considered significant when P-values were less than 0.05.

RESULTS

Experiment 1

Chicks fed the diet containing 400 mg of gossypol/kg weighed less ($P < 0.05$) at 21 and 42 d of age than chicks fed diets containing 0, 100 or 200 mg/kg gossypol (Table 2.2). At 21 and 42 d of age total feed consumption was lower ($P < 0.05$) for birds fed the diets containing 300 and 400 mg/kg gossypol than for birds fed the diet containing no gossypol (Table 2.2). As the level of dietary gossypol increased, there was a linear decrease in feed intake (Table 2.2). None of the birds died during the experiment. The free gossypol content of each of the diets did not decrease during the 24 h between the daily preparations (data not shown).

Organ weights relative to body weight (BW) were unaffected by gossypol supplementation at 21 and 42 d except for a decrease in relative heart size at 42 d for birds fed the 400 mg/kg diet (data not shown). Both gossypol enantiomers were detected in all the tissues examined (Tables 2.3 and 2.4). Although the diets contained equal concentrations of (-)- and (+)-gossypol, the concentration of (+)-gossypol was greater than (-)-gossypol in all tissues examined (Tables 2.3 and 2.4). With the exception of liver, the tissue concentrations of either enantiomer did not increase from 21 d to 42 d of age (Tables 2.3 and 2.4). As dietary gossypol concentrations increased, concentrations of (-)- and (+)-gossypol at 42 d of age increased linearly in the heart, kidney, muscle and plasma, however, in the liver, the concentration increased quadratically (Tables 3 and 4).

Experiment 2

Feeding either enantiomer depressed growth, but the highest dietary concentration of (-)-gossypol was the most detrimental (Table 2.5). Dietary supplementation with (+)-gossypol did not impact feed consumption, but the addition of (-)-gossypol did reduce feed intake of broiler chicks (Table 2.5). Water consumption adjusted for feed intake was not different between the dietary treatments (data not shown). Only one bird died during the experiment, on day 4 in the group fed the diet with 200 mg (-)-gossypol/kg of diet. The weight of testes, heart and kidney relative to BW were unaffected by dietary gossypol (Table 2.6). Compared to the control birds, relative liver weight increased ($P < 0.05$) in chicks fed the highest concentration of (+)-gossypol and decreased ($P < 0.05$) in chicks fed the highest level of (-)-gossypol (Table 2.6).

Liver had the highest accumulation of both gossypol enantiomers followed by bile, spleen, kidney, testes, heart, plasma and muscle (Table 2.7). The accumulation of the (+)-gossypol was higher ($P < 0.05$) than that of (-)-gossypol in all of the tissues examined except for the bile (Table 2.7). Only the enantiomer that was fed was detected in the tissues.

The amount of bile contained in the gallbladder was greater ($P < 0.05$) in birds fed the 200 and 400 mg levels of (+)-gossypol and 400 mg level of (-)-gossypol than from the control birds (Table 2.6). Bile concentrations as determined by dry matter (DM) and spectrophotometric analyses, however, were not different between the dietary treatments (data not shown). Packed cell volumes were 35.1 ± 0.8, 32.2 ± 0.4, 32.4 ± 0.8, 32.2 ± 0.4, and 32.3 ± 0.7 % respectively, for the birds fed diets containing 0, 200 (-), 400 (-), 200 (+) and 400 (+) mg of gossypol/kg of diet. Birds fed both dietary concentrations of

either enantiomer had lower ($P < 0.05$) packed cell volumes than birds fed the control diet.

DISCUSSION

The results of the GAA study were consistent with the widely reported idea that dietary gossypol from CSM negatively impacts weight gain and feed efficiency in broilers (Lillie and Bird, 1950; Milligan and Bird, 1951; Couch et al., 1955; Heywang and Bird, 1955; Phelps, 1966; Waldroup, 1981). The current results were inconsistent, however, with a previous experiment (Henry et al., 2001) where similar levels of partially purified gossypol were fed to broilers, but no decrease in BW and feed consumption was noted. Henry et al. (2001) did report, however, a significant decrease in BW gain and feed intake when they fed broilers diets containing 800 and 1600 mg gossypol/kg of diet.

Joseph et al. (1986) reported that the amount of (-)-gossypol required to produce cytotoxicity in human tumor-derived cells was approximately 10% of that required with (+)-gossypol. Furthermore, Wang and coworkers (1987) reported that (-)-gossypol had an antifertility effect in male rats while (+)-gossypol had neither an antifertility nor a toxicity effect at the same dose. In the present research, (-)-gossypol had a greater detrimental effect on feed consumption and BW gain than (+)-gossypol. Previous work utilizing CSM with a high (+)-to-(-) enantiomer ratio also indicated that (-)-gossypol might be more toxic to broilers than (+)-gossypol (Gamboa et al., 1997; Bailey et al., 2000). Therefore, it would be preferable to use a CSM with a high (+)-to-(-) enantiomer ratio in broiler diets as it would be less detrimental to feed intake. Our results indicate, however, that feeding diets containing

400 mg of (+)-gossypol per kg of diet also impact broiler growth, possibly due to its higher level of accumulation in the tissues of the birds. Thus, the development of a strain of cotton with a high (+)-to-(-) enantiomer ratio would decrease but not eliminate the negative impacts of gossypol on broilers fed diets containing CSM. Although gossypol plays a role in protecting the cotton plant from pathogens, altering the enantiomeric ratio in cotton plants may not adversely affect the resistance to certain fungal pathogens (Puckhaber et al., 2002).

Fish et al. (1995) reported that a pure preparation of either individual gossypol amine enantiomer was converted to an equilibrium mixture of both enantiomers after 48 h of exposure to sunlight. Racemization of gossypol amine condensates has also been observed to occur in the dark, albeit at a slower rate (Si et al., 1990). In the present study, no evidence of racemization was detected in any of the broiler tissues tested. This lack of gossypol racemization in broilers suggests that the conversion may occur for only specific gossypol amine complexes or that the reaction is inhibited in aqueous environments. The apparent lack of racemization of gossypol in tissues supports the idea of incorporating CSM with a high (+)-to-(-) ratio of gossypol in non-ruminant feed.

In the present research, accumulation of (+)-gossypol was typically twice that of (-)-gossypol in all broiler tissues examined. In lambs fed cottonseeds (Kim et al., 1996) and rats fed pure gossypol enantiomers (Chen et al., 1987) some tissues had higher levels of (+)-gossypol than (-)-gossypol but other tissues had either the reverse situation or equal concentrations of the two enantiomers. Chen et al., (1987) determined that (-)-gossypol had a shorter half-life and a higher clearance rate than (+)-gossypol in rats fed either pure (+)- or (-)-gossypol. Based on the shorter half-life, Chen and coworkers (1987) speculated that the toxicity observed with the (-)-gossypol might be attributed to

its metabolites. Thus, in broilers, the lower tissue concentrations of (-)-gossypol compared with (+)-gossypol may also result from a higher rate of clearance. In addition to possible differences in clearance, the lower tissue concentrations of (-)-gossypol in broilers might also be attributed to differences in intestinal absorption or in the rate of conversion to other metabolites between the two gossypol enantiomers.

Both experiments suggested that when broilers are fed a fixed dietary gossypol concentration, tissue gossypol levels rise until they reach a maximum threshold. With the exception of the liver, tissue concentrations of the enantiomers did not continue to accumulate between 21 and 42 d of age, and in fact in some tissues, the levels of (-)-gossypol were lower at 42 d than at 21 d. Previous research that involved long term feeding trials with diets containing gossypol in fish (Roehm et al., 1967), rats (Jensen et al., 1982) and broiler breeder pullets (Lordelo et al., 2004) also indicated that tissue gossypol levels plateau after a few weeks of feeding the treatment diets.

Gossypol accumulation was highest in the liver of broilers fed gossypol from GAA. This result agrees with previous reports in fish (Roehm et al., 1967), lambs (Kim et al., 1996), rats (Sharma et al., 1966; Chen et al., 1987) and broilers (Gamboa et al., 2001). Feeding diets containing gossypol also resulted in increased liver size in broilers (Henry et al., 2001), lambs (Morgan et al., 1988; Kandylis et al., 1998) and rats (Jensen et al., 1982). In the case of broilers, the current results indicate that the increased liver weight observed when feeding gossypol from GAA is due to (+)-gossypol and not (-)-gossypol. Finally, bile production was greater in broilers fed either (+)- or (-)-gossypol, which probably indicates that both enantiomers are eliminated from the liver via bile.

Sharma et al. (1966) also suggested that bile served as an important excretion route for gossypol in pigs.

The lower packed red blood cell volume in birds fed both enantiomers was not unexpected since dietary gossypol has been found to reduce iron absorption and its bioavailability for hemoglobin formation in several animal species (Herman and Smith, 1973; Clawson et al., 1975; Berardi and Goldblatt, 1980).

In summary, the present studies indicate that both enantiomers of gossypol can adversely affect performance in broilers, however, the toxicity is considerably more severe with (-)-gossypol due to its detrimental impact on feed intake. Unlike some species, (+)-gossypol accumulates in broiler tissues to a greater extent than (-)-gossypol, suggesting that (-)-gossypol may be cleared from the body more quickly, absorbed from the intestine at a lower rate or more rapidly converted to a metabolite than (+)-gossypol. Finally, the results suggest that utilizing CSM with a high ratio of (+)- to (-)-gossypol in broiler diets would be preferable for production efficiency.

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TABLE 2.1. Composition of the experimental diets (experiments 1 and 2)

Ingredient	Diet	
	Broiler starter [1]	Broiler grower [2]
	(g/100g of diet DM)	
Corn	56.12	60.80
Soybean meal, 48% CP	37.50	32.61
Poultry fat	3.07	3.43
Defluorinated phosphorus	1.75	1.56
Limestone	0.73	0.78
Salt	0.30	0.32
Vitamin mix [3]	0.25	0.25
DL- Methionine	0.20	0.17
Mineral mix [4]	0.08	0.08
Calculated analysis [5]		
M. E. (kcal/ kg)	3080.00	3150.00
Crude protein (%)	22.50	20.50
Crude fat (%)	5.28	5.76
Crude fiber (%)	2.53	2.50
Lysine (%)	1.26	1.12
Calcium (%)	0.95	0.90
Available phosphorus (%)	0.45	0.41
Methionine (%)	0.57	0.52

[1]Broiler starter diet utilized in experiments 1 and 2 from 0 to 3 wk of age.
[2]Grower diet utilized in experiment 1 from 3 to 6 wk of age.
[3]Vitamin mix provided the following per 100 g of diet: vitamin A, 551 IU; vitamin D_3, 110 IU; Vitamin E, 1.1 IU; vitamin B_{12}, 0.001mg; riboflavin, 0.44 mg; niacin, 4.41 mg; d-pantothenic acid, 1.12 mg; choline, 19.13 mg; menadione sodium bisulfate, 0.33 mg; folic acid, 0.55 mg; pyridoxine HCl, 0.47 mg; thiamin, 0.22 mg; d-biotin, 0.011 mg; and ethoxyquin, 12.5 mg.
[4]Mineral mix provided the following in milligrams per 100 g of diet: Mn, 6.0; Zn, 5.0; Fe, 3.0; I, 1.5; and Se, 0.5.
[5]Calculated analysis was based on Dale (2001).

TABLE 2.2. Body weight, feed consumption and feed conversion at 21 d and 42 d of age in broilers fed varying levels of gossypol from gossypol acetic acid (experiment 1)

Dietary gossypol	Body weight (g)[1]		Total feed consumption (g)[1]		Feed conversion ratio (g/g)[1]	
(mg/kg)	21 d	42 d	21 d	42 d	21 d	42 d
0	838 ± 10[a]	2545 ± 41[a]	1111 ± 7.8[a]	3967 ± 63.8[a]	1.44 ± 0.02[bc]	1.60 ± 0.02[a]
100	829 ± 14[a]	2379 ± 60[a]	1060 ± 21.5[ab]	3630 ± 40.4[ab]	1.39 ± 0.01[c]	1.57 ± 0.02[a]
200	807 ± 6[ab]	2313 ± 27[a]	1062 ± 7.6[ab]	3589 ± 93.4[abc]	1.44 ± 0.01[bc]	1.60 ± 0.03[a]
300	758 ± 12[bc]	2154 ± 138[ab]	1025 ± 11.3[bc]	3290 ± 115.8[bc]	1.49 ± 0.01[ab]	1.58 ± 0.02[a]
400	711 ± 17[c]	1921 ± 111[b]	967 ± 20.0[c]	3113 ± 28.8[c]	1.50 ± 0.01[a]	1.69 ± 0.06[a]
Regression analysis						
r^2	0.75	0.73	0.62	0.76	0.47	-
	---------- Coefficients ----------					
Intercept	836.79[†]	2557.17[†]	1109.21[†]	3927.65[†]	1.42[†]	-
Linear (gossypol)	-	-1.47[†]	-0.32[†]	-2.05[†]	-	-
Quadratic (gossypol)	0.00[†]	-	-	-	0.00[†]	-

[a-c]Values without a common letter superscript differ; $P < 0.05$.

[†]Regression coefficient value is significant; $P < 0.05$.

[1]Values are means ± SEM per bird with 6 replicate pens of 4 birds at 21 d and 3 replicate pens of 4 birds at 42 d for each diet.

TABLE 2.3. Concentrations at 21 d and 42 d of age of (-)-gossypol in tissues from broilers fed varying levels of gossypol from gossypol acetic acid (experiment 1)

Dietary gossypol (mg/kg)	Liver		Heart		Kidney		Muscle		Plasma	
	21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d
	(µg/g DM)[1,2]								(µg/mL)[1,2]	
100	61 ± 2.9[c]	82 ± 10.9[c*]	9 ± 1.2[b]	18 ± 4.3[b]	9 ± 0.4[c]	7 ± 0.6[c*]	2 ± 0.2[c]	2 ± 0.3[b]	4 ± 0.2[d]	5 ± 0.6[c]
200	101 ± 2.9[b]	140 ± 4.8[b*]	20 ± 1.9[b]	30 ± 5.7[ab]	20 ± 0.8[b]	16 ± 0.6[b*]	4 ± 0.4[bc]	2 ± 0.3[ab*]	9 ± 0.5[c]	8 ± 0.3[b]
300	154 ± 5.5[a]	203 ± 4.1[a*]	34 ± 2.8[a]	32 ± 1.6[ba]	32 ± 1.9[a]	20 ± 1.4[b*]	6 ± 0.5[ab]	4 ± 0.2[ab*]	13 ± 0.9[b]	11 ± 0.6[a]
400	191 ± 5.1[ab]	176 ± 10.2[a]	45 ± 3.9[a]	43 ± 3.3[a]	40 ± 3.2[a]	28 ± 2.2[a*]	7 ± 0.5[a]	5 ± 0.9[a]	16 ± 0.4[a]	12 ± 0.6[a*]
Regression analysis										
r^2	0.98	0.89	0.93	0.67	0.95	0.92	0.89	0.77	0.97	0.92
	Coefficients									
Intercept	16.62[†]	-42.36	-3.27	11.42[†]	-1.31	1.13	-0.02	0.18	-2.30	3.69[†]
Linear (gossypol)	0.44[†]	1.41[†]	0.12[†]	0.08[†]	0.11[†]	0.07[†]	0.02[†]	0.01[†]	0.07[†]	0.02[†]
Quadratic (gossypol)	-	0.00[†]	-	-	-	-	-	-	0.00[†]	-

[a-e]Values within a column without a common letter superscript differ; $P < 0.05$.

*Values for a tissue differ significantly between 21 and 42 d of age; $P < 0.05$.

[†]Regression coefficient value is significant; $P < 0.05$.

[1]Values are means ± SEM per bird with 6 replicate pens of 4 birds at 21 d and 3 replicate pens of 4 birds at 42 d for each diet.

[2]No gossypol was detected in the tissues of birds fed 0 mg of gossypol/kg of diet.

TABLE 2.4. Concentrations at 21 d and 42 d of age of (+)-gossypol in tissues from broilers fed varying levels of gossypol from gossypol acetic acid (experiment 1)

Dietary gossypol (mg/kg)	Liver		Heart		Kidney		Muscle		Plasma	
	21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d	21 d	42 d
	(µg/g DM)[1,2]								(µg/mL)[1,2]	
100	200 ± 4.7[d]	303 ± 24.2[c*]	14 ± 1.7[d]	27 ± 6.6[c]	32 ± 2.7[c]	30 ± 1.3[d]	4 ± 0.7[d]	5 ± 0.3[b]	8 ± 0.1[d]	11 ± 1.3[c]
200	353 ± 8.8[c]	554 ± 19.8[b*]	30 ± 1.9[c]	44 ± 5.4[cb]	61 ± 4.4[b]	58 ± 2.3[c]	9 ± 0.5[c]	8 ± 0.3[ab]	18 ± 1.2[c]	17 ± 0.5[b]
300	540 ± 21.7[b]	828 ± 16.9[a*]	49 ± 2.7[b]	54 ± 0.4[ba]	94 ± 4.4[a]	78 ± 4.9[b]	13 ± 0.6[b]	11 ± 0.8[ab]	25 ± 1.1[b]	23 ± 1.6[a]
400	686 ± 26.0[a]	827 ± 29.4[a*]	61 ± 1.4[a]	64 ± 2.5[a]	114 ± 8.3[a]	99 ± 4.6[a]	16 ± 0.3[a]	15 ± 3.0[a]	32 ± 0.4[a]	27 ± 1.4[a*]
Regression analysis										
r^2	0.98	0.96	0.97	0.81	0.94	0.96	0.97	0.73	0.98	0.91
	Coefficients									
Intercept	33.05	-148.87	-1.26	17.02[†]	5.13	9.07	-1.85	1.61	-4.20	6.00[†]
Linear (gossypol)	1.65[†]	4.99[†]	0.16[†]	0.12[†]	0.28[†]	0.23[†]	0.07[†]	0.03[†]	0.13[†]	0.05[†]
Quadratic (gossypol)	-	0.00[†]	-	-	-	-	0.00[†]	-	0.00[†]	-

[a-e]Values within a column without a common letter superscript differ; $P < 0.05$.

*Values for a tissue differ significantly between 21 and 42 d of age; $P < 0.05$.

[†]Regression coefficient value is significant; $P < 0.05$.

[1]Values are means ± SEM per bird with 6 replicate pens of 4 birds at 21 d and 3 replicate pens of 4 birds at 42 d for each diet.

[2]No gossypol was detected in the tissues of birds fed 0 mg of gossypol/kg of diet.

TABLE 2.5. Body weight, feed consumption and feed conversion at 21 d of age for birds fed (+)- or (-)-gossypol (experiment 2)

Dietary gossypol (mg/kg)	Body weight (g)[1]	Total feed consumption (g)[1]	Feed conversion (g/g) [1]
0	723 ± 9.0[a]	935 ± 15.57[a]	1.39 ± 0.02[a]
200 (-)	643 ± 13.6[b]	801 ± 21.04[bc]	1.35 ± 0.02[a]
400 (-)	512 ± 5.0[c]	632 ± 25.50[c]	1.37 ± 0.05[a]
200 (+)	666 ± 14.4[b]	845 ± 21.54[ab]	1.38 ± 0.01[a]
400 (+)	672 ± 5.1[b]	871 ± 11.61[ab]	1.40 ± 0.01[a]

[a-c]Values within a column without a common superscript differ; $P < 0.05$.
[1]Values are means ± SEM per bird with 3 replicate pens of 4 birds for each diet.

TABLE 2.6. Organ weights at 21 d of age for broilers fed (+)- or (-)-gossypol (experiment 2)

Dietary gossypol	Heart	Liver	Testes	Kidney	Spleen	Bile
(mg/kg)	----------(g/100 g BW)[1] ----------					---(mL/100 g BW)[1]---
0	0.61 ± 0.03	2.35 ± 0.09^{bc}	0.03 ± 0.005	0.34 ± 0.01	0.10 ± 0.01^{ab}	0.07 ± 0.01^{b}
200 (-)	0.60 ± 0.03	2.21 ± 0.08^{bc}	0.02 ± 0.002	0.31 ± 0.02	0.10 ± 0.02^{ab}	0.09 ± 0.01^{ab}
400 (-)	0.62 ± 0.04	2.07 ± 0.12^{c}	0.02 ± 0.002	0.33 ± 0.03	0.09 ± 0.01^{b}	0.11 ± 0.01^{a}
200 (+)	0.65 ± 0.07	2.50 ± 0.15^{ab}	0.02 ± 0.004	0.35 ± 0.04	0.10 ± 0.01^{ab}	0.11 ± 0.02^{a}
400 (+)	0.58 ± 0.02	2.73 ± 0.09^{a}	0.02 ± 0.002	0.36 ± 0.02	0.13 ± 0.01^{a}	0.11 ± 0.01^{a}

$^{a-c}$Values within a column without a common letter superscript differ; $P < 0.05$.

[1]Values are means ± SEM per bird with 3 replicate pens of 4 birds for each diet.

TABLE 2.7. Concentration at 21 d of age of (+)- and (-)-gossypol in tissues from broilers fed the pure gossypol enantiomers (experiment 2)

Dietary gossypol (mg/kg)	Liver	Spleen	Kidney	Testes	Heart	Muscle	Plasma	Bile
	----------(µg/g DM)[1,2,3]----------						------(µg/mL)[1,2,3]-----	
200 (-)	354 ± 11[b*]	52 ± 5[b*]	40 ± 3[*]	43 ± 13[*]	44 ± 3[b*]	10 ± 1[b*]	17 ± 1[b*]	291 ± 53
400 (-)	566 ± 58[a*]	113 ± 11[a*]	82 ± 19[*]	21 ± 12[*]	70 ± 7[a*]	15 ± 1[a*]	29 ± 2[a*]	377 ± 32
200 (+)	878 ± 57[x]	149 ± 9[x]	152 ± 27	137 ± 24	70 ± 6[x]	25 ± 1[x]	36 ± 1[x]	430 ± 42
400 (+)	1176 ± 69[y]	251 ± 16[y]	210 ± 4	195 ± 21	111 ± 4[y]	40 ± 0[y]	55 ± 0[y]	540 ± 72

[a-b] Values for (-)-gossypol for a given tissue without a common superscript differ; $P < 0.05$.
[x-y] Values for (+)-gossypol for a given tissue without a common superscript differ; $P < 0.05$.
[*] Values for (-)-gossypol are different than the corresponding values of (+)-gossypol; $P < 0.05$.
[1]Values are means ± SEM per bird with 3 replicate pens of 4 birds for each diet.
[2]No gossypol was detected in the tissues of birds fed 0 mg of gossypol/kg of diet.
[3]There was no (-)-gossypol detected in any of the tissues from birds fed (+)-gossypol and there was no (+)-gossypol detected in any of the tissues from birds fed (-)-gossypol.

CHAPTER 3

RELATIVE TOXICITY OF GOSSYPOL ENANTIOMERS IN LAYING AND BROILER BREEDER HENS[1]

[1] Lordelo, M. M., A. J. Davis, M. C. Calhoun, M. K. Dowd, and N. M. Dale. To be submitted to *Poultry Science*.

ABSTRACT

Gossypol, a natural but toxic component of cottonseed meal, exists in either a positive (+) or a negative (-) enatiomeric form. Two experiments were conducted to determine the relative toxicity of the individual gossypol enantiomers on laying and broiler breeder hens. In the first experiment, 25 individually caged Hy-Line W36, 43 wk-old, layers were fed a standard diet supplemented with either no gossypol or the individual pure enantiomers at 200 and 400 mg/kg of diet for 20 d (5 pens / treatment). In experiment 2, 15 Cobb 500 fast feathering, 44 wk-old, broiler breeder hens were fed a standard diet supplemented with either no gossypol or the individual pure enantiomers at 400 mg/kg of diet for 18 d (5 pens / treatment). In both experiments, feed intake, egg production and egg weight were monitored daily. All eggs were individually opened and scored for yolk discoloration. At the end of experiment 1, several organ and tissue samples were collected for gossypol analyses. In both experiments, egg production was lower and finally ceased in birds fed the diets containing (+)-gossypol. Only laying and broiler breeder hens fed (+)-gossypol produced eggs with severe yolk discoloration. Total feed intake was lower in layers fed the 400 mg/kg level of (+)-gossypol compared to laying hens fed the other dietary treatments. In contrast, broiler breeder hens consumed less ($P < 0.05$) of the diet supplemented with (-)-gossypol. In experiment 1, tissue accumulation of (+)-gossypol was higher than (-)-gossypol. The results indicate that although egg yolk discoloration is caused by ingestion of (+)-gossypol in both laying and broiler breeder hens, the effect of the gossypol enantiomers on feed consumption varies with bird genetics.

Key words: gossypol enantiomers, laying hens, broiler breeder hens, yolk discoloration

INTRODUCTION

Cottonseed meal (CSM) could be an attractive alternative protein source for poultry diets, but concern over the presence of the potentially toxic agent, gossypol, has limited its utilization. Gossypol [1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3, 3'-dimethyl-(2, 2'- binaphthalene)-8, 8'-dicarboxaldehyde] is a polyphenolic metabolite produced and primarily stored in the pigment glands that are distributed throughout the cotton plant (Heinstein et al., 1977). Gossypol is composed of two naphthalene rings with restricted rotation around the bond connecting the rings. As a result, gossypol occurs naturally as a mixture of two enantiomers [(+)- and (-)-gossypol] that differ in their optical properties (Huang et al., 1987).

Although CSM is substantially lower in energy and protein than soybean meal, it can still be used successfully in poultry diets. Based on its nutrient profile, CSM is actually a more valuable feed ingredient in laying hen diets than in broiler diets, since layers have lower energy and protein requirements than broilers. The laying hen, however, is more sensitive to the ingestion of CSM containing gossypol than broilers (Waldroup, 1981). Laying hens fed diets containing gossypol can produce eggs that have brown yolk discoloration (Phelps, 1966; Reid et al., 1984; Panigrahi, 1990; Panigrahi and Plumb, 1996; Davis et al., 2002).

The toxic effects of gossypol in poultry diets may be alleviated by the addition of highly soluble iron salts that bind gossypol (Withers and Brewster, 1917; Gallup, 1928; Eagle, 1949; Panigrahi et al., 1989; Panigrahi and Morris, 1991). The gossypol-related brown yolk discoloration of eggs produced by laying hens fed diets containing CSM can

be prevented when crystalline ferrous sulphate heptahydrate is added to the diet at a 4:1 weight ratio of iron to free gossypol (Panigrahi, 1990; Panigrahi and Morris, 1991; Panigrahi and Plumb, 1996). However, iron supplementation can depress bird performance by reducing the availability of dietary phosphorus (Panigrahi and Plumb, 1996), is costly and contributes to the heavy metal content of the feces.

Recently, Lordelo et al. (2005) reported that feeding broilers a diet supplemented with pure (+)-gossypol was less detrimental to broiler performance than feeding a diet containing (-)-gossypol. They suggested that developing genetic strains of cotton with a high (+) to (-) enantiomeric ratio would be beneficial for the utilization of CSM in broiler production. Since CSM is a more advantageous feed ingredient for laying hen diets, the goal of the present research was to determine if feeding the (+) enantiomer of gossypol to laying and broiler breeder hens has no impact on egg production and yolk quality.

MATERIALS AND METHODS

Experiment 1

Previous research has indicated that some genetic strains and individual laying hens are less susceptible to the effects of gossypol ingestion (Heywang et al., 1954; Panigrahi et al., 1989; Panigrahi and Morris, 1991; Davis et al., 2002). Therefore, in the present experiment, 100 individually caged, 32 wk-old, Hy-line W36 laying hens were fed a layer diet containing 30% CSM (Table 3.1) for 4 wk to determine their susceptibility to gossypol. Eggs were collected daily from each bird and then stored at 4 ºC for 14 d to enhance yolk discoloration (Heywang et al., 1954; Phelps, 1966). After

storage, eggs were individually opened and the degree of yolk discoloration caused by gossypol was estimated visually using a scale of 0 through 10 as previously described (Davis et al., 2002). Hens consistently producing eggs with a score above 3 were considered to be gossypol sensitive.

After the hens were fed the CSM diet for 4 wk, they were given a standard layer feed with 0% CSM (Table 3.1) for 7 wk. This period of time allowed for the excretion of gossypol from body tissues (Lordelo et al., 2004). At 43 wk of age, 25 hens that had been determined to be sensitive to gossypol were weighed and randomly assigned to one of 5 dietary treatments that consisted of the standard layer diet (Table 3.1) supplemented with either no gossypol or the pure individual enantiomers at 200 and 400 mg/kg of diet. Throughout the 20 d experimental period, hens were given free access to water and the mash experimental diets and maintained on their previous daily lighting schedule of 17 h light, 7 h dark. The Institutional Animal Care and Use Committee of the University of Georgia approved all animal procedures.

The individual gossypol enantiomers were prepared as previously described (Dowd, 2003). The optical purity of the gossypol enantiomers was at least 99.5% based on HPLC analysis. Each of the diets for the different dietary treatments was mixed on a daily basis to minimize binding of gossypol to other feed components. Feed consumption and mortality were recorded daily and BW was determined on d 1 and d 20 of the study.

At the end of the 20 d experimental period, all birds were killed and liver, heart, kidney, spleen, and a portion of the abdominal fat samples were collected. The contents of the gallbladder of each bird were collected with a needle and syringe. The individual organs were weighed and immediately frozen at -80 ºC for future gossypol analyses. The

developing preovulatory follicles were visually examined for color and signs of atresia. Blood was collected from the brachial vein of all birds and placed on ice. Heparinized blood samples were then centrifuged for 10 min at 3000 x *g*. Plasma was collected from each sample and then frozen at -80 ºC for future gossypol analyses. In addition, blood was collected in two heparinized capillary tubes from the brachial vein of each bird. The capillary tubes were immediately centrifuged in a microcapillary centrifuge and percent packed cell volume was determined with a microcapillary reader.[1]

Egg production and egg weight were monitored daily. Eggs were stored for 14 d and then the egg yolks were scored for discoloration (Davis et al., 2002). After the yolks were scored, the yolk and albumen of each egg were separated and placed in individual containers and frozen at -80 ºC for future gossypol analyses.

Experiment 2

To determine if feed consumption and egg yolk discoloration results obtained with laying hens fed the individual enantiomers of gossypol also occurred in broiler breeder hens, 15 individually caged Cobb 500 fast feathering, 44 wk-old, broiler breeder hens were randomly assigned to 3 dietary treatments. The treatments consisted of a standard breeder diet (Table 3.1) supplemented with either no gossypol or each gossypol enantiomer at 400 mg/kg of diet. The broiler breeder hens were maintained on a daily lighting schedule of 16 h of light and 7 h dark. Each broiler breeder hen was fed 145g of feed per day as recommended by the Cobb 500 Breeder Management Guide (2002). Egg production, egg weight, mortality and feed intake were monitored daily for the next 18 d. Eggs were collected daily and processed as described in experiment 1. At the end of the

[1] International Equipment Company, Needham Heights, MA.

18-day experimental period, the birds were killed and the developing preovulatory follicles were visually examined for color and signs of atresia.

Gossypol Determination

Tissues were freeze-dried for 48 h (which was sufficient to reach a constant dry weight). The concentration of (+)- and (-)-gossypol in organs, tissues, egg yolk and albumen were determined by HPLC as previously described (McMillan, 2000).

Statistical Analyses

Data from each experiment were subjected to ANOVA according to the General Linear Model procedure. Duncan's multiple-comparison procedure (SAS Institute, 2001) was used to detect significant differences among diets. All statistical procedures were done with SAS® statistical software package (SAS Institute, 2001) and differences were considered significant when p-values were less than 0.05.

RESULTS

Experiment 1

The addition of 400 mg (+)-gossypol/kg of diet reduced the feed consumption of laying hens (Table 3.2). Dietary supplementation with either gossypol enantiomer did not affect BW (Table 3.2). Only 1 bird died during the experiment, on d 19 in the group fed the diet with 200 mg (-)-gossypol/kg. The reason for the death was unrelated to the dietary treatment.

Compared to birds fed the control diet, liver and spleen weights relative to BW were increased ($P < 0.05$) in hens fed the diet containing the highest concentration of (+)-gossypol (Table 3.3). The weight of the heart and kidneys and total bile volume relative to BW was not affected by the ingestion of either gossypol enantiomer (Table 3.3). Percent blood packed cell volume was not different ($P > 0.05$) between any of the dietary treatments (data not shown).

In birds fed (-)-gossypol, the liver had the highest concentration of gossypol followed by the kidney, plasma, spleen and heart (Table 3.4). Interestingly, neither (+)- or (-)-gossypol was detected in any of the fat samples (data not shown). The accumulation of (+)-gossypol was higher ($P < 0.05$) than that of (-)-gossypol in all of the tissues examined (Table 3.4). Only the gossypol enantiomer that was fed was detected in any of the tissues analyzed.

Egg production was not significantly impacted by feeding (-)-gossypol (Table 3.5), but feeding (+)-gossypol decreased egg production (Table 3.5). Although egg weights were initially not affected by feeding either enantiomer of gossypol, during the last half of the experiment the eggs produced by birds fed (+)-gossypol were significantly smaller than the eggs produced by hens fed the other two dietary treatments (Table 3.6). At the end of the experiment 60% percent of the birds fed the 400 mg of (+)-gossypol/kg of diet had large, hierarchical, preovulatory follicles undergoing atresia. There was no evidence of hierarchical follicular atresia in any of the birds fed the other diets. Ingestion of (+)-gossypol caused hens to produce eggs with a high incidence of objectionable egg yolk discoloration (Table 3.7). Of the objectionable eggs produced 75% had a score of 5 to 10, which indicated severe discoloration (data not shown). Although feeding (-)-

gossypol did not cause discolored egg yolks, the accumulation of (-)-gossypol in the yolks was not significantly different or was numerically similar to the accumulation of (+)-gossypol (Table 3.8). No gossypol was detected in the albumen of eggs produced from birds fed any of the dietary treatments (data not shown).

Experiment 2

Although the broiler breeders hens in this experiment received a restricted amount of daily feed as recommended by the breeder guidelines, feeding a diet with (-)-gossypol still reduced feed intake in these birds (Table 3.9). Total egg production was not affected by the decrease in feed consumption in the birds fed (-)-gossypol, but the addition of (+)-gossypol to the diet did decrease egg production (Table 3.9). In fact, no eggs were produced by the broiler breeder hens fed (+)-gossypol after day 15 (data not shown). Furthermore, at the end of the experiment, 80% of birds fed (+)-gossypol had some hierarchical preovulatory follicles undergoing atresia. The remaining follicles were small in size and contained dark discolored yolk.

Egg weight was not affected by feeding (-)-gossypol, but egg weight was decreased after day 13 in birds fed (+)-gossypol (data not shown). The incidence of eggs having objectionable egg yolk discoloration (a score greater than 3) was significantly higher in birds fed (+)-gossypol in comparison to birds fed no gossypol.

DISCUSSION

Based on feeding broilers diets supplemented with pure gossypol enantiomers, Lordelo et al. (2005) suggested that the development of a genetic strain of cotton with only (+)-gossypol would be beneficial for the utilization of CSM by the poultry industry. In addition, the natural insecticide properties of gossypol that make it essential for optimal cotton production can apparently be met with just (+)-gossypol (Puckhaber et al., 2002). The present study, however, indicates that CSM made from a strain of cotton with only (+)-gossypol could not be universally utilized by the poultry industry since (+)-gossypol causes egg yolk discoloration and decreases feed intake and egg production in laying hens. Even though CSM produced from a cotton variety that contained (+)-gossypol would be more suitable in broiler production (Lordelo et al., 2005) and CSM produced from a cotton variety that contained (-)-gossypol would be suitable for laying hens, the prospect of cottonseed oil processing plants keeping the different varieties separate during processing for separate uses in the poultry industry is not feasible.

The sensitivity of laying hens to the ingestion of (+)-gossypol was unexpected. In rats (Wang et al., 1987) and broilers (Lordelo et al., 2005), it was only (-)-gossypol that caused severe toxic effects. Furthermore, it appears that it is (-)-gossypol that is responsible for the well-documented antifertility (Wang et al., 1987) antiviral (Lin et al., 1989) and anticancer (Liu et al., 2002) effects of gossypol. Our results suggest, however, that (+)-gossypol could have beneficial therapeutic properties as well since it can also clearly interfere with normal physiological functions in situations where (-)-gossypol has no or little effect.

Traditionally, gossypol has been assumed to cause egg yolk discoloration based on a chemical combination of gossypol with ferric iron released from yolk proteins (Swensen et al., 1942; Kemmerer et al., 1961; Kemmerer et al., 1966). In the present research, laying hens only produced egg yolks with objectionable discoloration when they were fed diets containing (+)-gossypol. Interestingly, the concentration of (-)-gossypol in the yolks of eggs produced by the hens fed the (-)-gossypol dietary treatments was similar to the levels of (+)-gossypol found in the discolored yolks of the eggs produced by the hens fed the (+)-gossypol dietary treatments. Furthermore, there were instances where the level of (+)-gossypol was very high in the yolk but the yolk was not discolored. As a whole, our results indicate that the mechanisms behind the egg yolk discoloration may be more complicated than originally thought.

Previous research indicated that feeding laying hens high levels of CSM containing gossypol reduced egg weight (Heywang et al., 1950; Narain et al., 1957; Reid et al., 1987; Davis et al., 2002). In the present research we determined that the reduction in egg weight is caused only by the ingestion of (+)-gossypol by laying and broiler breeder hens. Although the reduction in egg weight in response to gossypol ingestion was consistent in laying and broiler breeder hens, their response on egg yolk discoloration varied slightly. Unlike the laying hens fed (-)-gossypol, the broiler breeder hens fed (-)-gossypol did produce some objectionable eggs. Furthermore, the incidence of objectionable eggs was much lower in the broiler breeder hens fed (+)-gossypol than the layers fed the same level of (+)-gossypol.

Further variation was seen between the laying and broiler breeder hens in their feed consumption responses to gossypol enantiomers. The feed consumption of broiler

breeder hens was negatively impacted by (-)-gossypol, which agreed with a previous finding in young broilers (Lordelo et al., 2005). In contrast, the feed consumption of laying hens was not affected by the addition of the (-) enantiomer to the diet. There was, however, a significant decrease in feed consumption in laying hens fed (+)-gossypol. It is unclear if the reduced feed intake in the laying hens fed (+)-gossypol was a direct effect or an indirect effect related to their severe decrease in egg production. We believe it may have been a direct effect since feed consumption was significantly decreased by day 4 and egg production did not decrease until day 7 (data not shown). Furthermore, even though the broiler breeder hens were being feed restricted, the reduction in egg production caused by feeding (+)-gossypol was not followed by a decrease in feed consumption. The contradictory results between the feed intake responses of laying hens and meat-type birds to the different gossypol enantiomers may be explained by genetic differences. There are previous reports that indicated that the response to gossypol in CSM (therefore, a mixture of both enantiomers) differed based on bird breed differences (Heywang et al., 1954; Panigrahi et al., 1989; Panigrahi and Morris, 1991; Davis et al., 2002).

In the present research, accumulation of (+)-gossypol was always more than twice that of (-)-gossypol in the laying hen tissues examined. This difference in gossypol enantiomer accumulation was also reported in broilers (Lordelo et al., 2005). Lambs fed cottonseed (Kim et al., 1996) and rats fed gossypol enantiomers (Chen et al., 1987) had some tissues with higher levels of (+)-gossypol than (-)-gossypol while other tissues had either the reverse situation or equal concentrations of the two enantiomers. Chen et al. (1987) determined that (-)-gossypol had a shorter half-life and higher clearance rate than

(+)-gossypol in rats fed either pure (+)- or (-)-gossypol. Based on the shorter half-life, Chen and coworkers (1987) speculated that the toxicity observed with the (-)-gossypol might be attributed to its metabolites. Thus, in laying hens, the lower tissue concentrations of (-)-gossypol compared with (+)-gossypol may also result from a higher rate of clearance. In addition to possible differences in clearance, the lower tissue concentrations of (-)-gossypol in laying hens might also be attributed to differences in intestinal absorption or in the rate of conversion to other metabolites between the two gossypol enantiomers.

In summary, the poultry industry would not benefit from the development of a cotton variety with a higher (+) to (-) enantiomeric ratio. Both (+) and (-) forms of gossypol can cause detrimental effects in poultry depending on the genetics of the bird. Although further research is needed, it appears that Leghorn-type birds are more sensitive to (+)-gossypol while broilers are more sensitive to (-)-gossypol. Severe egg yolk discoloration was only caused by feeding hens (+)-gossypol and the mechanism by which (+)-gossypol causes egg yolk discoloration is unknown.

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TABLE 3.1. Composition of the experimental diets (experiments 1 and 2)

Ingredient	Diet		
	CSM layer [1]	Layer [2]	Broiler breeder[3]
	----------(g/100g of diet)----------		
Corn	53.78	61.75	63.77
Soybean meal, 48% CP	0.00	26.02	20.74
Cottonseed meal	30.00	0.00	0.00
Wheat middlings	-	-	4.30
Limestone	9.06	8.98	7.97
Dicalcium phosphate	1.15	1.29	1.39
Poultry fat	4.60	1.15	1.00
Salt	0.44	0.42	0.40
Vitamin mix [4]	0.25	0.25	0.3
DL- Methionine	0.12	0.06	0.07
Mineral mix [5]	0.06	0.06	0.06
L-Lysine, HCl	0.54	0.02	0.00
Calculated analysis [6]			
M. E. (kcal/ kg)	2,840.00	2,840.00	2,850.00
Crude Protein (%)	17.30	17.30	15.50
Calcium (%)	3.70	3.70	3.40
Available phosphorus (%)	0.38	0.38	0.37
Methionine and cystine (%)	0.66	0.66	0.60
Lysine (%)	0.95	0.95	0.79

[1]30% CSM layer diet utilized in experiment 1 to determine gossypol susceptibility.
[2]Layer diet utilized in experiment 1.
[3]Broiler breeder diet utilized in experiment 2.
[4]Vitamin mix provided the following per 100 g of diet: vitamin A, 551 IU; vitamin D_3, 110 IU; vitamin E, 1.1 IU; vitamin B_{12}, 0.001mg; riboflavin, 0.44 mg; niacin, 4.41 mg; d-panthotenic acid, 1.12 mg; choline, 19.13 mg; menadione sodium bisulfate, 0.33 mg; folic acid, 0.55 mg; pyridoxine HCl, 0.47 mg; thiamin, 0.22 mg; d-biotin, 0.011 mg; and ethoxyquin, 12.5 mg.
[5]Mineral mix provided the following in mg per 100 g of diet: Mn, 6.0; Zn, 5.0; Fe, 3.0; I, 1.5; and Se, 0.5.
[6]Calculated analysis was based on Dale (2001).

TABLE 3.2. Body weight change and feed consumption of layers fed (+)- or (-)-gossypol for 20 d (experiment 1)

Dietary gossypol (mg/kg)	Body weight change	Total feed consumption
	----------(g) [1]	----------
0	-53.6 ± 26.1[ab]	1801.8 ± 58.8[a]
200 (-)	-53.8 ± 9.0[ab]	1722.7 ± 71.2[a]
400 (-)	-93.8 ± 38.1[b]	1641.8 ± 55.3[a]
200 (+)	-22.0 ± 25.5[ab]	1697.0 ± 62.9[a]
400 (+)	8.2 ± 28.7[a]	1342.6 ± 68.4[b]

[a-b]Values within a column without a common superscript differ; $P < 0.05$.
[1]Values are means ± SEM per bird for the 20 d experimental period with 5 replicate birds for each dietary treatment except for the 200 (+) mg/kg treatment where n = 4 replicate birds.

TABLE 3.3. Organ weights for layers fed (+)- or (-)-gossypol for 20 d (experiment 1)

Dietary gossypol (mg/kg)	Heart	Liver	Kidney	Spleen	Bile
	----------(g / 100 g BW)[1] ----------				---(mL/ 100g BW)[1] ---
0	0.40 ± 0.03	2.04 ± 0.08[b]	0.29 ± 0.018[ab]	0.09 ± 0.001[b]	0.07 ± 0.010
200 (-)	0.39 ± 0.04	2.14 ± 0.11[b]	0.27 ± 0.013[b]	0.08 ± 0.007[b]	0.08 ± 0.012
400 (-)	0.41 ± 0.02	2.41 ± 0.13[b]	0.32 ± 0.014[a]	0.09 ± 0.010[b]	0.07 ± 0.007
200 (+)	0.44 ± 0.04	2.30 ± 0.09[b]	0.29 ± 0.019[ab]	0.09 ± 0.006[b]	0.09 ± 0.009
400 (+)	0.43 ± 0.02	2.93 ± 0.23[a]	0.34 ± 0.022[a]	0.21 ± 0.032[a]	0.08 ± 0.021

[a-b]Values within a column without a common superscript differ; $P < 0.05$.

[1]Values are means ± SEM per bird for the 20 d experimental period with 5 replicate birds for each dietary treatment except for the 200 (+) mg/kg treatment where n = 4 replicate birds.

TABLE 3.4. Concentration of (+)- and (-)-gossypol in tissues from layers fed pure individual gossypol enantiomers for 20 d (experiment 1)

Dietary gossypol (mg/kg)	Liver	Spleen	Kidney	Heart	Plasma
	----------(µg/g DM)[1,2,3]----------				----(µg/mL)[1,2,3]----
200 (-)	243 ± 39[b*]	31 ± 5[b*]	43 ± 4[b*]	7 ± 2[*]	33 ± 7[b*]
400 (-)	477 ± 60[a*]	54 ± 4[a*]	92 ± 8[a*]	21 ± 7[*]	70 ± 14[a*]
200 (+)	930 ± 117[x]	108 ± 6[x]	145 ± 15[x]	34 ± 6[x]	79 ± 6[x]
400 (+)	1852 ± 118[y]	283 ± 41[y]	397 ± 72[y]	55 ± 5[y]	232 ± 2[y]

[a-b] Values for (-)-gossypol for a given tissue without a common superscript differ; $P < 0.05$.
[x-y] Values for (+)-gossypol for a given tissue without a common superscript differ; $P < 0.05$.
[*] Values for (-)-gossypol are different than the corresponding values of (+)-gossypol; $P < 0.05$.
[1]Values are means ± SEM per bird for the 20 d experimental period with 5 replicate birds for each dietary treatment except for the 200 (+) mg/kg treatment where n = 4 replicate birds.
[2]No gossypol was detected in the tissues of birds fed 0 mg of gossypol/kg of diet.
[3]There was no (-)-gossypol detected in any of the tissues of birds fed the (+)-gossypol and there was no (+)-gossypol detected in any of the tissues of birds fed the (-)-gossypol.

TABLE 3.5. Average egg production in 5 d increments for layers fed (+)- or (-)-gossypol for 20 d (experiment 1)

Dietary gossypol (mg/kg)	Experimental period				
	1-5 d	6-10 d	11-15 d	16-20 d	0-20 d
	---------- (%)[1] ----------				
0	96 ± 4	80 ± 6[a]	88 ± 5[a]	96 ± 4[a]	90 ± 2[a]
200 (-)	92 ± 8	72 ± 5[a]	76 ± 4[a]	96 ± 4[a]	84 ± 3[ab]
400 (-)	96 ± 4	80 ± 6[a]	80 ± 6[a]	92 ± 5[a]	87 ± 2[ab]
200 (+)	100 ± 0	75 ± 5[a]	70 ± 13[a]	55 ± 13[b]	75 ± 5[b]
400 (+)	80 ± 11	12 ± 8[b]	28 ± 14[b]	4 ± 4[c]	31 ± 8[c]

[a-c]Values within a column without a common superscript differ; $P < 0.05$.

[1]Values are means ± SEM per bird with 5 replicate birds for each dietary treatment except for the 200 (+) mg/kg treatment where n = 4 replicate birds.

TABLE 3.6. Average egg weight in 5 d increments for layers fed (+)- or (-)-gossypol for 20 d (experiment 1)

Dietary gossypol (mg/kg)	Experimental period				
	1-5 d	6-10 d	11-15 d	16-20 d	0-20 d
	---------- (g)[1] ----------				
0	61.4 ± 2.75	60.8 ± 2.34	59.3 ± 2.36[a]	58.8 ± 2.20[a]	60.1 ± 2.41[ab]
200 (-)	62.5 ± 0.77	60.6 ± 0.86	61.3 ± 0.92[a]	61.1 ± 0.48[a]	61.4 ± 0.76[a]
400 (-)	60.1 ± 1.30	57.7 ± 0.92	59.3 ± 1.46[a]	57.8 ± 0.96[a]	58.8 ± 1.16[ab]
200 (+)	59.4 ± 2.01	55.4 ± 1.03	51.7 ± 1.67[b]	49.9 ± 1.55[b]	54.1 ± 1.57[bc]
400 (+)	58.1 ± 2.38	57.7 ± 1.43	47.0 ± 2.59[b]	42.4 ± 0.00[c]	51.3 ± 1.60[c]

[a-c]Values within a column without a common superscript differ; $P < 0.05$.
[1]Values are means ± SEM per bird with 5 replicate birds for each dietary treatment except for the 200 (+) mg/kg treatment where n = 4 replicate birds.

TABLE 3.7. The incidence of objectionable egg yolks in eggs produced from layers fed the individual pure gossypol enantiomers (experiment 1)

Dietary gossypol (mg/kg)	Experimental period			
	1-5 d	6-10 d	11-15 d	16-20 d
	----------(%)[1,2]----------			
0	0	0	0	0
200 (-)	0	0	0	0
400 (-)	0	0	0	0
200 (+)	0	56	88	100
400 (+)	23	50	100	100

[1]Egg yolks scored individually as previously described (Davis et al., 2002).
[2]Scores above 3 were considered objectionable.

TABLE 3.8. Average concentration of (+)- and (-)-gossypol in the yolk of eggs produced by layers fed individual pure gossypol enantiomers (experiment 1)

Dietary gossypol (mg/kg)	Experimental period							
	1-5 d		6-10 d		11-15 d		16-20 d	
	μg/g DM[1,2,3]	n	μg/g DM[1,2,3]	n	μg/g DM[1,2,3]	n	μg/g DM[1,2,3]	n
200 (-)	55.3 ± 7.1[b]	22	190.1 ± 5.2[c]	18	212.1 ± 9.6[d]	19	236.34 ± 12.66[b]	17
400 (-)	113.8 ± 13.4[a]	25	320.3 ± 20.5[a]	20	377.6 ± 12.7[b]	21	385.49 ± 9.10[a]	17
200 (+)	62.3 ± 5.9[ab]	20	253.8 ± 10.6[b]	15	335.1 ± 8.4[c]	14	374.52 ± 14.98[a]	9
400 (+)	91.5 ± 35.9[ab]	20	364.5 ± NA[a]	1	474.2 ± 22.2[a]	6	414.1 ± NA[a]	1

[a-d]Values within a column without a common superscript differ; $P < 0.05$.
[1]Values are means ± SEM per bird.
[2]No gossypol was detected in the yolk of birds fed 0 mg of gossypol/kg of diet.
[3]There was no (-)-gossypol detected in the yolks from birds fed the (+)-gossypol and there was no (+)-gossypol detected in the yolks from birds fed the (-)-gossypol.

TABLE 3.9. Feed consumption, egg production and incidence of objectionable egg yolks produced for broiler breeder hens fed either (+)- or (-)-gossypol for 18 d (experiment 2)

Dietary gossypol (mg/kg)	Total feed consumption (g/bird)[1]	Total egg production (number of eggs/bird)[1]	Objectionable (% of total eggs)[1,2,3]
0	2590 ± 3.0[a]	12.0 ± 0.63[a]	0 ± 0.00[b]
400 (-)	2182 ± 139.7[b]	7.6 ± 2.46[ab]	10 ± 7.47[b]
400 (+)	2548 ± 25.8[a]	5.2 ± 0.86[b]	36 ± 9.64[a]

[a-b]Values within a column without a common superscript differ; $P < 0.05$.
[1]Values are means ± SEM per bird for the 18 d experimental period with n = 5 replicate birds.
[2]Egg yolks scored individually as previously described (Davis et al., 2002).
[3]Scores above 3 were considered objectionable.

CHAPTER 4

COTTONSEED MEAL DIETS IMPROVE BODY WEIGHT UNIFORMITY IN BROILER BREEDER PULLETS[1]

[1] Lordelo, M. M., A. J. Davis, J. L. Wilson, and N. M. Dale.
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SUMMARY

Due to its low nutrient density, cottonseed meal (CSM) may be a potential alternative feed ingredient for soybean meal (SBM) in broiler breeder pullet diets to improve flock body weight uniformity. A major concern when utilizing CSM in poultry diets, however, is the presence of gossypol. In an initial experiment it was determined when broiler breeder hens are fed a diet containing CSM, gossypol accumulates in the liver. When CSM is removed from the diet, however, hepatic gossypol levels dissipate within a few weeks. In a subsequent study, in order to achieve the breeder guideline ideal weight, broiler breeder pullets reared with a diet containing CSM from 2 to 18 weeks of age had to be fed a larger amount than birds consuming a standard SBM diet. The coefficient of variation of bird weight was significantly lower during the rearing period for birds reared with the corn/CSM diet. This difference in uniformity was maintained as the hens entered the breeding period. Egg weight, hatchability and fertility were equivalent for hens reared with a diet containing CSM and those reared with a standard diet throughout the growing period. The results suggest that broiler breeder pullets reared on a diet containing CSM as the major protein source have improved flock body weight uniformity, and normal subsequent reproductive performance.

Key words: broiler breeders, weight uniformity, cottonseed meal, gossypol.

DESCRIPTION OF PROBLEM

The use of cottonseed meal (CSM) in poultry rations is limited as this feed ingredient has lower crude protein, metabolizable energy and available lysine contents than soybean meal (SBM). Additionally, the presence of gossypol and cyclopropenoid fatty acids (CPFA) lowers the desirability of this feed ingredient.

High levels of gossypol in laying hen diets can lead to the formation of an iron-gossypol complex in the egg yolk that gives it a chocolate brown color (Schaible et al., 1934; Davis et al., 2002). The presence of more than 0.1% residual cottonseed oil in the laying hen diet may increase saturated fatty acids, such as CPFA in the yolk, which contribute to egg albumen discoloration (Evans et al., 1961; Reid, 1972; Reid et al., 1984). Today, however, the major cottonseed processors use an expander-solvent extraction process that likely destroys the heat labile CPFA.

Numerous researchers have reported that high levels of gossypol in broiler rations are associated with depressed weight gains (Lillie and Bird, 1950; Milligan and Bird, 1951; Phelps, 1966; Waldroup, 1981), and feed efficiency (Couch et al., 1955; Heywang and Bird, 1955).

Extensive research has been conducted to establish methods to decrease the negative impacts that the factors inherent to CSM might have on poultry. The objective of the present study, however, is to determine if some of the negative attributes of CSM could be advantageous in feeding broiler breeder pullets. Since CSM has only 58% of the total lysine and 43% of the digestible lysine of SBM (National Research Council, 1994), it could be a more appropriate dietary protein source when a low nutrient density

diet must be provided. The rearing period of broiler breeder pullets is unique in that it is necessary to restrict feed consumption to prevent excessive meat and body fat deposition and a subsequent decline in reproductive efficiency. Given the lower nutrient density of CSM, it may be possible to formulate diets based on this feed ingredient so that the severity of feed restriction can be reduced and improved body weight uniformity attained.

The present research was conducted to determine if feeding broiler breeder pullets a diet containing CSM during the rearing period could be an effective approach to improving body weight uniformity. In addition, studies were conducted to establish whether exposure to gossypol during the rearing period of breeder pullets had a negative impact on future reproductive fitness.

MATERIALS AND METHODS

During the refining of cottonseed oil, there is a portion of the extracted oil termed soapstock, which is rich in gossypol. In solvent extraction mills, soapstock is removed from the cottonseed oil and in most mills it is added back to the cottonseed meal. The CSM used in Experiment 1 was obtained by the expander-solvent extraction method and contained 1.5% total and 0.15% free gossypol. For Experiment 2, in order to ensure a low free gossypol content, soapstock was not added to the meal and thus, the CSM contained 1.356% total gossypol and 0.071% free gossypol.

Experiment 1

A preliminary trial was conducted to assess whether gossypol would accumulate in the liver of breeder hens fed a diet containing CSM, and then be depleted once the CSM was removed from the diet. A flock of 165 commercial Cobb broiler breeder hens (37 weeks of age) was divided into 3 pens (3 × 3.5 m) of 55 birds each (pens A, B and C). Six 37-week-old roosters were placed in each of the experimental pens. All groups received 15 hours of light during the 8-week experimental trial. The pens were equipped with nipple drinkers and pan feeders. The birds in pen A were fed a standard SBM based broiler breeder diet (Table 4.1) throughout the study. The birds in pens B and C were fed for the initial 4 weeks a diet containing CSM (Table 4.1), and the SBM diet for the following 4 weeks. Daily feed allotments were the same for all pens and based on body weight. Water was provided ad libitum. Livers were collected for gossypol analysis from 5 broiler breeder hens in pen C at the end of every week for the duration of the experiment. After removal, livers were immediately frozen at -80 ºC for future gossypol analysis.

Egg production, egg weight, fertility, hatchability and mortality were closely monitored in pen A and B throughout the experimental period. Individual egg production was recorded for the determination of weekly egg production. Egg weight was recorded on a daily basis prior to storage. Eggs produced from 3 days of the week (Monday, Wednesday and Friday) were kept for incubation. Weekly hatchability and fertility rates were calculated for each pen throughout the experimental period.

Experiment 2

This experiment was conducted to determine if rearing broiler breeder pullets with a diet containing CSM would improve body weight uniformity without negatively impacting future reproductive performance. Fifteen hundred day old commercial Hubbard Hi-Y broiler breeder pullets were randomly divided into 6 groups of 250 birds each and placed in separate rooms (9.14 × 7.31 m). Pullets were wing banded for individual weight identification. Three hundred day old Hubbard Hi-Y cockerels were also placed in a 9.14 × 7.31 m room. All the groups were reared in floor pens in a light-tight, environmentally controlled facility under 24 hours of light per day for the first 3 days post hatch, 12 hours of light per day from day 4 to day 13 and 8 hours of light per day until 21 weeks of age. Rooms were equipped with automatic chain feeders and nipple drinkers.

From hatch to 2 weeks of age, the pullets received a standard pullet starter diet fed ad libitum. From 2 to 18 weeks of age, 3 groups of pullets received a commercial type corn/SBM grower diet while the other 3 groups of pullets were fed a grower diet containing 20% CSM as the main protein source (Table 4.1). From 18 to 21 weeks of age, all the groups of pullets were fed the standard grower diet (Table 4.1). Cockerels were fed the corn/SBM starter and grower diet throughout the rearing period. Cockerel diets were of the same nutrient and ingredient composition as the pullet starter and grower diets (Table 4.1).

During the rearing period, feed allocation was based on the mean weekly body weight of the pullets from each treatment and how closely these values matched the primary breeder target weight at that specific age. The amount of feed allocated per week

was divided by 4 days to determine the amount of feed to be given on the 4-3 feeding program. The birds were fed on Monday, Wednesday, Friday and Saturday of each week, and were not fed on the other days of the week. Individual bird weight was monitored every other week to calculate the coefficient of variation of bird weight. In the weeks between, sample weights of 30% of the birds were taken to determine the amount of feed to be provided. Mortality was monitored daily. Birds were subjected to a standard commercial vaccination schedule during the rearing period.

At 21 weeks of age the weight profile was determined for the entire population of pullets remaining in the 3 rooms for each dietary treatment. Based on this weight profile, 480 pullets were selected from each of the original treatment groups. The selected birds were split into 8 replicate breeder pens for each of the original treatments. The selection of the birds for each breeder pen was done such that the weight profile and uniformity was representative of the overall profile for each treatment. Ten roosters randomly selected from their grower room were placed into each of the breeder pens. One additional group of 60 hens and 10 roosters for each treatment was placed into separate extra pens. These extra birds were kept to provide replacements for any birds that died in the experimental pens during the breeding period. The breeding pens were 3 x 3.5 m, and the pen space consisted of 2/3 slat and 1/3 scratch area. The breeder pens were located in an environmentally controlled house equipped with an evaporative cooling pad system. Each pen was equipped with an automatic chain feeder sized specifically for hens and nipple drinkers. Roosters had access to a breeder diet that was manually fed in height specific pan feeders. From 21 to 32 weeks of age all groups received a standard SBM based broiler breeder diet (Table 4.1) daily and had access to water ad libitum. Feed

allocation was identical for all treatments and was based on the average weekly body weight of the birds.

Egg production, egg weight, fertility, hatchability and mortality were closely monitored throughout the breeding period, until 32 weeks of age. Eggs were collected 3 times a day and daily records of egg production per pen were recorded for the determination of weekly and total egg production. During the first 2 weeks of egg production all eggs were collected and incubated. During the following weeks, only eggs produced from 3 days of the week (Monday, Wednesday and Friday) were kept for incubation. The eggs were weighed after storage on a weekly basis. Weekly hatchability and fertility values were calculated through the 32nd week of age.

The entire liver was collected from 5 birds every 4 weeks during the rearing period from the birds fed the corn/CSM based diet and once a week during the breeding period from the birds previously reared with a diet containing CSM. Liver samples were collected from 5 birds fed the corn/SBM diet at 6, 19, and 32 weeks of age. Livers were immediately frozen at -80 ºC until gossypol analysis could be conducted.

Hatchability and Fertility Determination

After collection, eggs were placed in an 18.3 °C cooler. At the end of each week, 30 eggs from each of the collection days were randomly selected from each pen and incubated. Eleven days after placement in the incubator, all eggs were candled and those not appearing viable were opened for investigation of possible early dead, middle dead, contamination, infertiles and cracks. On day 19, the eggs were transferred to a hatcher. Temperature settings from 0 to 18 and 19 to 21 days of incubation were 37.8 and 37.2 °C,

respectively. Relative humidity settings at 0 to 19 and 20 to 21 days of incubation were 53 and 70%, respectively. On day 21 the cull chicks, dead chicks, live pips, dead pips and live chicks were counted. Hatchability and fertility were then calculated for each pen.

Gossypol determination

CSM samples were assessed for free and total gossypol content by the official methods of the American Oil Chemists Society (AOCS, 1985 a; AOCS, 1985 b). Total gossypol content, negative gossypol enantiomer and positive gossypol enantiomer concentrations were determined on liver samples by HPLC (McMillan, 2000). The hepatic concentration of the specific gossypol enantiomers was determined to investigate if accumulation and depletion of both enantiomers of gossypol occurred at the same rate.

Statistical Analyses

Weekly differences between mean coefficient of variation of body weight, egg weight, egg production, fertility and hatchability among treatments were summarized and analyzed by one-way analysis of variance (SAS, 2001). Regression analysis for these parameters was also used to test for differences over time between the dietary treatments (SAS, 2001). Differences between mean weekly contents of gossypol in the liver were tested using Duncan's test by the General Linear Model Procedures of SAS (2001). All statements of significance were based on testing at the $P < 0.05$ level.

RESULTS AND DISCUSSION

Experiment 1

The objective of the preliminary experiment was to assess whether gossypol accumulates in liver tissue when broiler breeders are fed a diet containing CSM and if hepatic gossypol levels would decrease once CSM was removed from the diet. Liver gossypol concentrations increased significantly in response to the consumption of a diet containing CSM (Table 4.2). There was a steady increase in hepatic gossypol concentration for the initial 2 weeks. However, total liver content of gossypol at 3 and 4 weeks was not significantly different from the 2-week value. After the broiler breeder hens were returned to the standard SBM based diet there was a steady and significant decline in total liver gossypol content (Table 4.2). Interestingly, the negative enantiomer of gossypol, which is believed to be more toxic than the positive enantiomer (Joseph et al., 1986), was removed from the liver at a quicker rate ($P < 0.05$) than the positive enantiomer.

Although the experimental design precluded a statistical analysis of reproductive performance, egg production, egg weight, hatchability and fertility were monitored. Even though gossypol did accumulate in the liver of broiler breeder hens fed a diet containing CSM, there were no apparent detrimental effects on reproduction (data not shown).

Experiment 2

The purpose of this experiment was to determine whether feeding broiler breeder pullets a diet containing CSM would improve body weight uniformity without being detrimental to reproduction. Weekly body weight gain of pullets during the rearing period closely followed the Hubbard breeder guidelines regardless of the dietary treatment (Figure 4.1). However, in order to maintain these ideal body profiles, more feed had to be given to the birds consuming the corn/CSM based pullet grower diet (Table 4.3). The birds could be fed more of the corn/CSM grower diet without increasing body weight gain over that of controls because of the very low levels of total and available lysine in CSM relative to SBM.

Since more feed was provided to the CSM pullets, it was not surprising that these birds had a lower ($P < 0.01$) overall average coefficient of variation for body weight throughout the rearing period than pullets fed the control corn/SBM diet. The linear regression equation for the coefficient of variation for body weight for the birds fed SBM equaled 14.24 + 0.096(week of age) while it equaled 14.93 – 0.049(week of age) for the birds fed CSM. Individual weekly coefficients of variation for the pullets fed the 2 diets were not always significant (Table 4.4). This was not unexpected given the low number of replicates (n=3) for each treatment and that a cumulative difference was anticipated.

During the breeding period, weekly egg production over time was not different between hens reared with different dietary regimes (Figure 4.2). Control groups started laying eggs 3 days prior to the onset in egg production of the groups previously reared with CSM. Hens from both treatment groups reached 25% production between the 26^{th} and the 27^{th} week of age, 50% production between the 27^{th} and the 28^{th} week of age and

peak egg production at 31 weeks of age (Figure 4.2). Nevertheless, it was noted that the CSM reared birds reached peak production a few days sooner and laid more eggs than the control birds (Figure 4.2). Although not significantly different, by 32 weeks of age birds reared on the CSM diet had produced on average about 1 more egg per bird than those reared on the standard diet. This accounted for 430 more eggs laid overall by the hens reared on a diet containing CSM.

Since published data indicates that gossypol has a depressive effect on egg weight (Davis et al., 2002), this parameter was monitored. However, average weekly egg weights did not differ between the hens reared with the different dietary treatments (data not shown). In addition, the average egg weight for all eggs weighed from week 25 through week 32 was 52.7942 g and 52.8011 g for eggs produced by hens reared on SBM and CSM, respectively. This would indicate that CSM was in fact, removed from the diet early enough before the onset of lay to prevent a depression in egg weight. Weekly fertility and hatchability values as well as those over the entire experimental duration were not significantly affected by consumption of gossypol during the rearing period (Table 4.5).

Mortality was similar between treatments throughout the experimental period. Thirty-five birds died during the rearing period and 8 birds during the breeding period for birds fed the standard corn/SBM based diet. For the pullets reared on the diets containing CSM, 39 birds died during the rearing period and 5 during the breeding period.

As the liver is the site of synthesis for most of the egg yolk components, hepatic gossypol levels were closely monitored in the birds consuming the CSM based broiler breeder pullet diet. Once CSM was incorporated in the diet at 2 weeks of age, the levels

of gossypol in the liver quickly increased with time until they reached a plateau at 10 weeks of age (Table 4.6). A sharp decrease in liver gossypol was observed between 14 and 18 weeks of age. Since CSM was not removed from the diet until after 18 weeks of age, the reason for this sharp decrease is unclear. When the livers were collected from the pullets at 18 weeks, it was observed that follicular growth had begun and the ovaries contained several small white follicles. Thus, liver gossypol may have been transferred to the yolk with the lipid synthesized in the liver. Once the CSM was removed from the diet, the levels of gossypol in the liver quickly decreased with time (Table 4.6). As was found in the preliminary experiment, the negative enantiomer of gossypol was depleted from the liver at a faster rate ($P < 0.05$) than the positive enantiomer.

CONCLUSIONS AND APPLICATIONS

1. Feeding a diet with CSM as the major protein source during the rearing period of broiler breeder pullets improves body weight uniformity without adversely affecting future reproductive fitness. Pullets with similar body weights commence egg production at the same time, and lay eggs of uniform weight.
2. Although gossypol accumulated in the liver of broiler breeder pullets and hens fed a diet containing CSM, upon removal of the CSM from the diet, hepatic gossypol levels declined to very low levels in 4 to 5 weeks. Therefore, CSM should be removed from the diet of broiler breeder pullets at least 4 weeks prior to the onset of egg production to ensure that gossypol does not affect fertility and hatchability.

3. These findings establish a niche for CSM in poultry diets in which the lower nutrient density of CSM compared to SBM is actually advantageous.

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TABLE 4.1. Composition of the experimental diets (experiments 1 and 2)

Ingredient	Diet				
	Pullet starter (SBM)[A]	Pullet grower (SBM)[B]	Pullet grower (CSM)[C]	Broiler breeder (SBM)[D]	Broiler breeder (CSM)[E]
	----------(g/100g of diet)----------				
Corn	55.20	66.52	67.68	63.77	56.61
Soybean meal, 48% CP	25.74	18.84	1.83	20.74	4.25
Cottonseed meal, 40% CP			20.00		25.00
Soybean meal hulls		6.08	1.61		
Wheat midds	15.00	5.00	5.00	4.30	
Dicalcium phosphate[F]	1.65	1.50	1.41	1.39	
Limestone	1.25	1.21	1.43	7.97	7.61
Defluorinated phosphorus					1.33
Salt	0.45	0.46	0.45	0.40	0.26
Poultry fat	0.31			1.00	4.26
Vitamin mix[G]	0.25	0.25	0.25	0.30	0.25
DL- Methionine	0.09	0.08	0.08	0.07	0.12
L- Lysine HCl			0.20		0.24
Mineral mix[H]	0.06	0.06	0.06	0.06	0.07
Calculated analysis[I]					
M. E. (kcal/ kg)	2860	2860	2860	2850	2909
Crude Protein (%)	19.00	15.50	15.50	15.50	17.00
Crude fat (%)	3.21	2.92	2.80	3.76	6.21
Crude fiber (%)	3.20	5.01	5.23	2.52	1.54
Lysine (%)	0.99	0.76	0.72	0.79	0.85
Calcium (%)	0.92	0.90	0.90	3.40	3.40
Available Phosphorus (%)	0.45	0.40	0.40	0.37	0.38
Methionine (%)	0.41	0.35	0.33	0.35	0.38

Continued on the following page

[A] Starter diet fed to all groups of Experiment 2 from 0 to 2 weeks of age.
[B] Grower diet fed to control groups of Experiment 2 from 2 to 18 weeks of age and fed to all groups from 18 to 21 weeks of age.
[C] Grower diet fed to CSM groups of Experiment 2 from 2 to 18 weeks of age.
[D] Breeder diet fed to control groups of Experiment 1 during the entire duration of the trial and to all the pullets of Experiment 2 from 21 to 32 weeks of age.
[E] Breeder diet fed to CSM groups of Experiment 1.
[F] The dicalcium phosphate contained 22% calcium and 18.5% phosphorus
[G] Vitamin mix provided the following per 100 g of diet: vitamin A, 551 IU; vitamin D_3, 110 IU; Vitamin E, 1.1 IU; vitamin B_{12}, 0.001mg; riboflavin, 0.44 mg; niacin, 4.41 mg; d-panthotenic acid, 1.12 mg; choline, 19.13 mg; menadione sodium bisulfate, 0.33 mg; folic acid, 0.55 mg; pyridoxine HCl, 0.47 mg; thiamin, 0.22 mg; d-biotin, 0.011 mg; and ethoxyquin, 12.5 mg.
[H] Mineral mix provided the following in milligrams per 100 g of diet: Mn, 6.0; Zn, 5.0; Fe, 3.0; I, 1.5; and Se, 0.5.
[I] Calculated analysis was based on Feedstuffs Ingredient Analysis Table (2001).

TABLE 4.2. Accumulation and depletion of hepatic gossypol in broiler breeder hens fed a diet containing CSM for 4 weeks (experiment 1)

Week of age	Total gossypol	Positive gossypol enantiomer	Negative gossypol enantiomer
	----------(µg/g liver gossypol)[A]----------		
37	192.2 ± 18.19^{bc}	147.4 ± 14.45^{bc}	44.8 ± 3.76^{b}
38	291.4 ± 12.58^{a}	223.8 ± 8.97^{a}	67.8 ± 4.49^{a}
39	340.4 ± 17.18^{a}	264.2 ± 12.66^{a}	75.8 ± 4.79^{a}
40	305.2 ± 25.16^{a}	240.0 ± 20.08^{a}	65.4 ± 6.17^{a}
41	202.0 ± 27.97^{b}	163.6 ± 21.55^{b}	38.2 ± 6.61^{bc}
42	145.6 ± 6.69^{cd}	120.0 ± 6.66^{c}	25.6 ± 0.81^{cd}
43	93.4 ± 19.59^{de}	77.6 ± 12.69^{d}	17.8 ± 6.53^{de}
44	59.8 ± 13.47^{e}	49.6 ± 10.57^{d}	10.0 ± 2.77^{e}

[A]Values are means ± SEM, n = 5 replicates. Means with different letter superscripts within a column differ significantly ($P < 0.05$).

TABLE 4.3. Feed allocation for broiler breeder pullets between treatments (experiment 2)

	Treatment	
Week of age	SBM	CSM
	----------(g/bird/week)[A]----------	
2	120	128
3	124	132
4	132	140
5	140	152
6	164	176
7	184	200
8	208	228
9	228	252
10	244	272
11	256	288
12	260	292
13	264	296
14	268	300
15	280	304
16	286	304
17	286	304
18	290	290
Total	3734	4058

[A]Weekly feed allocation was split into 4 feedings per 7-day period, as in a 4-3 feeding program schedule.

TABLE 4.4. Coefficient of variation of body weight between broiler breeder pullets fed a diet with either SBM or CSM as the major dietary protein source during the rearing period (experiment 2)

	Treatment	
Week of age	SBM	CSM
	----------(%)[A]----------	
2	14.30 ± 0.41	14.53 ± 0.49
4	13.83 ± 0.22	13.12 ± 0.79
6	14.32 ± 0.24	16.11 ± 1.5
8	15.32 ± 0.53	15.07 ± 1.27
10	16.10 ± 0.46	15.72 ± 0.37
12	16.38 ± 0.40	15.45 ± 0.55*
14	15.92 ± 0.59	15.02 ± 0.49
16	15.74 ± 0.52	14.83 ± 0.60
18	15.81 ± 0.54	14.78 ± 0.26*
20	15.27 ± 0.91	13.37 ± 0.53

[A]Values are means ± SEM, n = 3 replicate pens of 250 birds each for each dietary treatment.
*Significantly different from the corresponding control value ($P < 0.05$) (SAS, 2001).

TABLE 4.5. Fertility and hatchability of eggs produced by broiler breeder hens fed a diet containing either SBM or CSM during the rearing period (experiment 2)

	Fertility[A]			Hatchability[A]		
Week of age	SBM	CSM	p-value	SBM	CSM	p-value
	----------(%)----------			----------(%)----------		
25	87.12 ± 5.25	88.63 ± 4.89	0.82	70.27 ± 6.11	56.79 ± 9.49	0.25
26	93.32 ± 1.63	93.13 ± 1.47	0.70	74.43 ± 2.62	73.81 ± 2.83	0.85
27	94.78 ± 0.64	94.61 ± 1.24	0.79	80.36 ± 1.77	79.48 ± 2.81	0.79
28	96.91 ± 0.56	96.73 ± 0.50	0.87	85.28 ± 2.10	82.39 ± 2.09	0.35
29	97.53 ± 0.80	96.37 ± 0.51	0.21	87.13 ± 1.67	86.91 ± 0.83	1.00
30	97.78 ± 0.51	97.22 ± 0.94	0.51	85.83 ± 1.58	86.81 ± 1.23	0.72
31	98.05 ± 0.62	97.63 ± 0.97	0.67	89.55 ± 1.42	87.73 ± 1.36	0.38
32	98.19 ± 0.51	98.87 ± 0.43	0.31	89.56 ± 1.18	87.92 ± 1.64	0.43

[A] Values are means ± SEM, n = 8 replicate pens.

TABLE 4.6. Hepatic gossypol content in broiler breeder pullets fed a diet containing cottonseed meal from 2 to 18 weeks of age (Experiment 2)[A]

Week of age	Total gossypol	Positive gossypol enantiomer	Negative gossypol enantiomer
	(µg/g liver gossypol)[B]		
6	203.4 ± 8.99[d]	165.5 ± 8.17[c]	37.9 ± 1.25[c]
10	385.8 ± 12.22[a]	324.8 ± 10.42[a]	60.4 ± 2.72[b]
14	416.0 ± 36.22[a]	346.1 ± 31.54[a]	69.9 ± 4.72[a]
18	272.8 ± 24.94[bc]	235.8 ± 21.57[b]	36.9 ± 3.67[c]
19	294.1 ± 29.34[b]	251.2 ± 23.80[b]	43.0 ± 5.78[c]
20	226.3 ± 40.07[cd]	192.3 ± 34.16[c]	34.0 ± 6.21[c]
21	139.7 ± 13.90[e]	119.3 ± 11.35[d]	16.2 ± 4.44[de]
22	134.2 ± 16.43[e]	111.9 ± 13.24[d]	22.4 ± 3.19[d]
23	77.1 ± 10.83[f]	64.9 ± 9.75[e]	12.2 ± 1.12[ef]
24	46.9 ± 6.84[gf]	39.3 ± 5.35[ef]	7.7 ± 1.54[efg]
25	55.5 ± 12.65[gf]	46.9 ± 10.25[ef]	8.5 ± 2.41[efg]
26	37.4 ± 5.54[gf]	32.5 ± 5.07[ef]	4.9 ± 0.61[fg]
27	45.4 ± 7.49[gf]	40.4 ± 6.26[ef]	5.0 ± 1.30[fg]
28	23.7 ± 3.90[gf]	22.9 ± 3.12[ef]	0.9 ± 0.79[g]
29	21.0 ± 6.00[gf]	19.8 ± 5.32[ef]	1.2 ± 0.73[g]
30	13.6 ± 2.65[g]	13.6 ± 2.65[f]	0.0 ± 0.00[g]
31	13.2 ± 3.66[g]	13.2 ± 3.66[f]	0.0 ± 0.00[g]
32	8.6 ± 2.98[g]	8.6 ± 2.98[f]	0.0 ± 0.00[g]

[A]Control levels of hepatic total, positive enantiomer and negative enantiomer of gossypol were less than 10.44, 9.36 and 1.08 µg/g, respectively.

[B]Values are means ± SEM, n = 5 replicates. Means with different letter superscripts within a column differ significantly ($P < 0.05$).

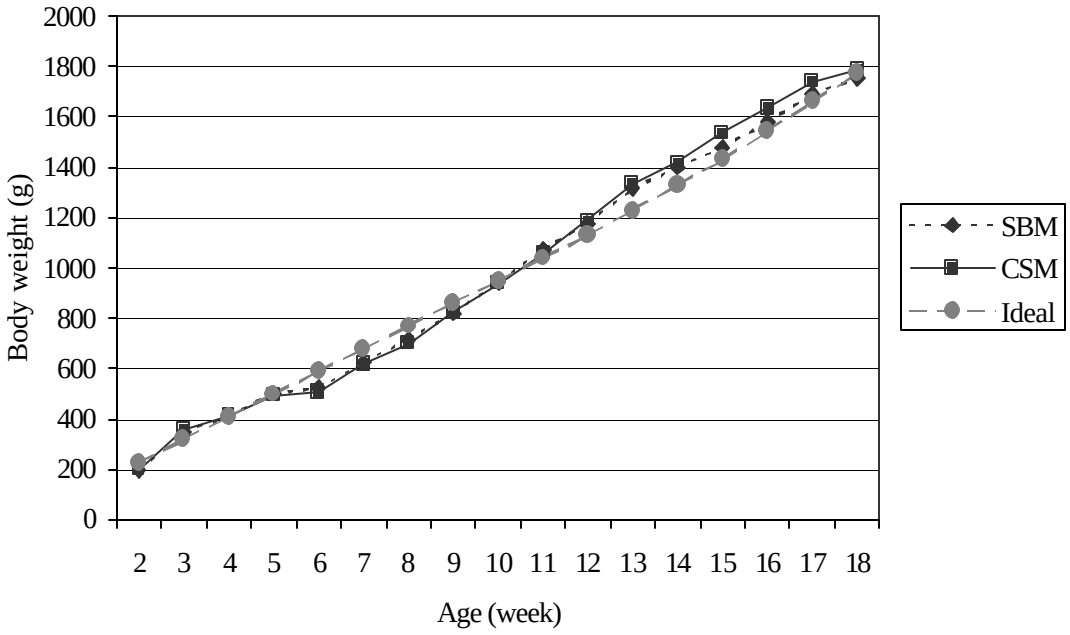

FIGURE 4.1. Weekly body weights of female broiler breeder pullets fed diets containing either SBM or CSM as the major protein source and the ideal weekly body weights as suggested by the breeder guideline (Experiment 2)

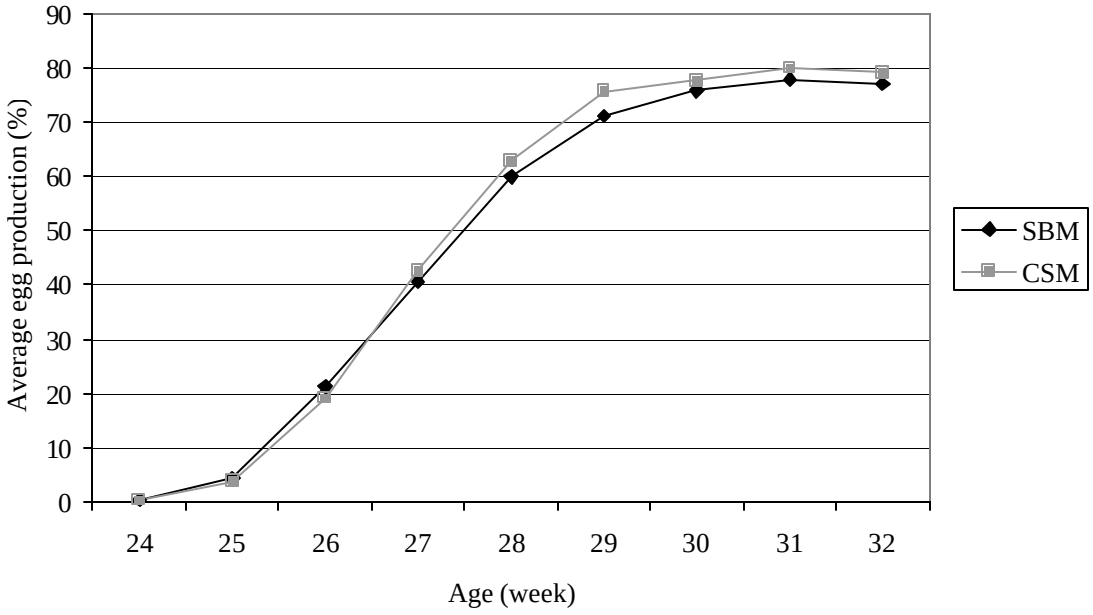

FIGURE 4.2. Egg production of broiler breeder hens fed a diet containing either SBM or CSM as the major protein source during the rearing period (Experiment 2)

CHAPTER 5

NEAR INFRARED REFLECTANCE SPECTROSCOPY FOR THE DETERMINATION OF FREE GOSSYPOL IN COTTONSEED MEAL[1]

[1] Lordelo, M. M., S. A. Shaaban, A. J. Davis, M. C. Calhoun, P. F. Vendrel, and N. M. Dale. To be submitted to *Journal of Applied Poultry Research*.

SUMMARY

Gossypol is a toxic polyphenolic compound that is produced and located in the pigment glands of the cotton plant. The gossypol content of cottonseed meal (CSM) is commonly determined by the American Oil Chemists' Society (AOCS) wet chemistry method. The AOCS method, however, is laboratory intensive and time consuming and therefore not practical for quick field analyses. To determine if the gossypol content of CSM could be predicted by near infrared reflectance spectroscopy (NIRS), CSM samples were collected from all over the world. All of the CSM samples were ground and a portion of each was analyzed for gossypol content by the AOCS procedure (reference data) and by NIRS (reflectance data). Both reflectance and reference data were combined in a calibration. The coefficient of determination (r^2) and standard error of prediction (SEP) were used to assess the calibration accuracy. The r^2 was 0.728 and the SEP was 0.034 for the initial calibration that included samples from all over the world. However, the r^2 and SEP improved to 0.921 and 0.014, respectively if the calibration was made using CSM samples only obtained from the United States. These results indicate that a general prediction equation can be developed to predict the gossypol content of CSM by NIRS. From a practical standpoint, NIRS technology provides a method for quickly assessing whether a particular batch of CSM has a gossypol content low enough to be suitable for use in poultry diets.

Key words: cottonseed meal, gossypol, near infrared reflectance spectroscopy

DESCRIPTION OF PROBLEM

Cottonseed meal (CSM) is a potential protein source for poultry diets, however, the possibility that it contains high levels of gossypol discourages its use. Gossypol is a toxic polyphenolic compound produced by and located in the pigment glands of the cotton plant. The gossypol found in CSM exists in both a free and bound form. Because of its overall negative charge, gossypol tends to form complexes with positively charged molecules such as lysine in CSM (Waldroup, 1981). In chickens, free gossypol is assumed to be more readily absorbed from the gastrointestinal tract than bound gossypol and therefore, to be the primary cause of the observed negative effects associated with feeding gossypol (Heywang et al., 1952; Phelps, 1966; Waldroup, 1981).

In broiler production, feeding diets that contain CSM with a high level of gossypol has been attributed to poor weight gain (Lillie and Bird, 1950; Milligan and Bird, 1951; Phelps, 1966; Waldroup, 1981) and poor feed efficiency (Couch et al., 1955; Heywang and Bird, 1955; Henry et al., 2001). However, researchers have found that CSM containing low levels of gossypol may be used in broiler diets, which contain adequate levels of all essential nutrients, with no adverse effects on body weight or mortality (Waldroup et al., 1968, Watkins et al., 1994). Furthermore, the lower nutrient density of CSM, when compared to soybean meal, makes it a desirable protein source for broiler breeder pullets (Lordelo et al., 2004). In fact utilizing CSM in broiler breeder pullet diets reduces the need for severe feed restriction, which results in better flock body weight uniformity (Lordelo et al., 2004).

Although CSM can be successfully utilized for poultry diets, it typically is not used because the gossypol content is unknown. The gossypol concentration of CSM can vary considerably based on the genetic variety of the cotton (Boatner et al., 1949; Santos et al., 2002; Sullivan et al., 2002). In addition, Pons et al. (1953) reported that the gossypol content of cottonseeds was negatively correlated with the temperature and positively correlated with the rainfall that the cotton plants were exposed to while producing the seed. Furthermore, methods of cottonseed oil extraction that achieve maximum oil production will yield CSM with the lowest gossypol content (Waldroup, 1981). Finally, CSM which contains soapstock has a higher concentration of gossypol than a meal without added soapstock since soapstock, a waste by-product from the cottonseed oil extraction process, contains a high concentration of gossypol (Dowd, 1996; Davis et al., 2002).

For CSM to be utilized as a feed ingredient for poultry diets, a quick and reliable method for the determination of gossypol is needed. The official methods for the quantification of free and total gossypol in CSM are the American Oil Chemists' Society methods (AOCS, 1985a; AOCS, 1985b). However, these procedures are time consuming as well as laboratory and labor intensive. A quicker alternative method for determining the gossypol content of CSM, might be near infrared reflectance spectroscopy (NIRS). As reviewed by Van Kempen (2001), NIRS technology has been used for the past several years to quickly and efficiently determine moisture, protein, fat, metabolizable energy, total amino acid and digestible amino acid contents in feed ingredients and feeds.

Previous attempts have been made to measure the gossypol content of whole cottonseeds by NIRS (Birth and Ramey, 1982; Wadsworth and Richard, 1993). There are

no reports, however, of predicting the gossypol content of CSM by NIRS. Therefore, it is the goal of the present study to develop a NIRS calibration prediction equation to analyze the free gossypol content of CSM.

MATERIALS AND METHODS

Experiment 1

Eighty-six CSM samples were obtained from various locations around the world (Table 5.1). All of the CSM samples were ground through a 1 mm sieve in a Wiley Laboratory Intermediate Mill[1] to obtain a uniform particle composition across all the samples. The free gossypol content of a portion of each CSM sample was then chemically determined by the AOCS (1985a) method.

A portion of each CSM sample was also scanned for gossypol using a model 6500 monochromator NIRS system[2]. The NIRS system continuously scanned wavelengths from 1100 to 2500 nm. Reflectance data were recorded at 2 nm intervals. The wavelengths used for free gossypol calibration were based on the analysis of the pure gossypol sample[3] and previous reports of free gossypol determination in whole cottonseeds by NIRS (Birth and Ramey, 1982; Wadsworth and Richard, 1993). The NIRS spectra data was then processed using the software WinISI 11 - version 1.50. The spectra data from 56 randomly selected samples of the original 86 samples was then combined with the AOCS reference data to generate a calibration prediction equation using a modified partial least-square regression method (WinISI 11 - version 1.50).

[1] Thomas Scientific, Swedesboro, NJ.
[2] FOSS North America, Eden Prairie, MN.
[3] Sigma Chemical Company, St. Louis, MO.

Once the prediction equation was obtained it was validated with the remaining 30 CSM samples. In order to validate the prediction equation, a linear regression analysis was used to compute the correlation between the gossypol content obtained for these samples by the AOCS method and the NIRS method. The validation procedure was used to estimate the coefficient of determination (r^2) and SEP as parameters for calibration accuracy. The validation was also used to determine the average difference between the gossypol reference method and predicted values (bias), and the linear regression of the gossypol reference method against predicted values (slope).

Experiment 2

Many of the international samples used in experiment 1 appeared to be poorly processed based on the presence of a large amount of residual cotton fibers and the very dark appearance of the meal. Furthermore, the oil extraction procedures for the international samples were not known. Therefore, in experiment 2 only the more uniformly processed CSM samples obtained from the United States were used for the NIRS calibration. Thus, a total of 51 samples from the original 86 were used in this experiment. Since the sample population was small, the validation sample set was comprised of the same 51 samples used for the calibration.

RESULTS AND DISCUSSION

Experiment 1

As determined by the AOCS method, the free gossypol content of the obtained CSM samples had a wide distribution from 0.03 to 0.39 percent (Figure 5.1). Obtaining CSM samples with this high variability was critical since a NIRS prediction equation should be constructed from samples that represent the range of values that will be encountered in field testing. The CSM samples obtained from Egypt and Peru contained a high concentration of gossypol while the samples from China and Iran had lower gossypol levels (Table 5.1).

The regression analysis for the validation of the reflectance values for gossypol in the international CSM samples yielded a r^2 of 0.728 and SEP of 0.034 (Figure 5.2). Although this coefficient of determination is statistically acceptable, a higher correlation between the reference and reflectance values was desirable.

Experiment 2

Cottonseed oil extraction in the U.S. is very efficient and yields a very uniform high quality CSM that is devoid of a lot of excess lint and residual oil from the cottonseed (Jones, 1981; Waldroup, 1981). Therefore, it was not surprising that when only the samples obtained from the U.S. were utilized for the NIRS prediction equation, the regression analysis of the gossypol determinations between the AOCS and the NIRS methods yielded a r^2 of 0.921 and a SEP of 0.014 (Figure 5.3).

Since we had only 51 CSM samples from the U.S. the same samples had to be used to formulate and validate the NIRS free gossypol prediction equation. Ideally, the validation would have been done as in experiment 1, with new CSM samples. For comparison purposes, we also determined that if the validation sample set consisted of the same 56 CSM samples used to formulate the prediction equation for the international samples (experiment 1), the r^2 decreased to 0.652 and the SEP remained at 0.034. Therefore we feel confident that the NIRS free gossypol prediction equation that was constructed can be utilized to accurately determine the free gossypol content of CSM produced in the U.S.

In order to obtain an accurate free gossypol concentration in CSM samples using NIRS, the results from the two current experiments suggest that NIRS prediction equations for free gossypol content of CSM may have to be constructed for different geographical regions of the world. The need for different free gossypol prediction equations reflects the differences in CSM oil extraction processing in different parts of the world, which result in CSM that varies tremendously in quality and appearance. Many of the international CSM samples contained an excessive amount of residual lint compared to the CSM produced in the U.S. The residual lint in CSM may interfere with accurate free gossypol determinations by NIRS. Wadsworth and Richard (1993) reported a very high standard error of prediction (SEP) when they developed a NIRS calibration for gossypol in whole fuzzy (covered in lint) cottonseed.

The main purpose of constructing a NIRS prediction equation in one NIRS system is to then transfer that prediction equation to other NIRS instruments. However, no two NIRS systems are the same, and even subtle differences between them may cause

variation in the spectral data. Such differences can make the transfer of calibration equations ineffective. Fortunately, there are mathematical manipulations that use the slope and bias factors obtained when each prediction equation is constructed, which can be used to correct spectral data between NIRS analyzers. For the international samples, the slope and bias values were 1.034 and –0.003 respectively, while for the U.S. samples the slope and bias were 1.0 and 0.0 respectively. Additionally, instead of using the bias and the slope correction method, a new calibration equation for free gossypol could also be quickly established for a different NIRS system by utilizing a pre-established CSM sample set in which the reference (AOCS) were already determined.

CONCLUSIONS AND APPLICATIONS

1. A NIRS calibration was successfully developed for the determination of the free gossypol content of CSM.
2. Due to differences in CSM processing around the world, separate prediction equations for individual regions of the world may be needed to confidently predict the free gossypol content of CSM by NIRS.
3. Cottonseed processing plants in the U.S. could use NIRS as a rapid means of quantifying the gossypol content of the CSM they produce. Once the gossypol content was determined, poultry nutritionists and feed mill operators could decide whether to purchase the CSM for use in poultry diets.

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TABLE 5.1. Gossypol content of cottonseed meal samples obtained from several countries

Country	Number of samples	Range of gossypol concentration[1]	Average gossypol concentration[1]
United States	51	0.057 - 0.238	0.123
China	10	0.036 - 0.101	0.064
Egypt	6	0.075 - 0.392	0.236
Turkey	6	0.071 - 0.124	0.080
Peru	5	0.093 - 0.116	0.105
Iran	4	0.030 - 0.088	0.055
Burkina Faso	2	0.131 - 0.261	0.196
Brazil	1	0.207 - 0.207	0.207
Zimbabwe	1	0.037 - 0.037	0.037
Total	86	0.030 - 0.392	0.113

[1]Gossypol concentrations determined by the official methods of the AOCS (1985a).

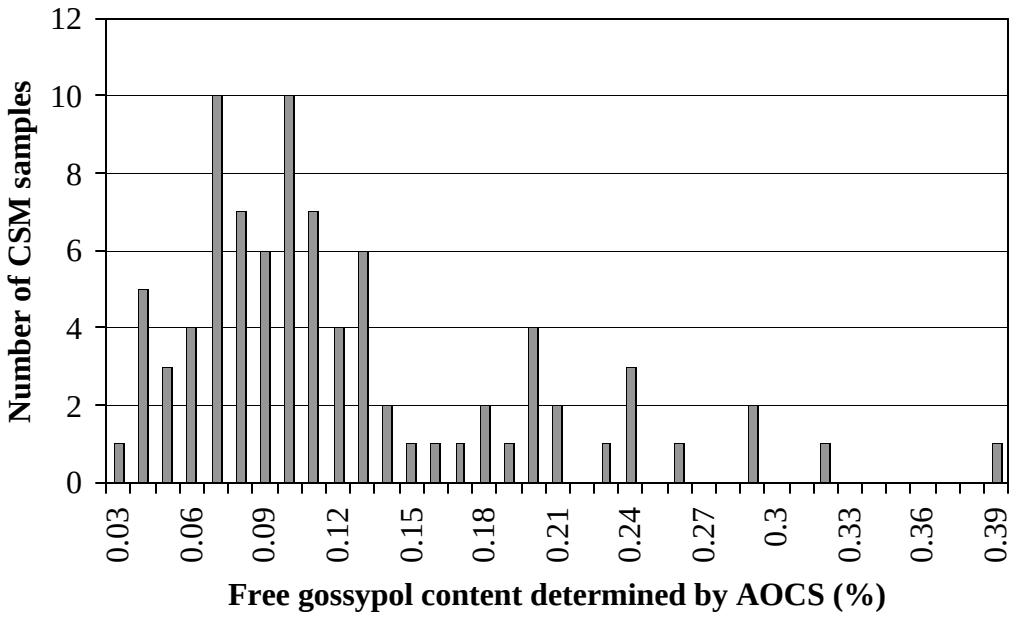

FIGURE 5.1. Distribution of the free gossypol content determined by the official methods of the AOCS (1985a) in the cottonseed meal (CSM) samples analyzed from several countries

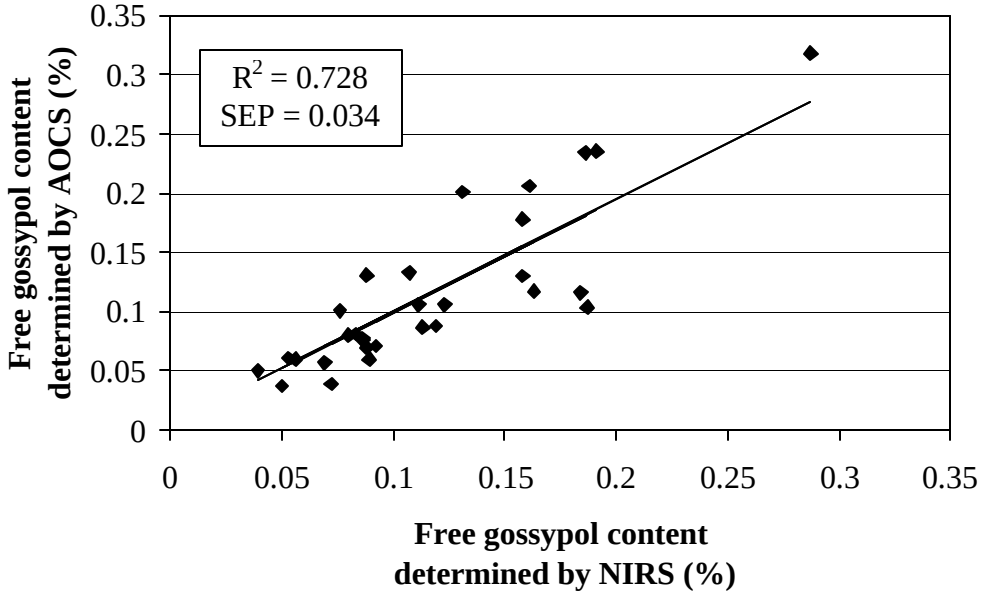

FIGURE 5.2. Correlation between the free gossypol content of the international cottonseed meal samples determined by the AOCS (1985a) official method and the predicted content based on near infrared reflectance (NIRS) measurements

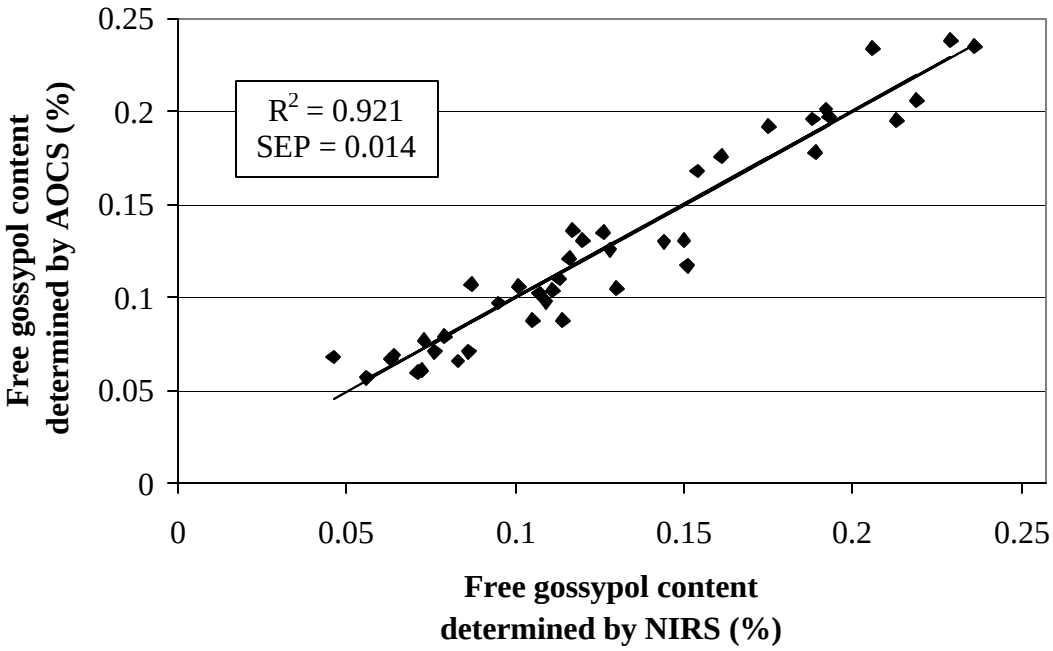

FIGURE 5.3. Correlation between the free gossypol content of the U.S. cottonseed meal samples determined by the AOCS (1985a) official method and the predicted content based on near infrared reflectance (NIRS) measurements

CHAPTER 6

GENERAL CONCLUSIONS

There are many areas in the world where cotton and poultry are major agriculture commodities. Thus, it is not surprising that about seven decades of research exists on the topic of utilizing cottonseed meal (CSM) in poultry diets. The reason for the continuous research in this field revolves around the fact that CSM contains gossypol, which is a toxic compound. Feeding diets containing CSM and thus gossypol is associated with poor body weight gain and poor feed efficiency in broilers and with egg yolk discoloration in laying hens. Due to the negative effects of gossypol in poultry, plant geneticists developed a cotton strain that produced almost no gossypol. Cotton plants from this strain, however, produce poor cotton yields and the quality of the cotton fiber is reduced since gossypol acts as a natural insecticide.

Gossypol occurs naturally as a mixture of two enantiomers that differ in their optical properties: (+)-gossypol and (-)-gossypol. Recently, Puckhaber et al. (2002) reported that both (+)- and (-)-gossypol are equally effective insecticides. This finding combined with reports that indicated that (-)-gossypol is more toxic to mammalian species than (+)-gossypol (Joseph et al., 1986; Wang et al., 1987; Kim et al., 2000) stimulated interest in the possibility that poultry could be fed CSM made from cottonseeds that only contained (+)-gossypol.

When diets containing the individual pure gossypol enantiomers were fed to broilers, only the diet containing (-)-gossypol depressed feed intake. Although birds fed the diets containing (+)-gossypol had a small reduction in their growth rate, feed conversion was not affected. Therefore, the initial results from the current research suggested that the development of a cotton cultivar that contained only (+)-gossypol could yield a CSM that might be more suitable for poultry production.

Laying hens are highly sensitive to gossypol (Heywang et al., 1954; Kemmerer et al., 1961; Reid et al., 1984; Panigrahi, 1992), therefore, a complete assessment of the relative toxicity of the gossypol enantiomers in poultry required a study with laying hens. When diets containing the pure individual gossypol enantiomers were fed to laying hens, feed intake, egg production, egg weight and egg yolk quality were all negatively affected by just (+)-gossypol. Therefore, in contrast to broilers, (+)-gossypol was more toxic to laying hens than (-)-gossypol. This difference in the relative toxicity of gossypol enantiomers between laying hens and broilers indicated that altering the enantiomeric ratio of gossypol in the cotton plant would not be beneficial for poultry industry. In addition, the value of utilizing CSM in poultry diets would not outweigh the logistics of cottonseed processors trying to ensure the production of CSM that contained only either (+)- or (-)-gossypol.

In order to clarify whether the difference in gossypol sensitivity between laying hens and broilers was due to an age or a genetic difference, broiler breeder hens were fed diets containing the individual pure gossypol enantiomers. The broiler breeder hens were the same genetic line of the hens that produced the broiler chicks for the previous experiments and the same age of the laying hens used in the previous experiments. Feed consumption in the broiler breeder hens decreased only when (-)-gossypol was added to the diet. Egg production declined and severe yolk discoloration occurred only when (+)-gossypol was consumed by broiler breeder hens. These results suggest that (-)-gossypol is detrimental to feed intake in broilers of any age, while (+)-gossypol is detrimental to egg yolk quality in both broiler breeder and laying hens. Further research is needed to determine if (+)-gossypol is detrimental to feed intake in Leghorn breeds of laying hens

of any age, but our results strongly suggest that this may be the case. In addition, further research should be conducted with broilers to determine if all commercial strains exhibit the same response to (-)-gossypol as was observed in the present experiments with birds from the Cobb fast feathering strain.

Although the current research with the individual gossypol enantiomers established that the development of a cotton strain with only one gossypol enantiomer would not be beneficial to the poultry industry, a use for CSM in the poultry industry was established. In broiler breeder pullet diets, CSM is a more suitable protein source than the most commonly used protein source, soybean meal, because CSM has a lower nutrient density. Specifically, CSM contains more fiber, has lower protein content and lower available lysine content than soybean meal. To prevent excessive meat and fat deposition that will be detrimental to future reproductive performance, broiler breeder pullets are feed restricted. A problem with feed restriction is that the more aggressive birds consume more than their share of the food to the detriment of the more passive birds, which ultimately leads to poor flock body weight uniformity. If a protein source with a lower nutrient density than soybean meal is employed in the diet, such as CSM, the severity of the feed restriction can be diminished.

In the current research, in order to achieve the same weight as the broiler breeder pullets fed soybean meal, pullets fed a diet formulated with CSM as the main protein source, had to consume more feed. Since more feed had to be made available to the pullets receiving the CSM diet, there was less competition among the pullets and therefore, there was a significant improvement in flock body weight uniformity.

Gossypol can affect reproductive performance (Heywang et al., 1950; Phelps, 1966). Therefore, the broiler breeder pullets were not fed a diet containing CSM beyond 18 weeks of age, which allowed adequate time for the birds to eliminate gossypol from their body tissues prior to the onset of egg production. Not surprisingly, birds fed CSM during the rearing period had similar egg production, fertility and hatchability rates as birds fed the standard soybean meal diet throughout the experimental period. The free gossypol content of the CSM utilized in broiler breeder pullet diets was 0.071% and until further research can be conducted with higher free gossypol contents, our recommendation to broiler breeder pullet managers would be not to exceed a dietary level of 0.075% free gossypol.

Even though CSM may be successfully utilized in broiler breeder pullet diets, its gossypol content should be below the recommended level. Gossypol concentrations in CSM may vary based on the genetic variety of the cottonseeds, the environmental conditions under which the cottonseeds developed and the method by which the oil was extracted from the cottonseed. Therefore, before a decision can be made to utilize a certain batch of CSM for broiler breeder pullet diets, the gossypol content must be determined.

The conventional American Oil Chemists' Society (AOCS) chemistry-based method for gossypol determination is too slow and laboratory intensive for quick field determinations. The near infrared reflectance spectroscopy (NIRS)-based method for free gossypol determination developed for part of the current research, however, is suitable for quickly determining the free gossypol content of CSM. The NIRS method for determining the free gossypol content of CSM is easy to perform but more

importantly the results are highly accurate when compared to results obtained from the conventional AOCS method.

In summary, the poultry industry will not benefit greatly if genetic strains of cotton are produced that have a high level of (+)-gossypol relative to (-)-gossypol. A niche for CSM, however, was found in the poultry industry. Cottonseed meal can be successfully utilized in broiler breeder pullet diets. In broiler breeder pullet diets, CSM is a better feed ingredient than the typically utilized soybean meal, since diets containing CSM improve flock body weight uniformity. To facilitate the use of CSM in broiler breeder pullet diets a NIRS-based method was developed to measure the free gossypol content of CSM.