EFFECT OF FEEDING GOSSYPOL FROM COTTONSEED MEAL AND VITAMIN E TO CATTLE

By

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To my parents

Jose Velasquez Delgadillo

Olga Pereira de Velasquez

To my brothers, sisters and their families

Jose Luis

Marco Antonio

Aurora Maria

Rodrigo Eugenio

Carlos Alfonso

Francisco Javier

Alvaro Fernando

Olga Natalia

Diego Vicente

To my wife and my unborn child

To my country

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EFFECT OF FEEDING GOSSYPOL FROM COTTONSEED MEAL AND VITAMIN E TO CATTLE

By

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A series of experiments were conducted to evaluate the value of supplemental vitamin E on the physiological response to free gossypol (**FG**) from cottonseed meal (**CSM**) fed to ruminants and preruminants. Experiments 1 (yearling heifers) and 2 (newborn bull calves from 2 wk to 6 mo) used the following dietary treatments: **CON**: soybean meal-based diet; **GOS**: CSM-based diet; **G+2E**: CSM-based diet + 2,000 IU vitamin E · animal^{-1} · d^{-1}; **G+4E**: CSM-based diet + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Experiment 3 (bulls from 6 to 16 mo) lacked treatment G+2E. Erythrocyte osmotic fragility (**EOF**) was increased ($P < .05$) in animals fed CSM; however, vitamin E supplementation lowered EOF in experiment 1. Vitamin E supplementation did not influence plasma or tissue gossypol concentration. Cottonseed meal seemed to aid in the absorption and deposition of α-T in animals fed a normal level of vitamin E. In heart,

neck muscle, and testis (-)-gossypol was higher ($P < .05$) than (+)-gossypol, while the reverse was true for liver. In vitro lipid peroxidation of tissue indicates that gossypol acts as an antioxidant in lipid peroxidation systems and its role as antioxidant may be dose or tissue dependant. In calves, hemoglobin and hematocrit were decreased ($P < .05$) in GOS calves, and vitamin E supplementation counteracted ($P < .05$) this effect. Ten calves died, 6 from the GOS and 2 each from the G+2E and G+4E treatments. Necropsy findings were compatible with gossypol toxicity. Histopathological examination revealed centrilobular necrosis in the liver and atrophy and vacuolation of cardiocytes. In bulls, percentage motility, normal, and live sperm, and daily sperm production were lower ($P < .1$), while percentage of primary abnormalities, and abnormal midpieces were increased ($P < .05$) in the GOS bulls. There was a trend of gossypol to decrease and vitamin E to improve libido score. The results of the GOS libido test may indicate lack of sexual maturity which agrees with sperm production data.

In experiment 1, gossypol (4 g FG/d for 112 d) did not have any adverse effect on animal performance, nor did it cause any sign of gossypol toxicity. In experiment 2, feeding CSM at a level to provide 400 mg FG/kg of feed DM caused death of some calves with gossypol related toxicity signs. Vitamin E supplementation increased performance and may have conferred some protection against gossypol toxicity. In experiment 3, feeding gossypol to Holstein bulls negatively affected some reproductive measurements; however, vitamin E supplementation improved these conditions. Gossypol did not decrease plasma or tissue α-tocopherol in any of the three experiments.

CHAPTER 1
INTRODUCTION

By-products of the cotton fiber and cottonseed oil industry such as whole cottonseed (**WCS**) and cottonseed meal (**CSM**) are an important source of economical protein and energy in livestock rations. Approximately six million tons of cottonseed are produced per year in the United States (NCPA, 1990). Gossypol [(2,2'-binaphthalene)-8,8'-dicarboxaldehyde-1,1',6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl] is a yellow polyphenolic pigment found in cottonseed. Gossypol found in cottonseed is referred to as free gossypol (**FG**), which is the form that is toxic to livestock. Bound gossypol (**BG**) is found in by-products of the cottonseed oil industry along with FG. Bound gossypol has not been found to be a major toxicant for livestock, but its role in animal toxicity is not yet fully understood (Jones, 1991). Free gossypol is transformed to BG as the cottonseed is treated with heat and pressure in the process of extracting the oil. Cottonseed meal, or any other by-product of the cottonseed oil industry may be less toxic to livestock as their concentration of FG is lower, but Calhoun and Holmberg (1991) suggested that BG may be released during the digestive process and have a physiological effect in the animal.

In the past several years, FG in ruminant diets has increased due to an increased consumption of WCS compared with CSM. Lusby et al (1991) reported that WCS is

being fed to ruminants more than any other by-product of the cottonseed oil industry (e.g. 2.1 million tons of WCS vs 1.5 million tons of CSM). In Florida, the WCS used in dairy rations has increased in the past several years, and the daily consumption of FG may be above 15 g per cow.

Although ruminants seem to have a large capacity to detoxify gossypol, toxicity resulting from consumption of this compound has been observed. Gossypol has been found to affect liver function, erythrocyte oxygen carrying or releasing capacity, respiration rate, feed intake and productive and reproductive capacity (Lindsey et al., 1980; Calhoun et al., 1990; Gray et al., 1990). It is also believed that gossypol may compromise the immune response of ruminants (Holmberg et al., 1988; Hudson et al., 1988). Gossypol intake by mature ruminants may overwhelm ruminal detoxification capacity and become absorbed at potentially toxic levels (Randel et al., 1992). Lindsey et al. (1980) suggested that these toxicity signs can be intensified when ruminants are under physiological, nutritional, and/or environmental stress.

Preruminants are especially susceptible to gossypol toxicosis. It seems that the undeveloped young ruminant rumen is unable to detoxify free gossypol (Morgan et al., 1988). Calhoun et al. (1990) found that osmotic fragility of erythrocytes was increased when gossypol was fed to lambs. Holmberg et al. (1988) reported that high dietary concentrations of gossypol for calves caused death with lesions compatible with gossypol toxicity. Risco et al. (1992) found that feeding 800 mg FG/kg to calves resulted in the death of four animals as a result of circulatory failure associated with gossypol toxicity.

Gossypol has been found to have an antifertility effect. In humans, which are very sensitive to gossypol; it can provoke damage to the reproductive tract that often is irreversible. In China, dosage of 60 to 70 mg per day of gossypol for 35 to 42 days has been reported to produce sterility in human males, as well as resulting in some side effects (Qian and Wang, 1984). The concentration of gossypol in meat for human consumption has been very low in muscles of lambs fed high amounts of gossypol, although significant amounts were found in liver and kidney. The amount of gossypol that can be found in meat from livestock fed cottonseed products has not been yet clearly established.

Gossypol can interact directly with biological membranes by promoting the formation of highly-reactive oxygen-containing free radicals (Janero and Burghardt, 1988). Free radicals provoke oxidative injury and compromise the antioxidant system of living organisms (Bender et al., 1988). These authors found that systemic levels of alpha-tocopherol, ascorbate and glutathione peroxidase, and other antioxidants were reduced by feeding rats high amounts of gossypol. In dairy cattle, Lane and Stuart (1990) found that feeding gossypol decreased plasma alpha-tocopherol concentrations, indicating a possible relationship between gossypol toxicity and vitamin E.

There are few data showing the effect of supplemental vitamin E in counteracting gossypol toxicity. Thus, at the University of Florida, a series of experiments were undertaken to evaluate the value of supplemental vitamin E on the physiological response to CSM (gossypol) fed to ruminants and young ruminants prior to rumen development.

CHAPTER 2
LITERATURE REVIEW

Gossypol is a naturally occurring polyphenolic substance present in various parts of plants belonging to the Malvaceae family. Gossypol content of the plant varies depending on environmental factors and plant species. It is probably the most important antiquality compound in cottonseed and its by-products and tends to limit their utilization when fed to livestock (especially nonruminants and preruminants).

This chapter addresses some aspects of biosynthesis, chemistry, metabolism, and toxicity of gossypol with reference to ruminants and preruminants.

Gossypol Biosynthesis

The biosynthesis of gossypol occurs in various parts of the cotton and other plants that belong to the Malvaceae family. In cotton root homogenates, Heinstein et al. (1962) reported that according to the labeling pattern and incorporation of ^{14}C-labeled acetate the biosynthesis of gossypol may occur via the isoprenoid pathway. This pathway of biosynthesis was later confirmed when Heinstein et al. (1970) observed that six molecules of mevalonate-2-^{14}C were incorporated stereospecifically into one molecule of gossypol.

Chemical Structure

Gossypol is a yellow polyphenolic pigment that has a molecular weight of 518.54 and a molecular formula of C_{30} H_{30} O_8. Gossypol occurs in three tautomeric forms: aldehyde, hemiacetal and phenolic quinoid tautomers (Figure 2-1). It is a highly reactive substance due to the two phenolic hydroxyl and the aldehyde groups that allow gossypol to react with a variety of compounds (Abou-Donia, 1989). It is a cherical molecule because of steric hindrance about the internaphthy bond (Cass et al., 1991). When gossypol is isolated from plants, it is a mixture of (+)-, and (-)-gossypol isomers. There are differences in the amount of each isomer isolated from different plants. In *Thespesia polpunea* the isolation of gossypol yields an excess of (+)-gossypol, while *Gossypium barbadense* yields more (-)-gossypol (Cass et al., 1991). The stereospecificity of gossypol is in part involved in the toxic effect seen with this compound. The (-)-gossypol isomer appears to be responsible for the antifertility effect observed in rats (Wang et al., 1992).

Gossypol in whole cottonseed (**WCS**) is found in the pigment glands which appear as small black dots on the cut surface of the seed (Jones, 1991). Its concentration in the seed is related to environmental conditions as well as agricultural practices. Three terms are commonly used to describe gossypol: free (**FG**), bound (**BG**), and total (**TG**) gossypol.

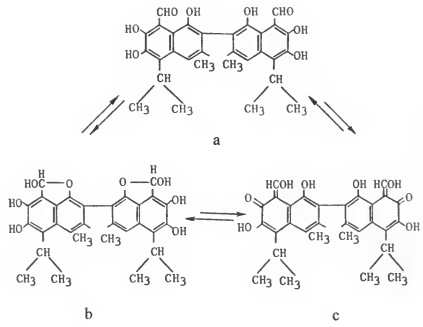

Figure 2-1. Tautomer forms of gossypol. (a) aldehyde, (b) hemiacetal, and (c) phenolic quinoid

Bound gossypol is used to describe the amount of the substance that binds to proteins and other compounds during WCS oil extraction process. Different procedures of extracting the oil from WCS yield different amounts of BG and FG. The direct solvent extraction of cottonseed oil yields the highest FG content in the cottonseed meal (**CSM**) while the screw press process yields the lowest. All gossypol found in the undisrupted seed is in the free form, which plays an important role in the toxicity of the compound. Total gossypol is equal to the sum of FG and BG and, therefore, is not affected by the processing of the seed, but differences in varieties and environmental factors affecting the growth of the cotton plant.

Metabolism of Gossypol

Gossypol is absorbed through the intestine as well as the epithelial lining of the stomach with the small intestine being the main site (Abou-Donia et al., 1970). Before reaching the site of absorption gossypol undergoes some changes. Bressani et al. (1964) reported greater FG excretion than intake in dogs, suggesting that some bound gossypol may have been released during its passage throughout the gastrointestinal tract (**GIT**). In ruminants, although these changes have not been quantified, higher amounts of gossypol are suspected to react not only in the stomach and intestine but in the rumen. In lambs, Calhoun and Wang (1995) found that absorption of gossypol from cottonseed meats was greater when it was fed to bypass the rumen than when rumination was allowed. In the rumen, gossypol may react with soluble proteins and, therefore, reduce its later

absorption (Reiser and Fu, 1962). Calhoun (1995) suggested that although the binding of gossypol to soluble proteins in the rumen is important in the detoxification of this compound, other mechanisms of rumen detoxification may render gossypol unavailable (i.e. binding to metals and (or) microorganism membranes).

The first action of gossypol upon the animal system would be to decrease bioavailability of nutrients, including minerals and proteins. The next detrimental effect of gossypol before absorption may be related to its effect on a variety of enzymes including digestive enzymes. Abou-Donia (1989) reported that in vitro digestion of proteins by pepsin and trypsin was reduced when gossypol was added prior to enzymatic digestion. If gossypol has an effect on rumen microorganisms, rumen digestibility could also be reduced. It is unknown whether these effects exist and(or) to what extent they affect animals.

There are differences in the amount of gossypol absorbed from different gossypol containing feedstuffs (i.e. CSM and WCS). Free gossypol from CSM seems to be more available than free gossypol in WCS (Chase et al., 1994). Several studies found reproductive impairment in bulls fed CSM that provided between 14 to 16 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} (Velasquez-Pereira et al., 1996a; Chenoweth et al., 1994; Risco et al., 1993) but not when fed greater amounts from WCS (Cusack and Perry, 1995; Chase et al., 1994; Smith et al., 1991). Several theories have been postulated to explain this controversy. Calhoun (1995) suggested that FG analysis may not represent in the ruminant the fraction believed to be correlated with toxicity in nonruminants. Bound gossypol from CSM may become FG in the GIT and be available for absorption. Free gossypol in WCS may be

released at a slower rate and may become permanently bound and not available for absorption in the small intestine.

After absorption, most gossypol is excreted via bile in rats, suggesting a biliary circulation of gossypol between the liver and GIT. Qian and Wang (1984) suggested that gossypol fits the criteria for a type of compound excreted via the bile, such as compounds of high molecular weight containing anionic groups and two or more aromatic rings. Abou-Donia (1989) suggested the following pathway for the metabolism of gossypol: gossypol is absorbed from the gastrointestinal tract, mainly the small intestine; it enters the liver via the hepatic artery or through the lymph into the hepatic sinusoid and is taken up by Kupffer cells. In the liver, gossypol is metabolized, conjugated, and excreted with the bile into the duodenum. Some of the gossypol excreted is reabsorbed, completing an enterohepatic cycle that may be repeated several times which involves a gradual excretion via feces. The mechanism of excretion seems to require an active secretory process that can be saturated.

Gossypol isomers have been found to have the following pattern of accumulation in ruminant tissues (Kim et al., 1996; Velasquez-Pereira et al., 1996b): in the liver total gossypol is higher than any other tissue, but (+)-gossypol concentration is greater than (-)-gossypol. The contrary is found in muscle and heart, where (-)-gossypol is greater than (+)-gossypol. Total gossypol accumulation pattern is liver>heart>muscle. The fact that gossypol is accumulated in the liver may be related to the tissue susceptibility to gossypol toxicity or to the fact that the liver is the main route of its metabolism. Also, gossypol is a lipophilic compound, therefore, it tends to accumulate in tissues or cell

fractions containing high amounts of lipid membrane e.g. mitochondria, lysosomal and microsomal fractions (Baran and Ismailov, 1993).

Gossypol has been reported to be cardiotoxic, causing degeneration of cardiac muscle. The (-)-gossypol has been shown to be the optically active form that induces fertility impairment in male animals (Wang et al., 1987). Accumulation of this isomer in liver and heart tissues is similar, which may be related to the toxicity signs shown during gossypol toxicosis.

Lower accumulation of (-)-gossypol in the liver relative to (+)-gossypol may be related to greater affinity of (-)-gossypol for plasma proteins (Wu and Reidenberg, 1990). The liver is perfused by a fluid similar to plasma which has greater protein concentration than the interstitial fluid found in heart and testis (Joseph et al., 1986). Therefore, (-)-gossypol could be bound to proteins at the time this protein rich fluid perfuses the liver and be redirected to other tissues where it can interact with cellular components that had greater affinity to bind (-)-gossypol than plasma protein. The (+)-gossypol may not be accumulated in tissues other than liver, where it seems to be eliminated, due to its low specific interaction with cellular components (Wang et al., 1992).

The Toxicity of Gossypol

There are several important factors that play a role in gossypol toxicity development, including the type of animal (ruminant vs. nonruminants); ruminants have greater capacity to detoxify gossypol before absorption than nonruminants. Among

ruminants, stage of rumen development would affect gossypol toxicity with young ruminants with undeveloped rumens behaving like nonruminant animals.

Composition of the diet (i. e. protein and mineral concentrations) is also important. The effect of gossypol is both time and dose dependant. It seems that a quantity of gossypol must be accumulated to a certain concentration in order to exert its effect and cause clinical signs of gossypol toxicity or death.

Young Ruminants

Young ruminants with undeveloped rumens are more susceptible to gossypol toxicity than older animals due to lack of a detoxification mechanism that occurs in the rumen of adult animals (Reiser and Fu, 1962). Calhoun and Wang (1995) demonstrated that the rumen plays an important role in detoxifying gossypol. These researchers fed 20 or 30 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} to two groups of animals. The first group consisted of weaned lambs fed a diet containing cottonseed meats as the FG source, while in the second group the cottonseed meats were mixed with the milk replacer in order to bypass the rumen. The weaned lambs exhibited no signs of gossypol poisoning whereas all milk-fed lambs died due to gossypol poisoning. Risco et al. (1992) conducted an experiment to define at what concentration FG is safe in young calves. Diets containing 0, 100, 200, 400, or 800 mg FG/kg of diet DM were fed to Holstein calves from 1 to 120 d of age. Clinical evidence of disease was limited to calves fed 400 or 800 mg FG/kg. Authors concluded that a ration containing up to 200 mg FG/kg is safe, 400 mg FG/kg approaches toxicity and 800 mg FG/kg causes death. Also, death losses from gossypol

toxicosis in calves has been reported in the following range (mg/kg of diet DM): 4000 total gossypol (Hudson et al., 1988), from 240 to 380 FG (Holmberg et al., 1988), and from 100 to 220 FG (Zelski et al., 1995).

Postmortem and histological findings of fatally intoxicated calves included widespread edema and congestion, particularly in the lungs and body cavities; straw-colored effusion noted in the abdominal and thoracic cavities; flabby and dilatated heart; and enlarged and congested liver with centrilobular degeneration (Risco et al., 1992). The clinical signs of gossypol toxicity are compatible with heart failure, although a direct effect of gossypol on the liver cannot be ruled out. Blood components and blood chemistry characteristics also may be affected by gossypol. Risco et al. (1992) reported that changes in erythrocyte characteristics such as hemoglobin concentration and hematocrit are not conclusive in young ruminants fed gossypol indicating normal hematopoietic response is maintained, thus the degree of anemia caused by gossypol is mild. Furthermore, chemistry panels designed to monitor serum proteins, energy status, serum electrolytes, renal function, liver function, and necrosis generally are not useful in demonstrating impending gossypol toxicity when used as a group monitoring test.

Ruminants

Reiser and Fu (1962) reported that binding of gossypol to soluble proteins within the rumen may confer ruminants the ability to withstand gossypol intakes greater than nonruminants. Furthermore, Calhoun (1995) suggested that ruminal bacteria also could play a role in gossypol detoxification.

Although ruminants seem to have a large capacity to detoxify gossypol, toxicity resulting from consumption of this compound has been observed. Gossypol has been found to affect liver functions, erythrocyte oxygen carrying or releasing capacity, respiration rate, feed intake and production and reproduction capacity (Calhoun et al., 1990; Gray et al., 1990; Lindsey et al., 1980). Gossypol also may compromise the immune response of ruminants (Holmberg et al., 1988; Hudson et al., 1988). These findings indicate that gossypol intake by mature ruminants may overwhelm ruminal detoxification and become absorbed at concentrations potentially toxic (Randel et al., 1992).

Lindsey et al. (1980) reported that these toxicity signs can be intensified when ruminants are under physiological, nutritional, and/or environmental stress. Some of the clinical signs observed by Lindsey et al. (1980) in mature dairy cattle fed 6.6 and 42.7 g FG $\cdot$ animal^{-1} $\cdot$ d^{-1} were reduced milk production and respiratory stress. Physiological changes included reduced hemoglobin concentration and increased erythrocyte osmotic fragility (**EOF**). Smalley and Bicknell (1982) reported that intake of 2.7 to 4.5 kg of ammoniated WCS by dairy cows resulted in 10% mortality rate. Calhoun et al. (1995b) reported that three dairy cows fed ammoniated WCS died of causes related to gossypol toxicity. Although most of the experiments reporting negative effects of gossypol on ruminants had used large amounts of cottonseed products, some special conditions such as treatment of the seed which makes gossypol more available have to be monitored very carefully.

Lane and Stuart (1990) in a field study reported that gossypol intake (up to 42 g FG/d) by mature lactating dairy cattle reduced plasma α-tocopherol (**α-T**) concentration, and suggested that the antioxidant system of animals fed gossypol may be compromised. In this study no statistical data were shown and gossypol analysis may not represent the real values. Willard et al. (1995) found that by d84, cows given 4 g FG · animal^{-1} · d^{-1} that showed elevated EOF, had lower plasma α-T concentration than control cows that showed low EOF. No supplemental vitamin E was given in the previous experiment. In that experiment, four animals with the highest EOF were chosen from 17 receiving 4 g FG · animal^{-1} · d^{-1} in a group fed experiment to determine plasma α-T concentration. It may have been possible that these animals consumed more supplement (high EOF) with low vitamin E content, and consumed less forage which would have been an excellent source of vitamin E. In a more controlled study, Mena et al. (1996) reported that gossypol from CSM, WCS or both (900 to 1800 mg TG/kg of diet DM) sources did not reduce concentrations of antioxidant vitamins, and that in fact it increased plasma α-T concentrations.

Erythrocyte fragility is a very sensitive indicator of systemic gossypol status, since it appears shortly after gossypol consumption has started. It has been reported to increase in several studies with gossypol-fed ruminants (Velasquez-Pereira et al., 1996c; Risco et al., 1993). The mechanism by which gossypol affects erythrocytes is not well understood. From in vitro experiments, the effect of gossypol on cells has been explained by the ability of gossypol to interact with proteins, especially membrane bound, and by

binding and modifying the properties of the lipid bilayer matrix (Reyes et al., 1984). These researchers found that gossypol binds strongly to bilayers of different lipid compositions and induces an electrical conductance that is accompanied by an increase in proton permeability. Furthermore, gossypol caused an increase in diffusion in lipid membranes in the presence of NaCl, which could explain the increase in EOF.

de Peyster et al. (1986) found an increase in membrane permeability due to gossypol treatment, and suggested that degeneration of cell membranes at all levels of organization may exist with acute prolonged exposure to gossypol in vivo. More evidence of the effect of gossypol on membranes was reported by Tanphaichitr et al. (1995) who found that gossypol at low concentrations intercalates in the phospholipid bilayer with the hydrophobic domains of the toxicant interacting with the phospholipid hydrocarbon chain, while its hydrophilic groups would be exposed to the aqueous phase. The intercalation of gossypol into a lipid bilayer, such as the erythrocyte membrane, would alter its fluidity and therefore may explain increased erythrocyte fragility.

Gossypol has been found to have an antifertility effect. Gossypol added to in vitro culture negatively affected embryo development (Zirkle et al., 1988), and in culture luteal cells decreased progesterone synthesis (Gu et al., 1990). In vivo experiments have demonstrated that feeding cows and heifers 20 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} did not affect reproductive performance (Willard et al., 1995; Gray et al., 1993).

In the male ruminant, gossypol seems to exert a unique and selective effect on the reproductive system. A reduction in sperm production and motility are the most common effects of gossypol toxicity.

A particular damage termed segmental aplasia of the mitochondrial sheath of the sperm midpiece has been reported in rats (Oko and Hrudka, 1982) and also in male ruminant sperm (Chenoweth et al., 1994).

Risco et al. (1993) found a lower percentage of normal sperm in the semen of bulls fed 16.6 mg FG · kg^{-1} BW · d^{-1} from CSM. Defects of sperm midpieces accounted for most of the abnormalities. These researchers observed this effect after 9 wk of feeding the gossypol-containing diet. Chase et al. (1994) did not find differences in sperm abnormalities when bulls were fed approximately 6 mg FG · kg^{-1} BW · d^{-1} from CSM or 60 mg FG · kg^{-1} BW · d^{-1} from WCS. Collectively, Risco et al. (1993) and Chase et al. (1994) demonstrated in ruminants that gossypol effect is both dose and time dependent, and that source of gossypol influences the development of toxicity.

The detrimental effect of gossypol on male bovine reproduction seems to appear when greater concentrations than those used by Chase et al. (1994) are fed and in the form of CSM, since feeding high gossypol diets in the form of WCS have yielded inconsistent results (Cusack and Perry, 1995; Smith et al., 1991; Arshami and Ruttle, 1988). Cusack and Perry (1995) reported that mature bulls fed 20.4 to 50.8 mg FG · kg^{-1} BW · d^{-1} from WCS for 8 mo did not show any sign of reproductive impairment. Similarly, Smith et al. (1991) reported that feeding high concentrations of gossypol (64 to 75 mg FG · kg^{-1} BW · d^{-1}) in the form of WCS for 4 mo did not affect sperm characteristics or cause testicular degeneration in bulls. Contrary to these two experiments, Arshami and Ruttle (1988) found that WCS, CSM and cottonseed hulls fed to bulls caused testicular histological changes, indicating a detrimental effect on the

spermatogenic tissue and associated cells. Several reasons have been proposed to explain these inconsistent results. 1) Cusack and Perry (1995) suggested that the feeding of WCS with gossypol chelating agents such as Ca, Na, protein, and trace minerals may have affected the availability of gossypol in their study and that of Smith et al. (1991). 2) Different concentrations of total gossypol and isomers are found in different species of cotton and even within the same species grown in different environments. 3) Calhoun (1995) suggested that treatment of the WCS (i.e. ammoniation) caused a different pattern of gossypol absorption. Therefore, differences in feeding practices and source of feed may account for different effects among experiments.

Sperm abnormalities, especially those associated with the midpiece, are increased due to gossypol ingestion. Segmental aplasia of the mitochondrial sheath of the sperm midpiece is a very characteristic defect caused by gossypol in nonruminants. Chenoweth et al. (1994) reported that sperm defects such as missing segments of the mitochondria helix, and irregular outlines and fractures in the sperm midpiece were caused by feeding bulls CSM (16.6 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}). Sperm motility also may be affected by gossypol feeding and (or) be associated with increase midpiece abnormalities. Chenoweth et al. (1994) found that feeding of relatively high concentration of FG to bulls did not affect adversely percentage motility at the same time that sperm abnormalities were increased (9 wk); however, some differences in sperm motility were apparent between the control and the gossypol-treated animals at the end of the experiment. Reduction in sperm motility has been reported in several animal species treated with gossypol (Randel et al., 1992; Wang et al., 1988).

Chenoweth et al. (1994) suggested that motility may be impaired by structural damage to the sperm midpiece in animals fed gossypol. Reduction in sperm motility may be caused by induction of abnormal midpiece structure and function (Cusack and Perry, 1995). Structural integrity of many sperm structures depend on S-S cross linked polypeptides. Disturbance of this bond may be associated with damage to the midpiece mitochondria (Baccetti et al., 1986). Mann and Mann (1981) reported that motility and metabolism of the sperm depends on interchange reactions between S-H and S-S groups. Gossypol may prevent the oxidation of S-H groups by chelating minerals or proteins responsible for this reaction (Chenoweth, P. J. Personal communication).

Sperm production is another male reproductive function that can be affected by gossypol. Chenoweth et al. (1994) reported that gossypol (16.6 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} from CSM) fed to Brahman bulls for 12 wk reduced daily sperm production to 50% of that of the control bulls. Chenoweth et al. (1994) suggested that this reduction may be caused by gossypol damage to the spermatogenic epithelium leading to reduced germinal cell layers. Mitochondria of Sertoli cells are more susceptible to gossypol than those from other cells (Tanpaichitr and Fitzgerald, 1989), and therefore may cause disfunction and abnormal structure of Sertoli cells. Chase et al. (1990) reported that bulls fed gossypol from WCS had damage to the basement membrane of the seminiferous tubules. Similarly, Arshami and Ruttle (1988) reported that bulls fed gossypol had damage to the basement membrane of the seminiferous tubules as well as decreased size of the Sertoli cells and decreased number of cell layers.

Cusack and Perry (1995) suggested that gossypol may reduce the incorporation of amino acids into spermatocytes and spermatids in the bovine testicles thus decreasing sperm production.

Gossypol appears not to affect production of reproductive hormones in bulls; however, in nonruminants testosterone production has been affected. Chase et al. (1990) reported that bulls fed gossypol (6 or 60 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}) from CSM and WCS did not show treatment differences in the basal mean concentration, maximal concentration, number of pulses or total testosterone released during 6 h of bleeding, but these amounts fed did not affect any other reproductive characteristic. Taylor et al. (1991) reported that feeding gossypol to male rats (from 5 to 20 mg gossypol $\cdot$ kg^{-1} BW $\cdot$ d^{-1}) for 11 wk provoked a dose-response decline in sexual motivation. Even at low dosage (5 mg gossypol $\cdot$ kg^{-1} BW $\cdot$ d^{-1}), over weeks of administration, males eventually showed signs of losing interest in seeking contact with a sexually receptive female. They concluded that the likely mechanism for the behavioral changes is a gradual suppression of serum testosterone. de Peyster and Srebnik (1988) found that rats administered 10 mg gossypol $\cdot$ kg^{-1} BW $\cdot$ d^{-1} subcutaneously every 5 d had reduced serum testosterone concentrations and decreased accessory organ weights. Possible explanations for these results were that gossypol may cause a direct suppression of steroidogenic enzymes, alter cAMP second-messenger function, or interfere with cell membrane properties which could result in a disruption of normal receptors in the interstitial cell membrane (de Peyster and Srebnik, 1988). Libido and hormone production in bulls have not been assessed when feeding gossypol at concentrations that could affect sperm characteristics.

Gossypol has been found to inhibit the generation of free radicals in some studies (Janero and Burghardt, 1988) and to stimulate it in others (de Peyster et al., 1984). Recently, Barhoumi and Burghardt (1996) reported that gossypol promoted the formation of reactive oxygen species and the depletion of glutathione in rat hepatocytes. Bender et al. (1988) reported a reduction in antioxidants in testes of rats fed gossypol. Damage due to lipid peroxidation in human sperm has been associated with loss of sperm motility, inactivation of enzymes, and loss of membrane integrity (Aitken and Fisher, 1994).

Gossypol appears to affect late spermatogenesis when spermatids discard the majority of their cytoplasm and thus they have lower cytoplasmic defensive enzymes (Aitken and Fisher, 1994). Therefore the effect of gossypol on semen characteristics may in part be related to increased free radical production and(or) inhibition of enzymes related to the antioxidant defense system.

Diagnosis and Recommendations

When suspecting or investigating a gossypol problem, the following factors should be considered (Risco and Velasquez, 1994): determine if cottonseed products are being fed; analyze sources of gossypol individually by an official gossypol testing laboratory; from these results calculate the amount of free gossypol in feeds and compare them to the recommended concentrations suggested by Calhoun and Holmberg (1991). There must be a clinical history which is suggestive of gossypol toxicosis.

Among other characteristics are involvement of multiple animals, sudden death syndrome, chronic respiratory problems that are unresponsive to antibiotics, and(or) history of infertility. Necropsy findings are compatible with cardiovascular and respiratory failure, and histopathology examination of liver reveals centrolobular hepatic congestion and necrosis related to hepatic anoxia.

Calhoun and Holmberg (1991) recommended that adult cows should be fed no more than 600 mg FG/kg of diet DM. Consumption of FG at this concentration should not adversely affect reproduction in females (Gray et al., 1993). In male ruminant used for breeding, the maximum intake allowed is 200 mg FG/kg of diet DM and for young ruminants, 100 mg FG/kg (Calhoun and Holmberg, 1991).

CHAPTER 3
FEEDING COTTONSEED MEAL AND VITAMIN E TO BEEF HEIFERS

Introduction

Gossypol is a yellow polyphenolic pigment found in the glands of roots, stems, and seeds of plants from the malvaceae family. This pigment is found in the cottonseed and its by-products, limiting its use in animal diets especially for nonruminant species. In ruminants, gossypol seems to have little effect when whole cottonseeds (**WCS**) and/or by-products are fed within safe limits (Calhoun and Holmberg, 1991; Rogers and Poore, 1995). There are three forms in which gossypol content of a product is expressed: free gossypol (**FG**) or the natural form which exists in the seed; bound gossypol (**BG**), and total gossypol (**TG**). Cottonseed meal (**CSM**) contains variable amounts of FG and BG, depending on the process used to extract the oil from the seed (Jones, 1991). Although toxicity effects are expected when high concentrations of FG are fed, ruminants have the capacity to detoxify large amounts by binding it to soluble proteins in the rumen (Reiser and Fu, 1962). Gossypol has been found to affect liver functions, erythrocyte oxygen carrying or releasing capacity, respiration rate, feed intake, and production and reproduction efficiency (Lindsey et al., 1980; Gray et al., 1990).

Gossypol effect on animals seems, at least to some extent, to be related to the formation of free radicals or to a decreased concentration of antioxidants such as α-tocopherol (**α-T**), and β-carotene (**β-C**). In a field study, Lane and Stuart (1990) reported a negative relationship between FG intake and serum retinol and tocopherol concentration of dairy cattle fed WCS at a concentration to provide from 10 to 41.1 g FG · animal^{-1} · d^{-1}. In a more controlled study, Willard et al. (1995) found that cows receiving 4 g FG · animal^{-1} · d^{-1} had lower ($P < .05$) serum α-T and β-C concentrations than cows fed a control diet with no gossypol. Bender et al. (1988) found that α-T, ascorbate and glutathione peroxidase, and other antioxidants were reduced by feeding rats high concentrations of gossypol. An in vitro experiment indicated that gossypol generated the superoxide radical in the presence of liver microsome and NADPH (de Peyster et al., 1984).

The effect of feeding high amounts of α-T on the gossypol status of heifers fed CSM has not been investigated. Therefore the objective of this experiment was to evaluate the effect of high concentrations of supplemental vitamin E on gossypol status and physiological response of beef heifers fed CSM.

Materials and Methods

Animals, diets and management. Thirty-two yearling Limousin and Angus crossbred heifers, daughters of two different sires and averaging 315 kg BW, were used in a 112-d experiment.

Animals were assigned randomly to one of four dietary supplements. Supplement one (**CON**) was based on soybean meal (**SBM**), corn, and adequate vitamin E (30 IU/kg of diet DM). Supplement two (**GOS**) was based on CSM, corn, and adequate vitamin E (30 IU/kg of diet DM). Supplement three (**G+2E**) contained CSM, corn, and 2,000 IU vitamin E · animal^{-1} · d^{-1}. Supplement four (**G+4E**) contained CSM, corn, and 4,000 IU vitamin E · animal^{-1} · d^{-1}. Treatments based on CSM provided 4 g FG · animal^{-1} · d^{-1}. Supplements (Table 3-1) were formulated to provide equal amounts of CP and TDN, and to meet NRC (1984) nutritional requirements. Heifers were fed the supplement daily via Calan gates and had free access to water and poor-quality mature bermuda grass (*Cynodon dactylon*) hay with an α-T concentration of 9.1 IU vitamin E/kg. The protocol for all heifer procedures had been approved by the University Animal Use Committee.

Blood and tissue sampling and analyses. Blood was collected every 2 wk via jugular venipuncture with an 18-gauge needle into heparinized vacutainer blood collection tubes for a total of 9 collections. Blood was analyzed for erythrocyte osmotic fragility (**EOF**), hemoglobin, and hematocrit. Erythrocyte osmotic fragility, measured as percentage hemolysis, was evaluated in .65 and .55% buffered saline solution as described by Risco et al. (1993). Hemoglobin was determined using a colorimetric procedure (Sigma Chemical Co., St. Louis, MO). Hematocrit was determined from blood using a micro hematocrit centrifuge (IEC MB Centrifuge, Needham Heights, MA). Blood was centrifuged for 25 min at 700 x g, and plasma was removed and stored at -20°C until analyzed for α-T (all collections), retinol (**RET**), retinol palmitate (**RETP**),

Table 3-1. Composition of dietary supplements[a].

	Supplement[b]			
Item	CON	GOS	G+2E	G+4E
Offered (kg/d)[c]	4.2	4.5	4.5	4.5
DM (%)	88.0	88.0	88.0	88.0
CSM (%)[c]	0.0	75.0	75.0	75.0
SBM (%)[c]	65.0	.0	.0	.0
Corn (%)[c]	33.0	23.0	23.0	23.0
Limestone (%)[c]	1.0	1.0	1.0	1.0
Trace minerals (%)[c]	1.0	1.0	1.0	1.0
CP (%)[d]	37 ± 2	36 ± 4	36 ± 2	37 ± 2
Vitamin E (IU/kg)[e]	47 ± 9	44 ± 10	488 ± 54	871 ± 138
Vitamin A (IU/kg)[e]	10600	6293	6293	6293
(+)-gossypol (%)[f]	.00	.35	.36	.36
(-)-gossypol (%)[f]	.00	.86	.88	.87
Free gossypol (%)[g]	.00	.10	.10	.10
Total gossypol (%)[g]	.00	1.10	1.13	1.14
FG intake[h]	.00	4.5	4.5	4.5
Ca (%)[d]	0.6 ± .06	0.6 ± .04	0.7 ± .03	0.7 ± .02
K (%)[d]	1.3 ± .14	1.2 ± .10	1.2 ± .09	1.2 ± .08
Mg (%)[d]	.2 ± .01	.5 ± .01	.5 ± .01	.5 ± .01
P (%)[d]	.6 ± .13	1.1 ± .07	1.1 ± .06	1.1 ± .06
Cu (mg/kg)[d]	12.5 ± 1.67	13.5 ± 3.26	16.1 ± 6.35	14.9 ± 2.89
Zn (mg/kg)[d]	45 ± 27	64 ± 32	66 ± 29	68 ± 34
Mn (mg/kg)[d]	44 ± 10	39 ± 7	38 ± 5	42 ± 8
Fe (mg/kg)[d]	149 ± 108	102 ± 18	107 ± 27	116 ± 30
Se (mg/kg)[d]	.28 ± .06	.33 ± .06	.30 ± .04	.34 ± .02

[a]Values are means of eight mixes ± standard deviation of the mean.

[b]CON = soybean meal (SBM) + corn + 30 IU vitamin E/kg; GOS = cottonseed meal (CSM) + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c] As fed basis.

[d] DM basis.

[e] As fed basis. A composite sample from all mixing dates. An IU/kg = retinol acetate (μg/kg) * 2.91

[f]As fed basis. Composite sample. HPLC procedure (Calhoun et al. (1995a), and Kim and Calhoun (1995)). Texas A&M.

[g]As fed basis. Composited sample. AOCS procedure. Texas A&M.

[h]As fed basis. FG = free gossypol. FG intake (g · animal^{-1} · d^{-1}) = Amount offered (g/d) * % FG.

β-C, alkaline phosphatase (**AP**), creatine kinase (**CK**) (every other collection), (+)-, (-)-, and total gossypol (collections 1, 3, 5, and 9). At the end of the experiment, animals were slaughtered and portions of liver, heart, and neck muscle (Sterno mandibularis) were collected and frozen at -20°C until analyzed for α-T, β-C, (+)-, (-)-, and total gossypol, Fe, Cu, and Zn. Liver samples were analyzed also for RET, RETP, and Se. Samples of supplements were collected and analyzed for α-T, RETP, Ca, K, Mg, Mn, P, CP, Fe, Zn, Cu, and Se.

Alpha-Tocopherol was determined following the procedure described by Njeru et al. (1992) for plasma, and by Njeru et al. (1995) for tissue and feed samples. Retinol, RETP, and β-C in plasma were analyzed as follows: 1 mL of plasma, in a 16 x 125 mm glass tube, was deproteinized with 1 mL of ethyl alcohol, and .5 mL of 25% ascorbic acid solution and vortexed; samples were then double extracted with 3 mL petroleum ether and kept in an ice bath. The petroleum ether extract was dried by evaporation under a stream of N_2 in a 35°C water bath, reconstituted in 750 μL of a solution of .1% acetic acid, 29.9% tetrahydrofuran, and 70% iso-octane with .2% added β-hydroxytoluene. The sample was separated in equal amounts in two sealed vials one for RET and RETP and the other for β-C analysis. Tissue and feed samples were prepared as follows: approximately 1 g of sample (fresh weight) and .1 g of ascorbic acid were homogenized in 10 mL of acetone; 1 mL of the homogenate was deproteinized with 1 mL of ethanol. After adding 2 mL of .9% saline solution, the mixture was vortexed for 6 min. The solution was double extracted and reconstituted as with plasma. Retinol and RETP were determined by injecting 20 μL of the reconstituted extracted sample (plasma, tissue or

feed) into the HPLC system. The HPLC system consisted of a Perkin Elmer 550 terminal (Perkin-Elmer, Analytical Instruments, Norwalk, CT), an ISS-100 auto sampler (Perkin-Elmer), a Series 4 Liquid chromatograph pump (Perkin-Elmer), and a Lichrosorb Si 60 5μm, 4 mm ID x 250 mm column (Hibar Fertigsaule RT pre-packed column RT 250-4 E, Merck, Darmstadt, Germany). The mobile phase consisted of 70% iso-octane, 29.9% tetrahydrofuran, and .1% acetic acid. The UV detector was an ABI Analytical Spectroflow 757 set at a wavelength of 325 nm and sensitivity of .005. Data were collected by a LCI-100 Laboratory Computing Integrator (Perkin-Elmer). Flow rate was 1 mL/min. The retention time of RET was 6.1 min and for RETP 2.47 min. Standards consisted of 10 ng of RET (Sigma Chemical Co., St. Louis, MO) and 10 ng of RETP (Sigma Chemical Co., St. Louis, MO). Analysis for β-C was similar except that the mobile phase was 90% iso-octane, 9.9% tetrahydrofuran, and .1% acetic acid; the wavelength was set at 450 nm, and the retention time was 3.05 min. Standards consisted of 100 ng β-C (Sigma Chemical Co., St. Louis, MO).

Cell mediated immune response was measured on d 50 by determining the animals ability to respond to phytohemagglutinin-P (**PHA**; Sigma Chemical Co., St. Louis, MO). Phytohemagglutinin-P was prepared in physiological buffered saline to a concentration of 150 μg/.1 mL. The hair on the right side of the neck was clipped, the skin-fold thickness was then measured using constant tension skin-fold calipers. A .1 mL of PHA solution was injected intradermally at the clipped site and skin-fold thickness was measured at 6, 12, 24, and 48 h after injection.

Stimulated lipid peroxidation was performed in heart and liver samples according to a modification of the procedure of Kornbrust and Mavis (1980). Briefly, approximately 1 g of fresh tissue was homogenized in 9 mL of 1.15% KCl solution. An aliquot (100 μL) was incubated in a water bath at 37°C for the specified length of time (0, 50, 100, and 200 min) with 500 μL of 8 mM Tris-malate buffer, 200 μL of 5 mM $FeSO_4 \cdot 7H_2O$ and 200 μL of 2 mM ascorbic acid solutions. Peroxidation was terminated by rapid addition of a 2 mL solution containing thiobarbituric acid (.375%) and 15% trichloroacetic acid in .25 N HCl, and boiling in a water bath for 15 min. Then samples were centrifuged for 15 min at 700 x g. The amount of colored product was measured spectrophotometrically at 535 nm. Lipid peroxidation was expressed in terms of nmol malondialdehyde (**MDA**)/mg of protein. Janero and Burghardt (1989) suggested that this test cannot be used as anything else other than an empirical indicator of membrane oxidative injury, therefore care should be taken in the interpretation of these results.

Enzymes were determined using kits (CK:Sigma Diagnostic Procedure No. 520; AP: Sigma Diagnostic Procedure No. 104). Minerals were determined according to the procedures described by Rojas et al. (1995) and Whetter and Ullrey (1978). Samples of plasma and tissue for gossypol analyses were shipped in dry ice to Texas A&M University where they were analyzed by HPLC according to the procedures described by Calhoun et al. (1995a), and Kim and Calhoun (1995).

Statistical analyses. Plasma, lipid peroxidation, and weight data were analyzed by repeated measures analysis of variance in a completely randomized design using GLM procedure of SAS (1988). The Greenhouse-Geiser Epsilon was used to determine

significant levels for the F-test. The model included the effect of sire, treatment and the interaction between sire and treatment. Sire was considered a random effect, hence the interaction between sire and treatment was used as the error term for the treatment effect. When the sire effect probability value was less than .2, PROC MIXED (SAS, 1996) was used to calculate the correct standard errors of the least square means for a mixed model. Tissue concentrations of (+)-, (-)-, and total gossypol were analyzed using PROC MIXED of SAS (1996), with a nested mixed model where a 3 x 3 factorial design (three tissues x three isomers) was nested within each animal, and animals were nested within a 4 x 2 factorial design (four treatments x two sires (random)). The nine measurements (three gossypol values for each of three tissues) per animal were assumed to be equally correlated with each other. Analyses of variables containing single or calculated observations where performed by ANOVA (SAS, 1988). These variables were tissue concentrations of α-T, β-C, RET, RETP, and minerals. The effect of treatment was tested using the interaction between sire and treatment. When the overall treatment effect was significant ($P < .05$) or tended to significance ($P < .1$), separation of means was done using the Duncan multiple range test for all the variables except gossypol tissue concentration for which the Tukey test (PROC MIXED) was used. Pooled standard errors were reported when all treatments had the same number of replicates, otherwise individual values for each least square mean were given.

Results and Discussion

Animal performance and blood parameters. Although the intake of CSM was high (3.4 kg · animal^{-1} · d^{-1}), calculated FG intake was approximately 600 mg FG · kg^{-1} of diet, (based on an average intake of 7.5 kg of DM/d, NRC, 1984) during the experimental period. This amount of FG intake was on the upper limit of the safe guidelines given by Rogers and Poore (1995) and Calhoun and Holmberg (1991). In order to achieve an intake of 4 g FG · animal^{-1} · d^{-1}, supplements had a high CP concentration which was not representative of normal diets. This high concentration of dietary CP could have affected the absorption and metabolism of gossypol by supplying a greater number of free ε-amino groups with which gossypol may have combined in the digestive tract and then excreted and (or) by facilitating the metabolism and detoxification of the absorbed gossypol (Abou-Donia, 1976). On average, supplements supplied the targeted amount of vitamin E (Table 3-1).

All supplements produced an increase ($P < .01$) in body weight over time (Table 3-2). Average daily gain did not differ ($P > .1$) among heifers fed the four supplements throughout the experiment. Likewise, weight of beef heifers supplemented with 0, .5, 2.5, 5, 10, and 20 g FG · animal^{-1} · d^{-1}, did not differ in a study conducted by Gray et al. (1993). Calk et al. (1992) reported that vitamin E or lysine supplementation to lambs fed a basal diet containing 20% CSM with .184% FG, increased ADG over those not receiving vitamin E. Supplements had been fed ad libitum, therefore an increase in ADG in lambs fed vitamin E could have been due to increased intake. Also a decrease in ADG

Table 3-2. Effect of cottonseed meal (CSM) and vitamin E on weight gain and average daily gain (ADG) of beef heifers[a].

	Supplement[b]				
Item	CON	GOS	G+2E	G+4E	SE[c]
Initial weight (kg)	317	299	314	328	13
Final weight (kg)	407	387	401	415	13
112 d gain (kg)	89	87	88	87	5
112 d ADG (kg)	.79	.78	.78	.78	.04

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

may have been caused by the effect of gossypol on digestive enzymes of animals fed the unsupplemented basal diet. In vitro digestion of proteins by pepsin and trypsin was reduced when gossypol was added prior to enzymatic digestion in an experiment reported by Abou-Donia (1989). The action of gossypol on the enzymes seems to be related to the binding of gossypol to the ε-amino group of lysine on the protein substrate or to the zymogen that could not be converted to the active enzyme (Abou-Donia, 1989). In our experiment, neither gossypol nor vitamin E supplementation affected body weight.

Alkaline phosphatase is a membrane bound enzyme used for diagnosis of bone and liver disorders or drug exposure. Production of AP is increased in response to primary or secondary hepatocellular disorders. Liver degenerative changes and hemorrhage are signs of gossypol toxicity. Although the reference range for AP is wide for ruminants, changes in concentration of AP would indicate impaired liver function or damage. In our experiment, we did not find any changes ($P > .1$) in plasma AP concentration due to dietary supplementation (Table 3-3). Also, there was no effect ($P > .1$) of supplementation on plasma CK concentration (Table 3-3). Creatine kinase is an enzyme used in the diagnosis of muscular disorders. Gossypol has been found to cause edema, hypertrophy and heart dilatation and degeneration of cardiac muscle in several species including swine (Abou-Donia, 1989) and cattle (Kerr, 1989). These damages to the heart are similar to damage caused by Se/vitamin E deficiency. Rats treated with 5 mg gossypol/kg BW did not have increased serum enzymes that are usually taken as indicators of liver damage; however, gossypol treatment depressed the activity of liver microsomal enzymes (Gawai et al., 1995). The concentrations of enzymes found in this

Table 3-3. Effect of cottonseed meal (CSM) and vitamin E on plasma alkaline phosphatase (AP) and creatine kinase concentrations (IU/L) of beef heifers[a].

		Collection				
Item	Supplement[b]	1	3	5	7	9
AP	CON	65.9	46.9	43.0	41.3	44.7
	GOS	45.1	21.1	17.6	21.6	18.4
	G+2E	55.9	33.0	26.2	25.9	26.8
	G+4E	41.0	26.7	26.4	27.8	24.8
SE[c]		7.7	7.4	6.9	5.8	9.1
CK	CON	29.2	32.9	29.5	39.1	34.2
	GOS	38.1	49.3	41.5	47.2	47.0
	G+2E	26.4	43.6	36.0	81.7	55.8
	G+4E	25.8	45.6	45.6	60.3	48.0
SE		4.0	13.5	4.5	17.0	7.2

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

experiment, do not suggest liver or muscle damage by feeding gossypol at the concentration provided in this experiment. Blood chemistry has been demonstrated not to be useful for monitoring gossypol toxicosis (Risco et al., 1992) and changes in enzyme concentrations may be indicative of liver failure only during terminal stages of the disease in calves.

Plasma α-T concentration (Figure 3-1) showed a time x treatment effect ($P < .01$). Animals supplemented with high amounts of vitamin E (G+2E and G+4E) had greater ($P < .05$) plasma α-T concentrations than animals supplemented with a more typical amount (CON and GOS) from collection 2 to the end of the experiment (Figure 3-1). Heifers fed CON or GOS supplements had equal amounts of supplemental vitamin E (30 IU/kg) and similar analyzed concentrations (Table 3-1). Therefore, at the estimated dietary vitamin E requirement for beef cattle, FG intake of 4 g/d did not decrease plasma α-T concentration. Although not statistically different, plasma α-T concentration appeared to be greater in animals in GOS than CON treatments. Animals in G+2E and G+4E did not show differences ($P > .1$) in plasma α-T concentration, but were greater ($P < .05$) than the low vitamin E supplemented treatments. Vitamin E absorption from the gastrointestinal tract is aided by the presence of dietary fats; therefore, higher fat content of CSM compared to SBM (150 vs 103 g) based supplement (NRC, 1984) could have increased vitamin E absorption. Since there was not a treatment with high vitamin E and no gossypol included in this experiment, it is not possible to speculate on the effect of gossypol on plasma α-T concentration at high levels of vitamin E supplementation. However, the fact that no differences were found between the two high concentrations

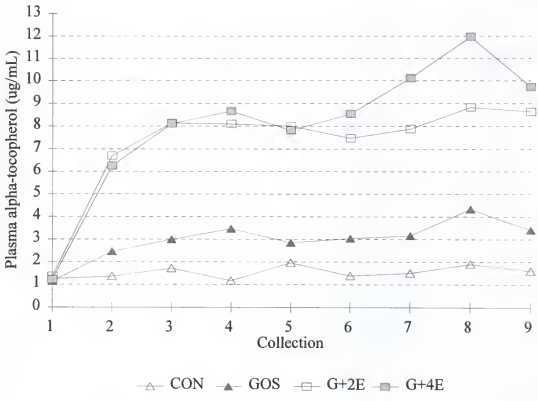

Figure 3-1. Effect of cottonseed meal (CSM) and vitamin E on plasma α-tocopherol concentration of beef heifers. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .16; C2, .71; C3, .90; C4, .82; C5, .99; C6, .90; C7, 1.20; C8, .86; and C9, .80.

(G+2E and G+4E), may indicate a reduced absorption of vitamin E either by gossypol effect or most likely saturation of vitamin E absorption. At high doses of vitamin E supplementation, the efficiency of vitamin E absorption decreased in rats (Traber et al., 1986). Furthermore, it has been reported that animals receiving excess dietary vitamin E absorbed only 1 to 5% of the dose (Hoffmann-La Roche Inc., 1994). Hydrolysis of vitamin E esters also could be reduced when feeding high concentrations of the vitamin. Absorption of tocopherols depends on various factors such as: a) pancreatic enzymes b) bile acids c) pH level of intestinal contents d) intestinal motility, and e) other food components, in particular fatty acids (Hidiroglou et al., 1992). A healthy liver is necessary for absorption and metabolism of vitamin E. The liver is targeted during gossypol toxicity, causing hemorrhage and degenerative changes.

Contrary to our results, Lane and Stuart (1990) reported that serum α-T and β-C concentrations were reduced in dairy cows fed high concentrations of gossypol (approximately 40 g/d). In this study, however, no statistical data were shown and gossypol analysis may not represent the real values. In a more controlled study, Mena et al. (1996) reported that gossypol from CSM, WCS or both (900 to 1800 mg total gossypol/kg) sources did not reduce plasma concentrations of antioxidant vitamins, and that in fact it increased plasma α-tocopherol concentrations.

Erythrocyte fragility is a very sensitive indicator of systemic gossypol status, since it appears shortly after gossypol consumption has started. Willard et al. (1995) found that by d 84, cows given 4 g FG $\cdot$ animal^{-1} $\cdot$ d^{-1} that showed elevated EOF had lower ($P < .05$) plasma α-T concentrations than cows not fed gossypol. No supplemental

vitamin E was given in this experiment. Four animals with the highest EOF were chosen from 17 receiving 4 g FG · animal^{-1} · d^{-1} in a group-fed experiment to determine plasma α-T concentration. It may have been possible that these animals consumed more supplement (high EOF) with low vitamin E content, and consumed less forage which would have been an excellent source of vitamin E.

In the present study, supplementation treatments did not affect ($P > .1$) plasma concentrations of RETP, RET, total retinoids, and β-C (Table 3-4). Analysis of a composite feed sample from each dietary supplement indicated a higher concentration of vitamin A in CON than in the other treatments (Table 3-1) even though all treatments had 20,000 IU vitamin A · animal^{-1} · d^{-1} added to the supplements. This was not reflected in greater plasma RET, RETP or total retinoids concentrations which agree with data reported by Eicher et al. (1994), who reported that plasma concentrations of RET and RETP did not reflect supplementation of vitamin A at normal doses. Similarly Westendorf et al. (1990) did not find increased plasma vitamin A concentration in steers fed normal concentrations of the vitamin. In a study with cottonseed products, plasma β-C concentration of cows supplemented with 4 g FG · animal^{-1} · d^{-1} and high EOF was lower than cows receiving 0 g FG · animal^{-1} · d^{-1}, by d 84 after initiation of supplementation (Willard et al., 1995). These authors related this change to seasonal availability of β-C in forages rather than any effect of gossypol on plasma β-C concentration.

In this study, EOF at .65 or .55% (Figure 3-2 and 3-3, respectively) saline solution, revealed a time x treatment effect ($P < .01$). Cottonseed meal supplementation

Table 3-4. Effect of cottonseed meal (CSM) and vitamin E on plasma retinol palmitate (RETP), retinol (RET), total retinoids, and β-carotene (β-C) concentrations (μg/mL) of beef heifers[a].

		Collection				
Item	Supplement[b]	1	3	5	7	9
RETP	CON	.18	.16	.12	.16	.15
	GOS	.26	.15	.18	.18	.16
	G+2E	.23	.14	.17	.18	.19
	G+4E	.22	.14	.17	.21	.19
SE[c]		.04	.02	.01	.01	.02
RET	CON	.62	.53	.52	.49	.43
	GOS	.53	.55	.45	.43	.41
	G+2E	.58	.48	.43	.41	.41
	G+4E	.64	.49	.43	.42	.40
SE		.06	.02	.03	.03	.02
Total Retinoids	CON	.84	.77	.70	.73	.69
	GOS	.84	.79	.70	.71	.74
	G+2E	.86	.70	.67	.68	.70
	G+4E	.88	.71	.68	.72	.76
SE		.05	.02	.03	.03	.03
β-C[d]	CON	1.15	---	1.29	1.56	1.61
	GOS	1.37	---	1.46	1.60	1.69
	G+2E	1.50	---	1.45	1.77	1.78
	G+4E	1.40	---	1.52	1.79	1.82
SE		.20	---	.07	.04	.05

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d] Missing samples in collection three.

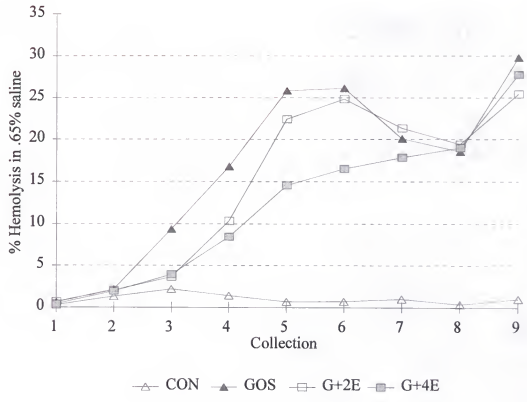

Figure 3-2. Effect of cottonseed meal (CSM) and vitamin E on erythrocyte osmotic fragility in .65% saline. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .35; C2, .92; C3, 3.32; C4, 4.60; C5, 3.10; C6, 2.50; C7, .80; C8, .93; and C9, 2.60.

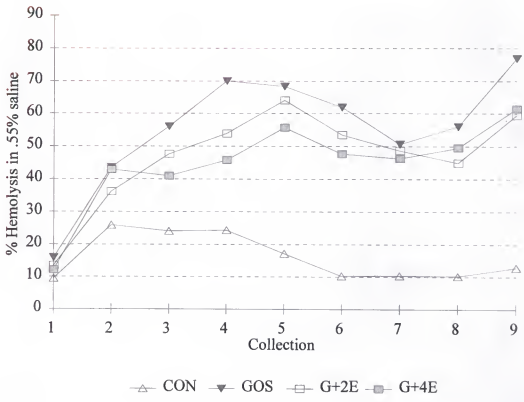

Figure 3-3. Effect of cottonseed meal (CSM) and vitamin E on erythrocyte osmotic fragility in .55% saline. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, 5.0; C2, 4.1; C3, 10.0; C4, 6.8; C5, 2.1; C6, 2.9; C7, 3.5; C8, 4.2; and C9, 5.3.

(GOS) tended to increase EOF ($P < .1$) over CON in collection 4. However, in vitamin E and gossypol supplemented animals (G+2E and G+4E) EOF did not differ ($P > .1$) from CON animals. On collections 5, 6 and 7, CON animals had lower ($P < .05$) EOF than the rest of the treatments, but the animals supplemented with 4,000 IU vitamin E · animal^{-1} · d^{-1} tended to have lower ($P < .1$) EOF than animals consuming gossypol with 30 IU vitamin E/kg (GOS). By the end of the experiment (collections 8 and 9), CON animals had lower ($P < .05$) EOF than the other treatments, and vitamin E supplementation no longer had any effect on EOF. A similar trend was observed with EOF measured at .55% saline. From these results, it appeared that vitamin E at high doses would slow down the increase in EOF for animals consuming gossypol. The EOF increase in animals consuming a gossypol containing feedstuff is an indicator of gossypol intake, but its use as a diagnostic tool for gossypol toxicity is questionable. In this study, the effect of gossypol on EOF was not accompanied by any other clinical sign of gossypol toxicity.

It is difficult to explain these results since the mechanism of gossypol effect on the erythrocyte is not well understood. From in vitro experiments, the effect of gossypol on cells has been explained by the ability of gossypol to interact with proteins, especially membrane bound, and by binding and modifying the properties of the lipid bilayer matrix (Reyes et al., 1984). These researchers found that gossypol binds strongly to bilayers of different lipid compositions and induces an electrical conductance that is accompanied by an increase in proton permeability. Furthermore, they showed that gossypol caused an increase in diffusion in lipid membranes in the presence of NaCl, which could explain the increase in EOF found in our experiment. de Peyster et al. (1986) found an increase in

membrane permeability due to gossypol treatment, and suggested that degeneration of cell membranes at all levels of organization may exist with acute prolonged exposure to gossypol in vivo. More evidence on the effect of gossypol on membranes was reported by Tanphaichitr et al. (1995) who found that gossypol at low concentrations intercalates in the phospholipid bilayer with the hydrophobic domains of the toxicant interacting with the phospholipid hydrocarbon chain, while its hydrophilic groups would be exposed to the aqueous phase. The intercalation of gossypol into a lipid bilayer, such as the erythrocyte membrane, would alter its fluidity and therefore may explain increased erythrocyte fragility. de Peyster et al. (1986) found that the effect of gossypol in lipid membranes is dependent on the lipid composition, with membranes composed of cholesterol showing very little gossypol effect. This finding led them to hypothesize that gossypol and cholesterol may occupy the same domains in the membrane, leaving little opportunity for insertion of gossypol into the cholesterol rich domains, or that gossypol may bind either more efficiently or exclusively to the fatty acid and(or) other non-cholesterol lipid components of membranes than it does to cholesterol. Alpha-tocopherol as well as cholesterol and gossypol are located in the lipid membranes of the cell, therefore, it may be possible that vitamin E had reduced the effect of gossypol on EOF by occupying the same domain in the membrane and thus reducing the intercalation of gossypol into the lipid membrane of the erythrocyte. However, at the end of the experiment this effect was not seen because gossypol effect is both time and dose dependant, with multiple oral doses gradually increasing the amount of the toxicant

retained in the body. Gossypol effect on the EOF has been also reported in several studies with cattle (Willard et al., 1995; Risco et al., 1993; Lindsey et al., 1980).

Treatment tended to have an effect ($P < .1$) on hemoglobin concentration (Table 3-5). In collections 7 and 9, heifers fed GOS and G+2E had lower ($P < .05$) blood hemoglobin concentrations than CON fed heifers. Blood hematocrit did not change ($P > .1$) during the experimental period (Table 3-5). All hematocrit values were within the normal range for cattle, although hemoglobin values were greater than the suggested normal range for this class of animals possibly due to hemolysis. Despite this, comparison may be valid since all samples were treated similarly. Lindsey et al. (1980) reported lower blood hemoglobin ($P <.07$) concentration in dairy cows fed 3.5 and 24.2 g FG $\cdot$ animal$^{-1} \cdot$ d^{-1}. Similar results were found in dairy calves (Risco et al., 1992). Our results agree with those of Risco et al. (1993) who found no clinical or hematological evidence of decreased survival of red blood cells.

Gossypol enantiomers have been found to have different toxicological and pharmacokinetic effects. Calhoun et al. (1995b) suggested that plasma gossypol reflects the availability of gossypol in the diet and the proportion of isomers in the source being fed. Furthermore, they suggested that plasma gossypol concentration plateau at about 4 to 6 wk and remains fairly constant until the diet is changed. Plasma (-)-, (+)- and total gossypol concentrations as affected by dietary supplement are presented in Figure 3-4, 3-5 and 3-6 respectively. Plasma (-)-, (+)-, and total gossypol concentrations in heifers of GOS, G+2E, and G+4E treatments increased ($P < .05$) from the initial sampling to the

Table 3-5. Effect of cottonseed meal (CSM) and vitamin E on blood hemoglobin and hematocrit of beef heifers[a].

		Collection								
Item	SUP[b]	1	2	3	4	5	6	7	8	9
Hemoglobin (g/dL)	CON	17.5	16.6	14.0	14.1	17.6	24.4	20.9[d]	17.5	17.0[d]
	GOS	16.5	14.5	13.1	13.3	16.0	18.0	18.7[ef]	15.3	15.1[e]
	G+2E	16.4	14.7	13.5	13.9	17.8	17.2	16.9[f]	18.7	15.2[e]
	G+4E	16.8	14.4	14.0	13.9	16.9	19.6	18.7[de]	20.4	16.1[de]
SE[c]		.2	1.1	.6	.4	.6	1.6	.4	2.0	.3
Hematocrit (%)	CON	33.3	38.1	38.9	38.8	39.4	39.3	39.5	41.6	40.3
	GOS	34.5	35.6	37.8	36.8	36.1	35.6	36.2	39.0	38.3
	G+2E	33.1	34.3	36.9	36.7	36.8	34.6	34.1	36.5	36.2
	G+4E	34.0	35.9	38.1	38.8	37.8	37.9	36.8	39.3	37.7
SE		.6	.6	.6	.3	.7	.7	.8	.8	1.0

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[def]Least square means with different superscripts in the same column differ $P < .05$.

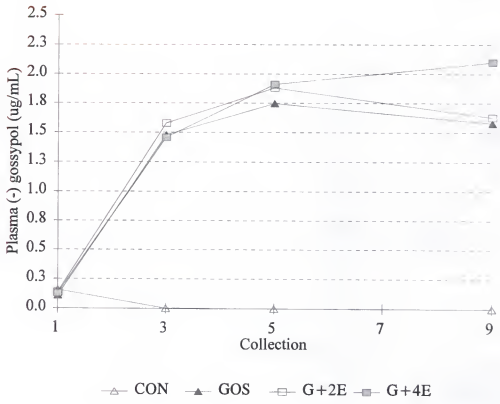

Figure 3-4. Effect of cottonseed meal (CSM) and vitamin E on plasma (-)-gossypol. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .03; C3, .30; C5, .30; and C9, .40.

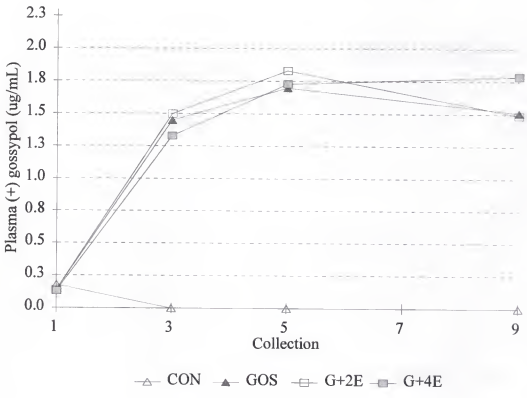

Figure 3-5. Effect of cottonseed meal (CSM) and vitamin E on plasma (+)-gossypol. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .03; C3, .30; C5, .20; and C9, .30.

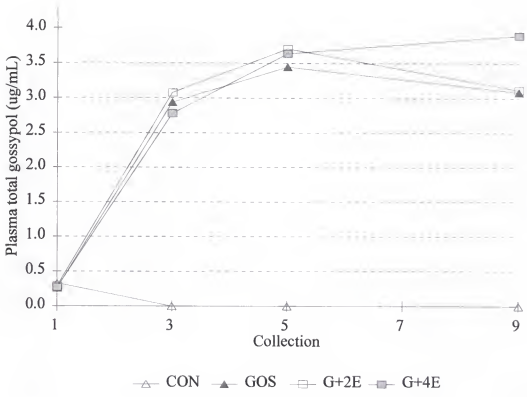

Figure 3-6. Effect of cottonseed meal (CSM) and vitamin E on plasma total gossypol. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (**C**) were: C1, .07; C3, .63; C5, .50; and C9, .73.

end of the experiment, and were similar ($P > .1$) among the three treatments receiving CSM. Heifers in the CON treatment had lower ($P < .05$) gossypol concentrations than the other three treatments after the first collection. Plasma total gossypol concentration was lower than the safer upper limit of 5 μg/mL proposed by Calhoun et al. (1995b). Lower total gossypol plasma concentrations were reported by Lindsey et al. (1980) when cows were fed 3.5 or 24.2 g FG · animal^{-1} · d^{-1}. Also, Lindsey et al. (1980) did not find differences in plasma gossypol concentrations between the two levels of FG intake, raising a concern as to whether FG in feed is a reliable indicator of bioavailability. Calhoun et al. (1995b) reported that dairy cows consuming from 27.2 to 33.8 g TG · animal^{-1} · d^{-1} from WCS had average plasma gossypol concentrations of 3.0 μg/mL with a range of 1.2 to 5.8 μg/mL.

The (-)-gossypol isomer has been found to disappear at a faster rate from the body than (+)-gossypol following a single oral dose in humans and dogs (Wu et al., 1986). This could either be due to a faster rate of elimination or that (-)-gossypol is more likely to be metabolized in vivo than (+)-gossypol, therefore explaining its higher biological activity (Matlin et al., 1985). Daily dosage of gossypol was eliminated five times slower than a single oral dose in rats and mice, which indicates that multiple dosage increased retention of gossypol (Abou-Donia et al., 1988). This agrees with our results where plasma gossypol concentration increased with time at the same dosage.

Tissue measurements. Tissue concentrations of (+)-, (-)-, and total gossypol shown in Figure 3-7. No differences ($P > .1$) were found among heifers fed the GOS, G+2E, and G+4E treatments in tissue gossypol concentrations, but they were greater

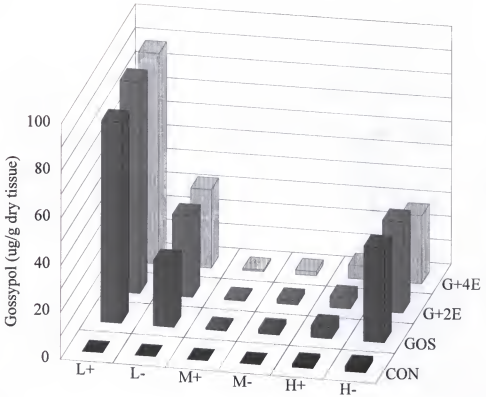

Figure 3-7. Effect of cottonseed meal (CSM) and vitamin E on liver (L), muscle (M), and heart (H), (+)-, and (-)-gossypol concentrations. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors of CON, GOS, G+2E and G+4E respectively were: L+: 1.2, 7.8, 7.3, and 7.3; L-: 3.0, 3.4, 3.1, and 3.1; H+: .5 for all; H-: 3.9, 4.2, 3.9, and 3.9; M+: .1 for all; and M-: .3 for all.

($P < .01$) than CON heifers. Liver (+)-gossypol was greater ($P < .01$) than (-)-gossypol concentrations in the heifers fed CSM (GOS, G+2E, and G+4E), while heart and muscle had greater ($P < .05$) (-)-gossypol than (+)-gossypol in the same treatments. The order of (+)-, and total gossypol accumulation in tissue was liver > heart > muscle ($P < .05$), and for (-)-gossypol liver and heart > muscle ($P < .01$). Supplemental vitamin E had no effect on total gossypol and the accumulation of its isomer in tissue.

Similar results where (+)-gossypol concentration was greater in liver while (-)-gossypol was greater in heart and muscle, were reported in lambs fed 20 to 30 mg FG · kg^{-1} BW · d^{-1} (Kim et al., 1996). In contrast to these results, the proportion of gossypol isomers in liver were similar in pigs fed a wide range of gossypol-containing diets (Knabe et al., 1995). In rats, similar deposition patterns were found by Jensen et al. (1982). The major excretory organ for gossypol appears to be the liver in several species including swine (Abou-Donia and Dieckert, 1974) and rats (Abou-Donia et al., 1970). Abou-Donia (1989) suggested a pathway of metabolism of gossypol. Gossypol is absorbed from the gastrointestinal tract, mainly the small intestine. It enters the liver via the hepatic artery or through the lymph into the hepatic sinusoid and is taken up by Kupffer cells. In the liver, gossypol is metabolized, conjugated, and excreted with the bile into the duodenum. Some of the excreted gossypol is reabsorbed, completing an enterohepatic cycle that may be repeated several times leading to a gradual excretion via feces. The mechanism of excretion seems to require an active secretory process that can be saturated. The fact that gossypol is accumulated in the liver may be related to the tissue susceptibility to gossypol toxicity or to the fact that the liver is the main route of gossypol metabolism.

Also, gossypol is a lipophilic compound, therefore it tends to accumulate in tissues or cell fractions containing high amounts of lipid membranes e.g. mitochondria, lysosomal and microsomal fractions (Baran and Ismailov, 1993). Gossypol has been reported to be cardiotoxic, causing degeneration of cardiac muscle. Our results suggest that since the heart is the second highest gossypol accumulator of the tissues studied, gossypol has the potential for causing cardiac aberrations. The (-)-gossypol has been shown to be the optically active form that induces fertility impairment in male animals (Wang et al., 1987). Accumulation of this isomer in liver and heart tissues was similar, which may be related to the toxicity signs shown during gossypol toxicosis. Gossypol isomers have been shown to differ in their pharmacokinetic in dogs and humans (Wu et al., 1986), and rats (Chen et al., 1987).

Liver RET and liver, heart, and muscle β-C concentrations did not differ ($P > .1$) among treatments (Table 3-6). However, liver RETP concentrations increased ($P < .05$) in the G+2E and G+4E treatments. It has been suggested that vitamin E protects retinyl esters in the storage globules of the liver cells, preventing a rapid breakdown of retinyl ester storage in the liver (Sondergaard, 1972). Olson (1991) suggested that retinyl ester hydrolase, an enzyme involved in the release of vitamin A from the liver, is regulated by vitamin E status, activated in vitamin E deficiency and inhibited in vitamin E sufficiency. Vitamin E supplementation (G+2E and G+4E) increased ($P < .05$) α-T concentration in liver, heart and muscle (Table 3-6). Feeding gossypol did not have an effect on α-T concentration in these tissues (CON vs GOS; $P > .1$) at a normal level of vitamin E supplementation (30 IU vitamin E/ kg). There was no difference between the two high

Table 3-6. Effect of cottonseed meal (CSM) and vitamin E on α-tocopherol (α-T), retinol (RET), retinol palmitate (RETP), and β-carotene (β-C) concentrations in tissue of beef heifers (μg/g of wet tissue)[a].

		Supplement[b]				
Tissue	Item	CON	GOS	G+2E	G+4E	SE[c]
Liver	α-T	5.1[e]	7.1[e]	19.9[d]	22.4[d]	.8
	RET	7.4	7.5	8.9	11.4	1.9
	RETP	39.0[f]	48.7[ef]	65.3[de]	74.7[d]	12.0
	β-C	15.4	16.9	15.5	16.3	.9
Heart	α-T	5.8[e]	7.6[e]	21.6[d]	23.1[d]	1.2
	β-C	5.8	5.3	5.5	5.4	.3
Muscle	α-T	2.6 [e]	3.3[e]	8.1[d]	8.3[d]	.6
	β-C	4.5	4.5	4.6	4.8	.4

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[def]Least square means with different superscripts in the same row differ $P < .05$.

concentrations of vitamin E supplementation on tissue α-T concentration. These results were similar to those found for plasma α-T concentration.

Trace minerals (i.e. Fe, Cu, Se, and Zn) have been implicated in gossypol metabolism. Heart failure, seen in gossypol toxicosis, is also a clinical sign of Se deficiency. Iron is chelated by gossypol reducing iron's bioavailability. Skutches et al. (1973) suggested that gossypol chelated Fe in the liver of pigs, and reduced Fe availability for hemoglobin synthesis. Gossypol also chelates Cu and Zn (de Peyster and Wang, 1993). Yu et al. (1981) reported that gossypol-treated rats had reduced Zn concentration in testis, and suggested that the chelation of Zn by gossypol may be the cause of its antispermatogenic activity. In our experiment, Cu, Fe, Se, and Zn concentrations in the experimental diets were within normal concentrations (Table 3-1), and feeding CSM did not affect ($P > .1$) concentrations of Cu, Fe, and Zn in heart, muscle and liver, and Se in liver (Table 3-7).

Gossypol has been demonstrated to inhibit free radical damage to lipid membranes in some studies (Janero and Burghardt, 1988) and to stimulate it in others (de Peyster et al., 1984). Bender et al. (1988) suggested that oxidative injury caused by the generation of reactive oxygen species may be the cause of gossypol toxicity. In this study, they found a decrease in the concentration of antioxidants, including α-T, in testis of rats fed gossypol acetic acid. Our data on in vitro stimulated lipid peroxidation in liver homogenate (Figure 3-8) indicated that from 50 min of incubation onward, liver from heifers fed CON supplement had greater ($P < .05$) MDA production than the other heifers. Feeding CSM (GOS) did not increase ($P > .1$) lipid peroxidation above the CSM

Table 3-7. Effect of cottonseed meal (CSM) and vitamin E on micromineral concentration of liver, heart, and muscle of beef heifers (mg/kg dry matter)[a].

		Supplement[b]				
Tissue	Mineral	CON	GOS	G+2E	G+4E	SE[c]
Liver	Cu	99.8	70.7	85.6	127.9	15.3
	Fe	150.6[e]	146.9[e]	165.5[e]	198.0[d]	5.0
	Se	1.4	1.7	1.3	1.2	.3
	Zn	114.5	102.4	114.0	115.0	8.2
Heart	Cu	13.8	13.2	12.8	11.8	1.4
	Fe	182.1	154.0	141.3	176.9	20.0
	Zn	54.4	66.3	64.0	62.0	2.5
Muscle	Cu	4.5	3.5	3.6	4.0	.6
	Fe	141.1	158.0	117.6	104.6	34.0
	Zn	167.2	173.0	196.8	192.9	19.0

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[de]Least square means with different superscripts in the same row differ $P < .05$.

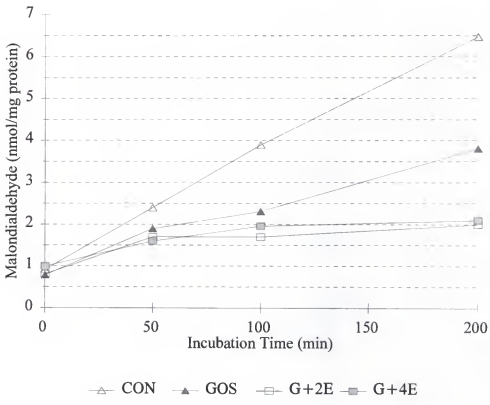

Figure 3-8. Effect of cottonseed meal (CSM) and vitamin E on in vitro stimulated lipid peroxidation of liver homogenates. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each time (T) were: T0, .14; T50, .13; T100, .51; and T200, 1.27.

and vitamin E supplemented animals (G+2E and G+4E). In vitro stimulated lipid peroxidation of heart tissue did not differ ($P > .1$) among treatments (Figure 3-9) possibly because of the resistance of this tissue to in vitro peroxidation (Kornbrust and Mavis, 1980). Sheriff et al. (1986) found similar results on the ability of gossypol to reduce lipid peroxidation in human spermatozoa and erythrocytes. Janero and Burghardt (1988) found that gossypol effectively prevented O_2^- dependant Fe-promoted peroxidation of myocardial membrane phospholipids and could attenuate the kinetics and extent of peroxidation. Furthermore, they suggested that the gossypol antioxidant effect is through the scavenging of lipid radical intermediates. Contrary to our results, de Peyster et al. (1984) found that gossypol when incubated with rat liver microsomes or human sperm, promotes the formation of O_2^-, causing oxidative injury. A possible explanation for the difference in results is that a metabolic product of gossypol (such as redox-cycling quinone) may be responsible for the ability of gossypol to generate oxygen radicals and cause the side effects associated with gossypol treatment (Janero and Burghardt, 1988; de Peyster et al., 1984).

Skinfold response to PHA intradermal injection on d 50 did not differ ($P > .1$) among treatments (Figure 3-10). Neither CSM alone nor with vitamin E affected the cell mediated immune response.

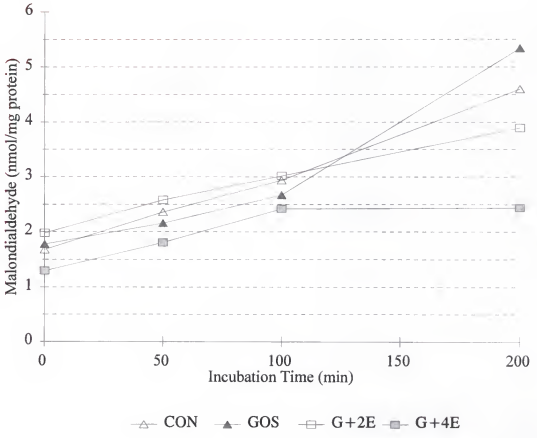

Figure 3-9. Effect of cottonseed meal (CSM) and vitamin E on in vitro stimulated lipid peroxidation of heart homogenates. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each time (T) were: T0, .3; T50, .3; T100, .3; and T200, .8.

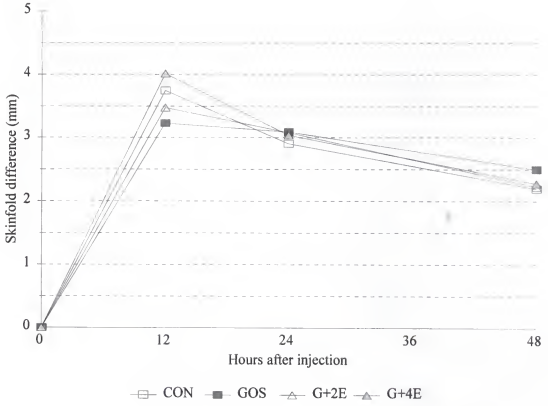

Figure 3-10. Effect of cottonseed meal (CSM) and vitamin E on skinfold thickness after PHA injection. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+2E = CSM + corn + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each time (T) were: T12, .4; T24, .4; and T48, .3.

Implications

Free gossypol fed at 4 g · animal^{-1} · d^{-1} at a low concentration of vitamin E supplementation (30 mg/kg of feed DM) did not decrease concentrations of antioxidant vitamins, including α-T, vitamin A and β-C or have any detrimental effect on growth performance of beef heifers.

CHAPTER 4
LONG TERM EFFECTS OF FEEDING COTTONSEED MEAL AND VITAMIN E TO DAIRY CALVES.

Introduction

Gossypol is a natural pigment found in the cotton plant (*Gossypium* spp.) and other plants belonging to the Malvaceae family. This pigment has been recognized to be toxic to nonruminant species and if fed at high enough concentrations also to ruminants, thus limiting the use of cottonseed products as feed for livestock.

Preruminants are especially susceptible to gossypol toxicosis. The undeveloped rumen appears unable to detoxify gossypol (Morgan et al., 1988). Soluble protein binding with gossypol has been proposed as a mechanism by which gossypol is detoxified in the rumen (Reiser and Fu, 1962). It is not known precisely at what age the rumen matures or becomes functional in calves. Therefore, young ruminants with undeveloped rumens appear to function as nonruminant animals, and gossypol cannot be detoxified effectively (Risco et al., 1992).

Toxicity signs and death of calves have been reported when feedstuffs containing gossypol were fed (Holmberg et al., 1988; Risco et al., 1992; Zelski et al., 1995). Risco et al. (1992) suggested that diets for dairy calves that contain up to 200 mg free gossypol (**FG**)/kg of diet DM are safe, 400 mg FG/kg of DM approaches toxicity, and 800 mg

FG/kg of DM results in death losses. In a more recent study (Zelski et al., 1995) calves fed from 100 to 220 mg FG/kg of feed resulted in more than 40% mortality. The authors also reported that gossypol toxicity signs ranged from preacute death during an acute phase, to poor growth during a chronic phase. There is a limited capacity for gossypol detoxification in preruminants and calves are susceptible to gossypol toxicity at concentrations comparable to those affecting nonruminant animals (i.e. swine).

Some gossypol toxicity signs have been related to a decrease in antioxidant concentrations and(or) an increase in the formation of reactive oxygen species. Janero and Burghardt (1988) suggested that gossypol can interact with biological membranes by promoting the formation of highly-reactive oxygen-containing free radicals. This and other studies (Willard et al., 1995; Lane and Stuart, 1990; Bender et al., 1988;) suggest that antioxidants play a key role in the metabolism of gossypol. Therefore, the objectives of this experiment were to evaluate the effect of feeding a diet containing 400 mg FG/kg of feed DM to dairy calves from 2 wk to 6 mo of age, and to evaluate the value of supplemental vitamin E on the effect of gossypol on preruminant animals.

Materials and Methods

This experiment was divided into two phases. Phase 1 describes animals individually fed and phase 2 describes those which were group fed.

Animals, diets and management. In phase 1 of this experiment, two groups of male Holstein calves were purchased from Florida dairies. The first group (group 1)

started the experiment when calves were approximately 2 wk of age (August, 1993), while the second group (group 2) started at approximately 4 wk of age (September, 1993). Phase 1 ended in October for both groups. The duration of this phase of the experiment was 82 and 50 d for groups 1 and 2, respectively. During the first two weeks of the experiment, approximately 30% of calves from group 1 died from causes determined to be unrelated to treatments (heat stress), therefore their data were not included in this experiment. Calves were fed 4 L/d of a commercial milk replacer (Ultra Suckle 2000® from Mama Pro Corporation) containing .454 kg of replacer in 4 L of water. Calves had free access to the following treatments (Table 4-1): 1) **CON**: a ration base on soybean meal (**SBM**) + 30 IU vitamin E/kg of feed; 2) **GOS**: a ration based on cottonseed meal (**CSM**) + 30 IU vitamin E/kg of feed; 3) **G+2E** a ration based on CSM + 2,000 IU vitamin E $\cdot$ calf^{-1} $\cdot$ d^{-1}; and 4) **G+4E** a ration based on CSM + 4,000 IU vitamin E $\cdot$ calf^{-1} $\cdot$ d^{-1}. Vitamin E in the G+2E and G+4E treatments was fed with the milk replacer. The number of animals per treatment in phase 1 were as follows: in group 1, CON had 8 calves, GOS had 11, G+2E had 6 and G+4E had 8 calves; in group 2, CON had 5, GOS had 4, G+2E had 5, and G+4E had 5 calves. Animals were individually weaned when they were consuming 1% of their body weight in dry feed. After weaning and while in phase 1, animals were fed individually the starter rations twice a day, and vitamin E (G+2E and G+4E) was fed in the morning with the dry feed, mixing it with half of the previous day intake.

In phase 2, calves from the two groups were moved to 16 pens (4 pens per treatment) and the amount of supplemental vitamin E changed. Calves fed G+2E

Table 4-1. Composition of calf diets.

	Treatment[a]			
Item	CON	GOS	G+2E	G+4E
DM (%)	91.00	91.00	91.00	91.00
CSM (%)[b]	.00	30.00	30.00	30.00
SBM (%)[b]	22.00	.00	.00	.00
Corn (%)[b]	19.00	22.00	22.00	22.00
Alfalfa meal (%)[b]	14.00	3.00	3.00	3.00
Oats (%)[b]	18.00	18.00	18.00	18.00
Beet pulp (%)[b]	22.00	22.00	22.00	22.00
Molasses (%)[b]	3.00	3.00	3.00	3.00
Minerals (%)[b]	2.00	2.00	2.00	2.00
CP (%)[c]	22.00	22.00	22.00	22.00
Vitamin E (IU/kg)[d]	41.00	45.00	45.00	45.00
Vitamin E (IU/kg)[e]	41.00	45.00	525.00	1291.00
(+)-gossypol (%)[f]	.00	.15	.15	.15
(-)-gossypol (%)[f]	.00	.35	.35	.35
Free gossypol (%)[g]	.01	.04	.04	.04
Total gossypol (%)[g]	.02	.50	.50	.50
Ca (%)[c]	.52	.50	.50	.50
K (%)[c]	1.10	.80	.80	.81
Mg (%)[c]	.21	.30	.34	.34
P (%)[c]	.51	.60	.60	.60
Cu (mg/kg)[c]	16.50	14.50	14.50	14.50
Zn (mg/kg)[c]	44.50	44.30	44.33	44.33
Mn (mg/kg)[c]	62.14	48.32	48.33	48.33
Fe (mg/kg)[c]	252.83	178.23	178.24	178.24
Se (mg/kg)[c]	.23	.21	.21	.21

[a]CON = soybean meal (SBM) + 30 IU vitamin E/kg; GOS = cottonseed meal (CSM) + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E· animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[b]As fed basis.

[c] DM basis .

[d] As fed basis. Phase 1.

[e] As fed basis. Phase 2.

[f]Composite sample. HPLC procedure (Calhoun et al. (1995a), and Kim and Calhoun (1995)). Texas A&M.

[g]Composited sample. AOCS procedure. Texas A&M.

received 700 IU vitamin E/ kg of feed and those fed G+4E received 1,400 IU vitamin E/ kg of feed. The number of animals per treatment were as follow: 13 (CON), 14 (GOS), 11 (G+2E), and 11 (G+4E). All diets were isocaloric and isonitrogenous and met the requirements of other nutrients for this class of animal (NRC, 1989). Diets based on CSM were formulated to provide 400 mg FG/kg of diet DM to ensure a mild level of toxicity, and to test the effect of vitamin E supplementation. Fresh feed and clean water were offered ad libitum. Sick calves were treated with antibiotics in the case of respiratory problems and oral fluid electrolyte replacement therapy for calves with diarrhea (under the supervision of a veterinarian).

Calves were weighed every two weeks in both phases, and individual (phase 1) and group (phase 2) feed intake data were recorded daily. Average daily feed intake was calculated by 14-d periods so as to match collection days. In phase 1, there were 6 periods and 7 blood and weight collections for group 1, and 4 periods and 5 collections for group 2. In phase 2 there were 3 periods and 4 weight and blood collections. The protocol for all calf procedures had been approved by the University Animal Use Committee.

Blood and tissue sampling and analyses. Blood samples were collected every 2 wk via jugular venipuncture with an 18-gauge needle into heparinized vacutainer blood collection tubes. Blood was analyzed for erythrocyte osmotic fragility (**EOF**), hemoglobin, and hematocrit. Erythrocyte osmotic fragility, measured as percentage hemolysis, was evaluated in .65% buffered saline solution as described by Risco et al. (1993). Hemoglobin was determined using a colorimetric procedure (Sigma Chemical

Co., St. Louis, MO). Hematocrit was determined from blood using a micro hematocrit centrifuge (IEC MB Centrifuge, Needham Heights, MA). Blood was centrifuged for 25 min at 700 x g. Plasma was removed and stored at -20°C until analyzed for α-T (all collections), alkaline phosphatase (**AP**) (phase 2), (+)-, and (-)-gossypol isomers (at approximately the beginning, middle and end of phase 1).

Complete necropsies were performed on dead calves. This consisted of a description of significant findings and collection of heart and liver tissues for histopathology and laboratory analyses. Tissues were frozen at -20°C until analyzed for α-T, β-carotene (**β-C**), (+)-, (-)-, and total gossypol concentration. Samples of diets were collected and analyzed for concentration of α-T, Ca, K, Mg, Mn, P, CP, Fe, Zn, Cu, and Se.

Alpha-Tocopherol was analyzed following the procedure described by Njeru et al. (1992) for plasma, and by Njeru et al. (1995) for tissue and feed samples. Tissue β-C was prepared as follow: approximately 1 g of sample (fresh weight) and .1 g of ascorbic acid were homogenized in 10 ml of acetone; 1 ml of the homogenate was deproteinized with 1 mL of ethanol. After adding 2 ml of .9% saline, the mixture was vortexed for 6 min. The solution was double extracted with 3 mL petroleum ether, and kept in an ice bath. The petroleum ether extract was dried by evaporation under a stream of N_2 in a 35°C water bath, reconstituted in 750 μL of .1% acetic acid, 29.9% tetrahydrofuran, and 70% iso-octane with .2% added β-hydroxy toluene. Beta-carotene was determined by injecting 20 μL of the reconstituted extracted sample into the HPLC system. The HPLC systems consisted of a Perkin Elmer 550 terminal (Perkin-Elmer, Analytical Instruments,

Norwalk, CT.), an ISS-100 auto sampler (Perkin-Elmer), a Series 4 Liquid chromatograph pump (Perkin-Elmer), and a Lichrosorb Si 60 5μm, 4 mm ID x 250 mm column (Hibar Fertigsaule RT pre-packed column RT 250-4 E, Merck, Darmstadt, Germany). The mobile phase consisted of 90% iso-octane, 9.9% tetrahydrofuran, and .1% acetic acid. The UV detector was an ABI Analytical Spectroflow 757 set at a wavelength of 450 nm and sensitivity of .005. Data were collected by a LCI-100 Laboratory Computing Integrator (Perkin-Elmer). Flow rate was 1 mL/min and the retention time was 3.05 min. Standards consisted of 100 ng β-C (Sigma Chemical Co., St. Louis, MO).

Alkaline phosphatase was determined using a kit (Sigma Diagnostic Procedure No. 104). Minerals were determined according to the procedures described by Rojas et al. (1995) and Whetter and Ullrey (1978). Samples of plasma and tissue for gossypol analyses were shipped in dry ice to Texas A&M where they were analyzed by HPLC according to the procedures described by Calhoun et al. (1995a) and Kim and Calhoun (1995).

Statistical analyses. In phase 1, group 1 and 2 data were analyzed separately. Continuous data such as body weight, feed intake, hematocrit, hemoglobin, and EOF, plasma concentration of (-)-, (+)-, and total gossypol, and α-T were analyzed by repeated measures analysis of variance in a completely randomized design using GLM procedure of SAS (SAS, 1988).

In phase 2, calves were group fed in 16 pens (4 pens/treatment) therefore pen (random) effect was included in the model. Continuous data such as body weight, feed

intake, hematocrit, hemoglobin, and EOF, plasma concentrations of AP, and α-T were analyzed by repeated measures analysis of variance in a completely randomized design using GLM procedure of SAS (SAS, 1988). The Greenhouse-Geiser Epsilon was used to determine significant levels for the F-test. Treatment and pen within treatment effects were tested using animal within pen and treatment effect. Pen within treatment effect was removed from the model when it was not significant ($P > .2$). When the pen within treatment effect was kept in the model PROC MIXED (SAS, 1996) was used to calculate the correct standard errors of the least square means for a mixed model. There were two sets of animals with an age difference of 2 to 4 wk, therefore age was used as a covariate. The time x treatment interaction was tested using time x pen within treatment interaction. When the overall treatment effect was significant ($P < .05$) or tended to be significant ($P < .1$) separation of means was done using Duncan multiple range test. Pooled standard errors were reported when all treatments had the same number of replicates, otherwise individual values for each least square mean were given.

Results and Discussion

Phase 1. In group 1, calves receiving the high vitamin E treatment (G+4E) had greater ($P < .05$) total weight gain and ADG than CON animals (Table 4-2). No differences in calf performance were observed in group 2. In group 1, average daily feed intake followed the same trend as body weight gain (Table 4-3). Calves fed large amounts of vitamin E (G+4E) had greater ($P < .05$) feed intake in periods 4, 5, and 6 than

Table 4-2. Effect of cottonseed meal (CSM) and vitamin E on weight gain and average daily gain (ADG) of dairy calves[a].

	Treatment[b]				
Item	CON	GOS	G+2E	G+4E	SE[c]
Phase 1[d], group 1					
No. of calves	8	11	6	7	
Days on milk	51	50	47	51	
Initial weight (kg)	37.0	38.9	39.4	41.1	2.0
Final weight (kg)	72.5[f]	77.1[f]	81.4[ef]	95.0[e]	5.7
82 d gain (kg)	35.5[f]	38.1[ef]	39.8[ef]	50.9[e]	4.4
82 d ADG (kg)	.4[f]	.5[ef]	.5[ef]	.6[e]	.1
Phase 1, group 2					
No. of calves	5	4	5	5	
Days on milk	24	26	20	26	
Initial weight (kg)	39.1	38.4	42.5	44.2	2.0
Final weight (kg)	61.2	63.8	74.5	71.7	4.7
50 d gain (kg)	22.0	25.4	31.9	27.5	4.4
50 d ADG (kg)	.4	.5	.6	.6	.2
Phase 2[d]					
No. of calves	13	14	11	11	
Initial weight (kg)	67.8[f]	74.8[ef]	77.0[ef]	83.3[e]	3.4
Final weight (kg)	97.8[f]	119.2[e]	121.5[e]	129.9[e]	6.3
43 d gain (kg)	30.1[f]	44.2[e]	46.2[e]	46.0[e]	3.6
43 d ADG (kg)	.7[f]	1.0[e]	1.1[e]	1.1[e]	.1

[a]Least square means.

[b]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Phase 1 individual feeding; Phase 2 group fed.

[ef]Least square means with different superscripts in the same row differ $P < .05$.

Table 4-3. Average intake of feed (kg · animal^{-1} · d^{-1}) and free gossypol (FG) (g · animal^{-1} · d^{-1}) of dairy calves in phase 1[a].

		Treatment[b]				
Intake	Period	CON	GOS	G+2E	G+4E	SE[c]
Group 1[d]						
Feed	1	.05	.04	.02	.05	.01
	2	.26	.24	.22	.23	.05
	3	.55	.53	.62	.68	.13
	4	.91[f]	1.08[ef]	1.33[ef]	1.59[e]	.20
	5	1.35[f]	1.85[ef]	2.06[e]	2.49[e]	.23
	6	1.98[f]	2.50[f]	2.52[f]	3.09[e]	.19
FG	1	.00[f]	.02[e]	.01[ef]	.02[e]	.00
	2	.00[f]	.10[e]	.09[e]	.09[e]	.02
	3	.00[f]	.21[e]	.25[e]	.27[e]	.05
	4	.00[f]	.43[e]	.53[e]	.64[e]	.08
	5	.00[g]	.74[f]	.82[fe]	1.00[e]	.08
	6	.00[g]	1.00[f]	1.07[f]	1.23[e]	.07
Group 2[d]						
Feed	1	.23[f]	.20[f]	.40[e]	.22[f]	.05
	2	.51[f]	.45[f]	.85[e]	.55[f]	.09
	3	1.14[f]	1.21[fe]	1.92[e]	1.48[e]	.15
	4	1.66[f]	1.96[f]	2.73[e]	2.24[fe]	.18
FG	1	.00[g]	.08[f]	.16[e]	.09[f]	.01
	2	.00[g]	.18[f]	.34[e]	.22[f]	.03
	3	.00[g]	.48[f]	.77[e]	.59[f]	.04
	4	.00[g]	.79[f]	1.09[e]	.90[f]	.06

[a]Least square means.

[b]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Individual feeding.

[efg]Least square means with different superscripts in the same row differ $P < .05$.

CON animals, and greater ($P < .05$) than calves on diets G+2E and GOS in period 6. In lambs, vitamin E supplementation (85 IU/kg of diet DM)) had been reported to improve weight gain and feed intake when fed a basal diet containing 20% CSM (Calk et al., 1992). Eicher et al. (1992) reported that calves fed supplemental vitamin E had greater ADG than control animals, and this difference occurred after 6 wk of feeding. Risco et al. (1992) did not find an effect of feeding gossypol (100 to 800 mg FG/kg of diet DM) on feed intake and body weight gain of calves in the first 83 d of age. Differences in ADG and weight gain could be explained by differences in feed intake. Results similar to the present experiment were found by Reddy et al. (1985) in which vitamin E supplementation tended to increase performance and intake of calves. Gossypol appeared not to have a negative effect on feed intake or gain of calves during phase 1.

Plasma α-T concentration of calves in groups 1 and 2 are presented in Figures 4-1 and 4-2, respectively. Calves supplemented with vitamin E (G+2E and G+4E) had greater ($P < .05$) plasma α-T concentrations than calves fed CON and GOS diets from collection 4 through collection 7 in group 1 and from collection 3 to 5 in group 2. Both groups of calves on CON and GOS treatments had similar plasma α-T concentrations during all collections. Therefore gossypol intake (Table 4-3) by calves fed 30 IU vitamin E/kg of diet DM did not decrease plasma concentrations of α-T. The lack of statistical difference between the two high concentrations of vitamin E supplementation could be the result of reduced vitamin E absorption at high levels of supplementation as shown with laboratory animals (Traber et al., 1986). Pancreatic and bile secretions are essential for adequate absorption and transport of vitamin E and pancreatic enzyme activity does

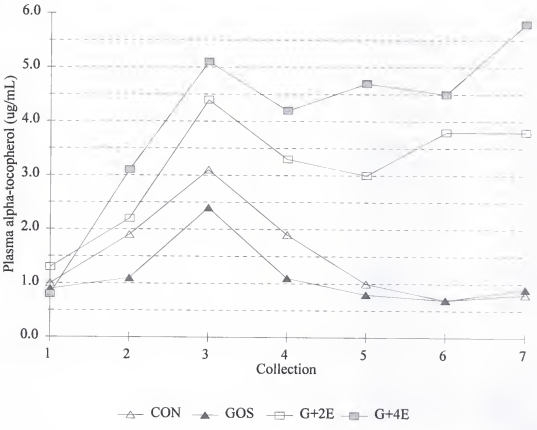

Figure 4-1. Effect of cottonseed meal (CSM) and vitamin E on plasma α-tocopherol concentration of dairy calves in phase 1 group 1. Diets were: CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .2; C2, .4 C3, .4; C4, .4; C5, .6; C6, .5; and C7, .4.

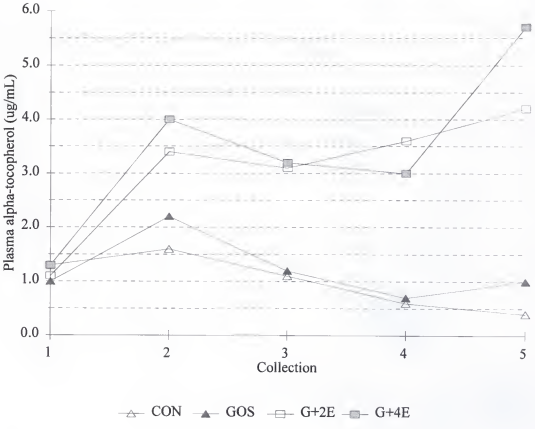

Figure 4-2. Effect of cottonseed meal (CSM) and vitamin E on plasma α-tocopherol concentration of dairy calves in phase 1 group 2. Diets were: CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .2; C2, .7; C3, .2; C4, .3; and C5, .5.

not peak until calves are about 1 mo old (Reddy et al., 1987). This could explain the lack of differences in plasma α-T concentration observed in the early periods.

Blood hemoglobin concentration (Table 4-4) and hematocrit (Table 4-5) were not affected by treatment in any of the two groups during phase 1. Risco et al. (1992) reported that overall changes in hematology and serum chemistry were modest and insufficient to be used for diagnosis of gossypol toxicity in calves fed from 0 to 800 mg FG/kg.

Erythrocyte osmotic fragility increased as feed intake increased, and was greater ($P < .05$) in calves receiving CSM than those receiving the CON diet in the last two collections in group 1 (Figure 4-3). In group 2, EOF did not differ ($P > .1$) among treatments (Figure 4-4). The effect of gossypol on EOF has been found to be dose and time dependent. The differences between groups 1 and 2 on EOF may have been the result of not only time of exposure but intake of FG (Table 4-3). It appeared that vitamin E supplementation at high doses (G+2E and G+4E) did not have an effect on reducing EOF increase as observed in heifers (Velasquez-Pereira et al., 1996c). Reyes et al. (1984) found that gossypol binds to lipid bilayers and induces an electrical conductance that is accompanied by an increase in proton permeability. Gossypol may have altered the permeability of the erythrocyte membrane and caused it to lyse when exposed to a .65% saline solution. de Peyster et al. (1986) suggested that alteration of cell membrane permeability may occur in vivo with prolonged exposure to gossypol. Furthermore, Morgan et al. (1988) speculated that increased membrane permeability may be responsible for myocardial injury detected in lambs fed gossypol acetic acid.

Table 4-4. Effect of cottonseed meal (CSM) and vitamin E on blood hemoglobin concentration (g/dL) of dairy calves[a].

		Treatment[b]				
Phase[d]	Collection	CON	GOS	G+2E	G+4E	SE[c]
Phase 1, group 1	1	13.6	11.6	10.8	13.8	1.1
	2	13.4	11.9	11.2	12.9	1.0
	3	13.2	11.4	12.3	13.4	.9
	4	14.1	10.6	10.9	12.4	.7
	5	12.8	10.4	10.8	11.0	.6
	6	12.1	10.3	10.9	11.5	.7
	7	11.5	10.8	11.7	11.2	.5
Phase 1, group 2	1	11.7	13.8	12.8	12.5	1.1
	2	10.6	12.7	11.8	11.2	1.0
	3	11.2	12.3	11.4	11.3	.8
	4	12.0	12.0	10.9	10.7	.7
	5	12.3	11.5	11.2	10.6	1.1
Phase 2	1	11.8	10.5	11.5	10.9	.5
	2	12.0[e]	10.5[f]	11.2[ef]	11.3[ef]	.3
	3	12.1[e]	10.5[f]	11.3[ef]	11.6[e]	.4
	4	12.2[ef]	11.2[f]	12.2[ef]	12.6[e]	.4

[a]Least square means.

[b]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Phase 1 individual feeding; Phase 2 group fed.

[ef]Least square means with different superscripts in the same row differ $P < .05$.

Table 4-5. Effect of cottonseed meal (CSM) and vitamin E on blood hematocrit (%) of dairy calves[a].

		Treatment[b]				
Phase[d]	Collection	CON	GOS	G+2E	G+4E	SE[c]
Phase 1, group 1	1	34.8	30.4	31.7	36.0	2.4
	2	37.1	32.4	33.1	36.4	2.2
	3	34.9	30.2	31.5	34.7	2.3
	4	35.6	29.8	30.2	32.1	2.1
	5	35.8	28.7	30.5	31.7	1.8
	6	34.5	28.4	30.6	31.8	1.8
	7	32.1	29.6	30.9	31.0	1.2
Phase 1, group 2	1	29.2	37.5	32.0	31.5	2.4
	2	29.1	35.5	31.0	31.6	2.4
	3	31.6	35.1	31.7	29.8	1.9
	4	32.4	33.2	31.2	30.5	2.1
	5	32.9	32.6	30.6	29.6	2.2
Phase 2	1	32.4	28.9	30.3	31.0	1.1
	2	33.6[e]	29.8[f]	31.7[ef]	33.2[e]	.9
	3	34.0[e]	29.7[f]	32.3[e]	33.8[e]	.7
	4	33.9[e]	30.9[f]	33.9[e]	35.3[e]	.9

[a]Least square means.

[b]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Phase 1 = individual feeding; Phase 2 group fed.

[ef]Least square means with different superscripts in the same row differ $P < .05$.

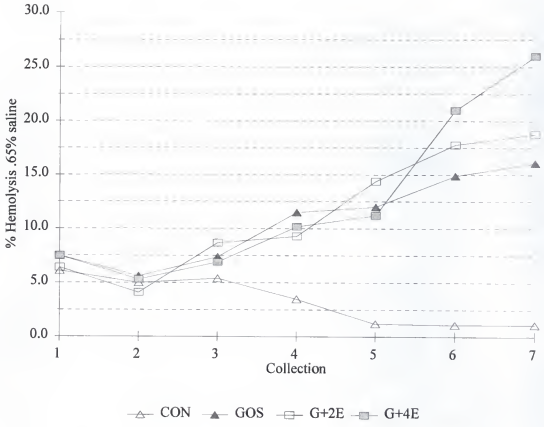

Figure 4-3. Effect of cottonseed meal (CSM) and vitamin E on erythrocyte osmotic fragility (.65% saline) of dairy calves in phase 1 group 1. Diets were: CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, 1.7; C2, 1.7; C3, 1.7; C4, 4.3; C5, 4.8; C6, 4.4; and C7, 4.2.

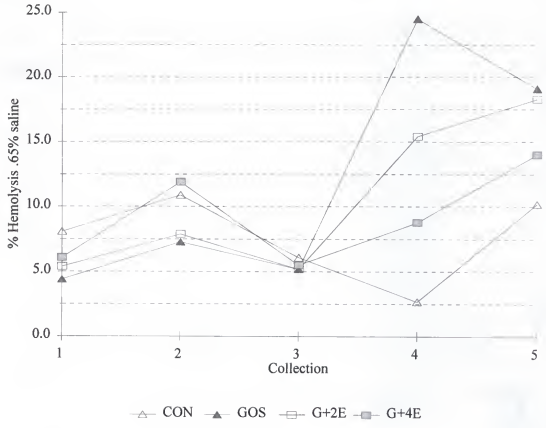

Figure 4-4. Effect of cottonseed meal (CSM) and vitamin E on erythrocyte osmotic fragility (.65% saline) of dairy calves in phase 1 group 2. Diets were: CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, 2.2; C2, 3.3; C3, 2.6; C4, 6.1; and C5, 6.7.

Erythrocyte osmotic fragility analysis is an in vitro procedure that has not been found to be related with any gossypol toxicity signs.

Plasma gossypol concentrations are shown in Table 4-6. In group 1, (+)-, (-)-, and total-gossypol were greater ($P < .05$) in CSM-fed calves than CON in collections 5 and 7. G+2E calves had lower plasma gossypol concentrations than G+4E and GOS treatments. In group 2, plasma concentrations of total gossypol and its isomers were greater in CSM fed calves than CON in the last collection only. Calhoun et al. (1995b) suggested that plasma gossypol reflects the availability of gossypol in the diet and the proportion of isomers in the source being fed. Furthermore, they suggested 5 μg/mL of total gossypol in plasma as a safe upper level. In this study, plasma gossypol concentrations were below this critical value. This range was proposed for adult dairy cattle and may not indicate the safe upper level for young ruminants. The rumen plays a key role in the metabolism of gossypol, and preruminants or ruminants fed gossypol in a manner which by-passes the rumen absorb FG similarly to nonruminants (Calhoun, 1995).

Phase 2. Phase 2 of this experiment included data on animals group fed in 16 pens (4 pens per treatment). Final weight, total gain and ADG (Table 4-2) were greater ($P < .05$) for calves receiving CSM diets than the CON treatment. Calves fed G+4E consistently had greater ($P < .05$) feed intake (Table 4-7) than CON calves but similar to calves fed GOS and G+2E diets. There was not differences ($P > .1$) in feed intake calculated as percentage of body weight. Calculated FG intake did not differ ($P > .1$) among animals receiving CSM. Calculated vitamin E intake was similar ($P > .1$) between calves fed the CON and GOS, but lower ($P < .05$) than calves fed the

Table 4-6. Effect of cottonseed meal (CSM) and vitamin E on plasma (+)-, (-)-, and total (T)-gossypol concentration of dairy calves (μg/mL)[a].

			Treatment[b]				
Group[d]	Isomer	Collection	CON	GOS	G+2E	G+4E	SE[c]
1	(+)	1	.0	.0	.0	.0	.0
	(+)	5	.0[f]	1.1[e]	.9[e]	1.3[e]	.3
	(+)	7	.0[f]	1.8[e]	.8[e]	1.7[e]	.2
1	(-)	1	.0	.0	.0	.0	.0
	(-)	5	.0[f]	.5[e]	.4[ef]	.6[e]	.2
	(-)	7	.0[g]	1.0[e]	.5[f]	1.0[e]	.1
1	T	1	.0	.0	.0	.0	.0
	T	5	.0[f]	1.6[e]	1.3[ef]	1.9[e]	.4
	T	7	.0[g]	2.8[e]	1.2[f]	2.7[e]	.3
2	(+)	1	.0	.0	.0	.0	.0
	(+)	3	.7	1.6	1.7	.7	.4
	(+)	5	.0[f]	1.3[e]	1.7[e]	1.3[e]	.3
2	(-)	1	.0	.0	.0	.0	.0
	(-)	3	.3	.9	.9	.4	.2
	(-)	5	.0[f]	.8[e]	.9[e]	.8[e]	.2
2	T	1	.0	.0	.0	.0	.0
	T	3	.9	2.4	2.6	1.1	.6
	T	5	.0[f]	2.1[e]	2.5[e]	2.2[e]	.5

[a]Least square means.

[b]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Individual feeding.

[ef]Least square means with different superscripts in the same row differ $P < .05$.

Table 4-7. Feed (kg · animal^{-1} · d^{-1}, and as %BW), and calculated free gossypol (g · animal^{-1} · d^{-1}) and vitamin E (IU · animal^{-1} · d^{-1}) intake of dairy calves in phase 2[a].

		Treatment[b]				
Intake[d]	Period	CON	GOS	G+2E	G+4E	SE[c]
Feed	1	2.14[f]	2.57[ef]	2.93[e]	3.15[e]	.22
	2	2.57[f]	2.91[ef]	3.27[ef]	3.61[e]	.25
	3	2.74[f]	3.95[e]	3.45[ef]	4.06[e]	.35
%BW	1	2.74	3.05	3.10	3.21	.15
	2	2.90	3.00	3.00	3.16	.15
	3	2.84	3.32	2.90	3.20	.19
FG	1	.00	1.03	1.17	1.26	.14
	2	.00	1.16	1.31	1.44	.12
	3	.00	1.58	1.38	1.62	.12
Vitamin E	1	64[g]	104[g]	2054[f]	4415[e]	116
	2	77[g]	108[g]	2288[f]	5060[e]	139
	3	82[g]	118[g]	2418[f]	5680[e]	150

[a]Least square means.

[b]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Feed and calculated free gossypol (FG) and vitamin E intake in phase 2.

[efg]Least square means with different superscripts in the same row differ $P < .05$.

G+2E and G+4E diets. Furthermore, G+4E calves had greater ($P < .05$) calculated vitamin E intake than G+2E. Performance of CON calves may have been affected by lower intake of the CON starter. Claypool et al. (1985) did not find any difference in performance between calves fed CSM and SBM. Holmberg et al. (1988) found no effect of FG on performance of dairy calves fed 250 to 380 mg FG/kg prior to the onset of clinical signs of toxicity. Risco et al. (1992) reported that FG intake had little effect on appetite and growth response of calves. In their diets SBM and CSM were in pelleted form which could have improved the physical form of the starter and therefore eliminated any effect of the main ingredients on intake. They also reported greater feed intakes than those found in this experiment.

Blood hemoglobin concentration (Table 4-4) was lower ($P < .05$) on calves on diet GOS than on CON in the second and third collections. Vitamin E supplementation at the highest concentration (G+4E) prevented the hemoglobin lowering effect of CSM at collection 3. Collection 4 values also were greater for calves on diet G+4E than on GOS. Blood hematocrit (Table 4-5) followed a trend similar to hemoglobin. It was lower ($P < .05$) in calves fed GOS than CON and G+4E diets after the initial collection and lower ($P < .05$) than calves fed G+2E after the second collection. Although differences existed among treatments in blood hemoglobin and hematocrit, these values were within the normal range for calves throughout the experiment. Iron and Cu deficiencies had been associated with lower hemoglobin and hematocrit values. In nonruminant animals, gossypol has been suggested to bind Fe and reduce its bioavailability for hemoglobin formation (Skutches et al., 1973). Ceppi and Blum (1994) reported that Fe deficiency in

calves decreased hemoglobin concentration and hematocrit percentage. However, Fe deficiency does not cause an effect on EOF similar to gossypol (Lindsay et al., 1980). They also suggested that gossypol effect on decreasing hemoglobin was independent of gossypol-induced Fe deficiency. Gossypol has been found to inhibit several enzymes including glucose-6-phosphate dehydrogenase, the rate limiting enzyme in the hexose-monophosphate pathway (Bender et al., 1988). Inhibition of this enzyme can cause a decrease in NADPH which is necessary to reduce glutathione, an important component of the cell antioxidant system. Enzyme inhibition in this pathway or excess oxidants may cause reduced hemoglobin and hematocrit values (Duncan et al., 1994). Therefore, the effect of vitamin E found in this experiment may be related to the antioxidant role of vitamin E.

Erythrocyte osmotic fragility at .65% saline solution as affected by treatment is presented in Figure 4-5. Animals receiving CSM-based starters had greater ($P < .05$) EOF in all collections than the CON calves. Feeding vitamin E did not reduce EOF. Calhoun et al. (1992) reported that the positive association between malondialdehyde and EOF indicated lipid peroxidation may be a factor in altering erythrocyte membrane fragility. Furthermore, they suggested the peroxidation effect to be secondary to changes in the structural integrity of the erythrocyte membrane. Results from this experiment indicated that gossypol effect on erythrocyte fragility may be related to alteration of membrane integrity through mechanisms not related to decreased antioxidant levels.

Alkaline phosphatase is a membrane bound enzyme used for diagnosis of bone and liver disorders or drug exposure. Production of AP is increased in response to

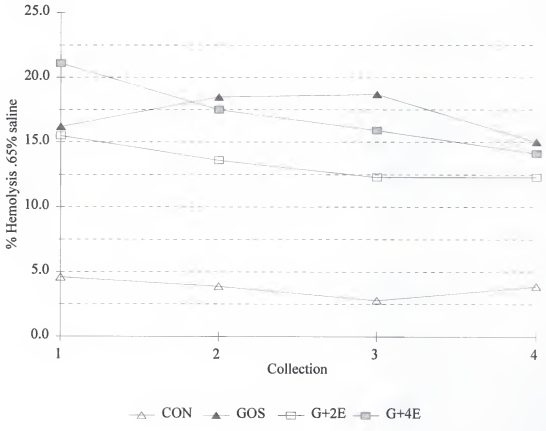

Figure 4-5. Effect of cottonseed meal (CSM) and vitamin E on erythrocyte osmotic fragility (.65% saline) of dairy calves in phase 2. Diets were: CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, 3.8; C2, 2.8; C3, 2.4; and C4, 2.3.

primary or secondary hepatocellular disorders. Liver degenerative changes and hemorrhages are signs of gossypol toxicity. Although the reference range for AP is wide for ruminants, changes in concentrations of AP would indicate impaired liver function or damage. In our experiment, we did not find any changes ($P > .1$) in plasma AP concentration due to treatment (Table 4-8).

Plasma α-T concentrations (Figure 4-6) were similar ($P > .1$) between calves fed CON and GOS diets, while lower ($P < .05$) than calves on G+2E and G+4E diets. Calves fed G+2E diet had lower ($P < .05$) plasma α-T concentrations than those fed G+4E in all collections except collection 2. Ranking of calculated vitamin E intake (Table 4-7) by diet was CON = GOS < G+2E < G+4E ($P < .05$). CON and GOS diets supplied equal amounts of supplemental vitamin E. Therefore, at this level of supplementation feeding CSM (400 mg FG/kg of diet DM) did not decrease plasma α-T concentration. Since there was no treatment of high vitamin E and no CSM included in this experiment, it is not possible to speculate on the effect of gossypol on plasma α-T concentration at high levels of vitamin E supplementation. Gossypol seemed not to be related to lowering plasma α-T concentrations as reported by Lane and Stuart (1990) and Willard et al. (1995).

Mortality. During the experimental period, 10 calves died by causes suspected to be treatment related. Six of these animals belonged to the GOS treatment and 2 each to G+2E and G+4E with no deaths in the CON treatment (Tables 4-9 and 4-10). Intake of FG by these animals at the time of death was from .8 to 2.2 g/d.

Table 4-8. Effect of cottonseed meal (CSM) and vitamin E on plasma alkaline phosphatase of dairy calves (IU/L)[a].

		Treatment[b]				
Phase 2[d]	Collection	CON	GOS	G+2E	G+4E	SE[c]
	1	58.1	43.5	55.4	68.1	7.8
	2	67.1	62.9	75.0	62.4	7.1
	3	55.6	44.4	57.8	62.2	6.1
	4	58.8	44.1	49.3	47.2	6.9

[a]Least square means.

[b]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Group feeding.

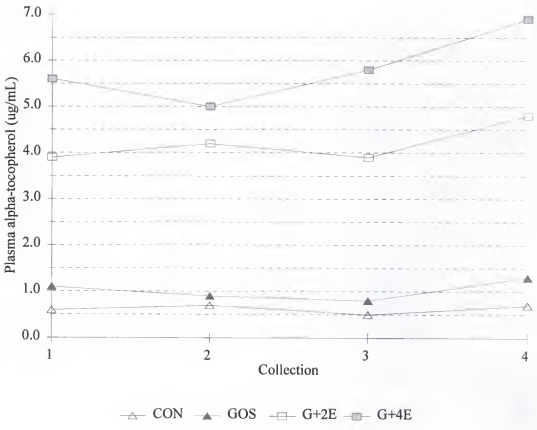

Figure 4-6. Effect of cottonseed meal (CSM) and vitamin E on plasma α-tocopherol concentration of dairy calves in phase 2. Diets were: CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .4; C2, .4 C3, .3; and C4, .4.

Table 4-9. Tissue concentrations of α-tocopherol (α-T) and β-carotene (β-C), plasma α-tocopherol, blood hemoglobin (hemo), hematocrit (hema), and erythrocyte osmotic fragility (EOF), and free gossypol (FG) intake of dead calves.

		Item											
		Liver[a]		Heart[a]		Testis[a]		Plasma[b]	Blood[c]			Intake[d]	
Animal	TRT[e]	α-T	β-C	α-T	β-C	α-T	β-C	α-T	Hemo	Hema	EOF	FG	/BW
3	GOS	1.4	4.5	12.0	5.1	1.2	5.8	.3	10.3	28.0	13.8	1.2	10.3
5	GOS	---	---	---	---	---	---	.9	11.9	35.0	10.0	1.4	12.0
12	GOS	2.2	7.6	2.9	6.7	.8	5.0	1.5	11.8	34.0	23.5	2.1	32.0
17	GOS	---	---	---	---	---	---	.4	8.7	29.0	1.3	1.1	35.5
42	GOS	---	---	---	---	---	---	.3	9.9	28.5	37.8	1.3	21.0
486	GOS	1.0	4.9	1.0	5.7	1.1	4.4	.5	10.1	26.5	25.0	1.1	15.5
33	G+2	11.4	8.3	6.1	4.6	11.1	7.5	2.8	10.8	29.5	15.3	1.4	12.2
636	G+2	19.3	4.9	6.3	5.5	8.2	5.7	2.5	10.9	30.0	3.3	.8	11.4
6	G+4	30.9	6.5	5.1	7.1	---	---	6.3	12.3	35.5	14.6	2.2	38.5
630	G+4	12.1	5.7	4.3	7.4	17.0	4.6	4.0	10.5	27.0	11.1	1.3	11.0

[a]μg/g wet tissue.

[b]Last collection before death (μg/mL).

[c]Last collection before death: Hemoglobin (g/dL); Hematocrit (%); EOF (%) in .65% saline.

[d]Intake at the time of death. FG= g FG/d; /BW = mg FG · kg^{-1} BW · d^{-1}.

[e]GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · $animal^{-1}$ · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · $animal^{-1}$ · d^{-1}.

Table 4-10. Effect of cottonseed meal (CSM) and vitamin E on tissue (+)-, (-), and total (T) gossypol of dead calves.

		Tissue											
		Liver[a]			Heart[a]			Testis[a]			Plasma[b]		
Animal	TRT[c]	(+)	(-)	T	(+)	(-)	T	(+)	(-)	T	(+)	(-)	T
3	GOS	60.7	26.5	87.2	12.6	26.0	38.6	21.7	11.9	33.6	1.7	1.0	2.7
5	GOS	37.0	17.8	54.8	5.3	16.1	21.4	11.9	8.5	20.4	.9	.5	1.4
12	GOS	76.4	57.6	134.0	5.1	19.0	24.1	8.7	5.6	14.3	1.9	1.1	3.0
17	GOS	---	---	---	---	---	---	---	---	---	2.1	1.3	3.4
42	GOS	59.3	50.3	109.6	7.5	42.3	49.8	10.4	7.0	17.4	1.3	.6	1.9
486	GOS	37.8	37.0	94.8	11.2	15.0	26.2	22.7	10.2	32.9	2.1	.9	3.0
33	G+2E	84.4	9.9	94.3	17.8	28.9	46.7	16.0	18.1	34.1	3.4	1.9	5.3
636	G+2E	51.8	42.1	93.9	10.4	49.5	59.9	8.4	14.2	22.6	1.6	.8	2.4
6	G+4E	75.3	53.1	128.4	7.1	26.5	33.6	17.4	13.7	31.1	3.2	1.5	4.7
630	G+4E	26.9	20.3	47.2	8.3	20.5	28.8	12.0	11.2	23.2	2.1	1.1	3.2

[a]µg/g dry tissue.

[b]Last blood collection before death (µg/mL).

[c]GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

Hematocrit and hemoglobin values were within normal range during the last blood collection, and EOF (.65%) varied between 1.3 to 37.8%. Calculated FG intake per kg BW/d before death was more than 10 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}. Calhoun and Wang (1995) reported that weaned lambs fed up to 30 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} exhibit no sign of gossypol poisoning while lambs fed 20 to 30 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} mixed with the milk replacer in order to by-pass the rumen died after 21 d of feeding. In another report, Kim et al. (1996) reported similar results to the previous study when lambs were fed at 20 to 30 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}. They also reported greater tissue gossypol concentrations of fatally intoxicated lambs than those reported in this study (Table 4-10). In agreement with the data of Kim et al. (1996), proportions of tissue gossypol enantiomers accumulation were similar, with (-)-gossypol accumulating more than (+)-gossypol in heart, and the reverse for liver. At this age, calves accumulated gossypol in testis which may have subsequently affected reproductive parameters at puberty.

Plasma gossypol concentrations were below 5 μg/mL in all animals except calf 33. The concentration of 5 μg/mL has been reported as a safe upper level for adult dairy cattle (Calhoun, 1995). Concentrations of plasma and tissue α-T seemed to agree with the treatment diets. Liver Cu concentrations ranged from 311.7 to 865.7 mg/kg dry tissue which were considered to be high for this class of animal (Netherlands Committee on Mineral Nutrition, 1973). de Peyster and Wang (1993) reported that gossypol may chelate Cu and Zn. Gossypol could have chelated Cu and Fe in the liver and reduced their availability. Lower hemoglobin concentration found in calves of the GOS group could have been the result of lower Cu and Fe availability or inhibition of enzymes

related with the antioxidant system; however, the increase in hemoglobin concentration due to vitamin E supplementation supports the hypothesis of decreased antioxidants other than α-T.

Bloat was determined to be the cause of death in calves 17 and 5 with no significant finding upon necropsy except that calf 5 had a large thymic mass. Calf 5 had the lowest concentration of heart and liver gossypol of all dead animals (Table 4-10). Histopathological examination of heart and liver of these calves revealed a normal condition for calf 17 and a mild lipoid vacuolation of cytoplasm of centrilobular hepatocyte in calf 5 (Table 4-11).

Calves 6, 42, 486, and 33 had substantial accumulation of straw-colored fluid in the abdominal and thoracic cavities. The heart of calf 42 had small areas of hemorrhage on the cardiac and pericardial surface. It was also slightly enlarged and the right ventricular surface appeared congested. There were small petechial hemorrhage on the tracheal mucosa surface. Similar findings were noted in calf 486 with pericardial fluid noted and hemorrhage in the trachea. The only significant findings on the gross necropsy of calf 3 was severe ascites.

Histopathological findings of heart and liver of the 10 dead calves are shown in Table 4-11. The heart microscopic findings ranged from insignificant to severe atrophy and fragmentation of cardiocytes, and vacuolation of cytoplasm. The liver examination revealed from insignificant damage to severe centrilobular necrosis.

In general, histological changes in the heart and liver were equivocal in most dead animals, and had no consistent pattern with gossypol accumulation in tissue. However,

Table 4-11. Heart and liver histopathology of dead calves[a].

D	AN	TRT	O	Organ findings	S
119	3	GOS	H	mild autolysis with several posmortem rods	
			L	marked autolysis	
124	5	GOS	H	eosinophilic and swollen cardiocytes with loss of striations; pyknotic nuclei	+
			L	mild lipoid vacuolation of cytoplasm of centrilobular hepatocytes	+
108	12	GOS	H	moderate vacuolation of sarcoplasm of cardiocytes	++
			L	normal	
95	17	GOS	H	normal	
			L	normal	
91	42	GOS	H	myofiber atrophy and eosinophilia of cardiocytes; pyknosis of nuclei	++
			L	moderate centrilobular necrosis of hepatocytes	++
80	486	GOS	H	moderate interfascicular edema thinning of myofibers	+
			L	moderate centrilobular and midzonal necrosis	++
84	33	G+2E	H	atrophy of cardiocytes;moderate interfascicular edema; vacuolation of cytoplasm and nuclei	+++
			L	severe centrilobular and midzonal necrosis of hepatocytes; vacuolation of cytoplasm of peripheral hepatocytes	+++
83	636	G+2E	H	normal	
			L	mild centrilobular vacuolation of hepatocytes	+
65	6	G+4E	H	normal	
			L	normal	
119	630	G+4E	H	atrophy and fragmentation of cardiocytes; interfascicular edema; vacuolation of cytoplasm	+++
			L	sever centrilobular and midzonal necrosis of hepatocytes	+++

[a]D = days on diet; AN = animal number; TRT = treatment: GOS = cottonseed meal (CSM) + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}; O = organ: H = heart, L = liver; S = degree of pathology: + mild, ++ moderate, +++ marked.

the livers of six of the ten dead calves revealed some type of centrilobular necrosis similar to the most prominent liver histological lesion reported in gossypol intoxicated calves (Zelski et al., 1995; Risco et al., 1992; Holmberg et al., 1988). This type of damage could be associated with congestive heart failure. Zelski et al. (1995) suggested that liver lesions in calves fed gossypol were probably the result of heart failure causing passive congestion and hypoxia, combined with a direct hepatotoxic effect. In this experiment, heart lesions consisted of atrophy and vacuolation of cytoplasm of cardiocytes. In addition, interfascicular edema was found in most of the affected calves which agrees with findings of Holmberg et al. (1988).

In the necropsy findings, the most common gossypol induced effect was the accumulation of a straw-colored fluid in the abdominal and thoracic cavities. Morgan et al. (1988) suggested that alteration of cellular membrane permeability by gossypol may be the cause of this effect. Accumulation of fluid in the abdominal and thoracic cavity also has been reported by Zelski et al. (1995), Risco et al. (1992), and Holmberg et al. (1988) as an indicator of gossypol toxicity.

Prior to death, calves did not exhibit health problems except for calves 17 (diarrhea and bloat) and 42 (pneumonia and rough coat). Similar results of sudden death of calves fed gossypol, were reported by Holmberg et al. (1988) and Hudson et al. (1988).

Our results agree with those of Zelski et al. (1995) in which necropsy and histopathological findings were not sufficiently specific and not useful in establishing a diagnosis. However, feeding 400 mg FG/kg of diet DM resulted in death of 10 animals when compared to 0 calves in the CON group. Therefore these data agree with the

hypothesis postulated by Risco et al. (1992) who reported that 400 mg FG/kg resulted in mild toxicity in calves. Supplementation of high vitamin E did not result in necropsy or histopathological findings different from those found in calves supplemented with normal concentrations. Lindsey et al. (1980) suggested that the effect of gossypol may be more pronounced if animals are exposed to some kind of stress. The role of vitamin E on disease resistance has long been recognized, therefore vitamin E could have increased resistance of these calves to diseases and therefore reduced the stress level (Table 4-12). However, a direct interaction between vitamin E and gossypol can not be ruled out.

Implications

Cottonseed meal fed at a concentration to provide 400 mg FG/kg of diet DM was associated with death in 10 calves. Necropsy findings were similar to those reported for preruminants fatally intoxicated with gossypol. Vitamin E supplementation at high concentrations increased feed intake and weight gain and may have conferred calves some protection against gossypol toxicity.

Table 4-12. Effect of cottonseed meal (CSM) and vitamin E on health of dairy calves.

	Treatment[a]			
Item	CON	GOS	G+2E	G+4E
Days at risk[b]	648	891	567	536
Respiratory treatment[c]	.017	.026	.018	.017
Diarrhea treatment[c]	.015	.015	.023	.007
Total treated days[c]	.032	.041	.041	.024

[a]CON = soybean meal + 30 IU vitamin E/kg; GOS = CSM + 30 IU vitamin E/kg; G+2E = CSM + 2,000 IU vitamin E · animal^{-1} · d^{-1}; G+4E = CSM + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[b]Sum of days each calf was alive in each treatment.

[c]Values for respiratory, diarrhea, and total treated days are number of treated days divided by days at risk for each treatment.

CHAPTER 5
LONG TERM EFFECTS OF FEEDING COTTONSEED MEAL AND VITAMIN E TO DAIRY BULLS. I. HEMATOLOGICAL AND TISSUE PARAMETERS

Introduction

By-products of the cotton fiber and cottonseed oil industry such as whole cottonseed (**WCS**) and cottonseed meal (**CSM**) are an important source of economical protein in livestock rations. Gossypol [(2, 2'-binaphthalene) -8, 8'-dicarboxaldehyde-1, 1',6, 6', 7, 7'-hexahydroxy -5, 5'-diisopropyl -3, 3'-dimethyl] is a yellow polyphenolic pigment found in cottonseeds. Gossypol found in cottonseeds is referred to as free gossypol (**FG**), which is the form toxic to livestock. Bound gossypol (**BG**) has not been found to be a major toxicant for livestock, but its role in animal toxicity is not yet fully understood (Jones, 1991). Free gossypol is transformed to BG as the WCS are treated with heat and pressure in the process of oil extraction. Cottonseed meal or any other by-product of the cottonseed oil industry may be less toxic to livestock as their concentration of FG is lowered. However, Calhoun and Holmberg (1991) suggested that BG may be released during the digestive process and have a physiological effect on the animal.

Janero and Burghardt (1988) suggested that gossypol can interact with biological membranes by promoting the formation of highly-reactive oxygen-containing free radicals. This and other studies (Willard et al., 1995; Lane and Stuart, 1990; Bender et

al., 1988) have suggested that antioxidants play a key role in the metabolism of gossypol. Therefore, the objective of this phase of the experiment was to evaluate the effect of long term feeding of CSM fed to provide 14 mg FG · kg^{-1} BW · d^{-1} and the use of vitamin E to counteract the negative effect of gossypol on hematological and tissue parameters in dairy bulls from 6 to 15 mo of age.

Materials and Methods

Animals, diets and management. In a previous phase of the experiment (Chapter 4), the effect of feeding CSM (400 mg FG/kg of diet DM) and supplemental vitamin E on calf performance from 2 wk of age to six mo was tested using four diets based on 1) soybean meal (**SBM**) + 30 IU vitamin E/kg, 2) CSM + 30 IU vitamin E/kg, 3) CSM supplemented with 700 IU vitamin E/kg of diet DM, and 4) CSM supplemented with 1400 IU vitamin E/kg of diet DM. A second phase of this experiment reported herein utilized eight bulls for each of the following dietary treatments (Table 5-1): 1) **CON** a supplement based on SBM + corn + 30 IU vitamin E/kg, 2) **GOS** a supplement based on CSM + corn + 30 IU vitamin E/kg, and 3) **G+4E** a supplement based on CSM + corn + 4,000 IU vitamin E · $bull^{-1}$ · d^{-1}. Supplements GOS and G+4E were formulated to supply 14 mg FG · kg^{-1} BW · d^{-1}. All supplements were isocaloric and isonitrogenous, and satisfied animal requirements for all other nutrients (NRC, 1989). Animals were selected to represent their previous diets. The bulls were housed in 12 pens, two animals per pen and four pens per treatment, from 6 to 15 mo of age. The diets consisted of the treatment

Table 5-1. Composition of dietary supplements[a]

	CON		GOS		G+4E	
Item	Initial	Final	Initial	Final	Initial	Final
Offered (kg/d)[b]	2.60	5.60	2.70	6.00	2.70	6.00
DM (%)	88.00	88.00	88.00	88.00	88.00	88.00
Ingredient[b]						
SBM (%)	59.0	71.0	----	----	----	----
CSM (%)	----	----	67.00	80.00	67.00	80.00
Corn (%)	38.00	27.50	30.00	18.50	30.00	18.50
Limestone (%)	1.00	.50	1.00	.50	1.00	.50
Minerals (%)	2.00	1.00	2.00	1.00	2.00	1.00
Vit.E (IU/kg)	30.00	30.00	30.00	30.00	1481.50	666.60
Analyses						
(+)-gossypol (%)[c]	.00	.00	.33	.40	.32	.40
(-)-gossypol (%)[c]	.00	.00	.79	.98	.77	.97
Free gossypol (%)[d]	.00	.00	.08	.11	.08	.11
Total gossypol (%)[d]	.00	.00	1.06	1.16	1.06	1.14
Average values[e]						
(+)-gossypol (%)	.00 ± .00		.32 ± .03		.32 ± .03	
(-)-gossypol (%)	.00 ± .00		.79 ± .08		.79 ± .08	
Free gossypol (%)	.00 ± .00		.08 ± .01		.08 ± .01	
Total gossypol (%)	.00 ± .00		1.02 ± .07		1.03 ± .07	
Vitamin A (IU/kg)[f]	2076.61		2759.10		2759.10	
Vitamin E (IU/kg)[b]	38.84 ± 11.55		44.73 ± 13.11		741.42 ± 182.90	
CP (%)[g]	38.59 ± 4.31		37.89 ± 2.03		37.09 ± 2.43	
Ca (%)[g]	.47 ± .17		.48 ± .13		.42 ± .12	
K (%)[g]	1.41 ± .44		1.42 ± .10		1.42 ± .36	
Mg (%)[g]	.22 ± .06		.57 ± .06		.55 ± .10	
P (%)[g]	.71 ± .08		1.12 ± .05		1.11 ± .08	
Cu (mg/kg)[g]	16.67 ± 7.65		15.97 ± 2.60		14.86 ± 3.17	
Zn (mg/kg)[g]	69.33 ± 28.39		73.29 ± 11.77		70.52 ± 13.85	
Mn (mg/kg)[g]	52.61 ± 26.95		36.22 ± 13.51		31.05 ± 13.55	
Fe (mg/kg)[g]	182.59 ± 91.51		161.71 ± 76.54		139.03 ± 40.00	
Se (mg/kg)[g]	.21 ± .11		.20 ± .04		.17 ± .04	

[a]CON = soybean meal (SBM) + corn + 30 IU vitamin E/kg; GOS = cottonseed meal (CSM) + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[b]As fed basis.

[c] As fed. HPLC procedure (Calhoun et al. (1995a), and Kim and Calhoun (1995)). Texas A&M.

[d]As fed. AOACS procedure. Texas A&M.

[e]Mean of 8 mixing ± SD.

[f] As fed. A composited sample from all mixing dates. An IU/kg = Retinol acetate (μg/kg) * 2.91

[g]DM basis.

supplement and a free choice low quality hay (vitamin E concentration of <9.0 IU/kg). Supplements were recalculated every month in order to provide 14 mg FG · kg^{-1} BW · d^{-1}. The protocol for bull procedures had been approved by the University Animal Use Committee.

Blood and tissue sampling and analyses. Blood and plasma samples were collected every 28 d (n = 10) via jugular venipuncture with an 18-gauge needle into heparinized vacutainer blood collection tubes. Blood was analyzed for erythrocyte osmotic fragility (**EOF**), hemoglobin, and hematocrit. Erythrocyte osmotic fragility, measured as percentage hemolysis, was evaluated in .65 and .55% buffered saline solution as described by Risco et al. (1993). Hemoglobin was determined using a colorimetric procedure (Sigma Chemical Co., St. Louis, MO). Hematocrit was determined from blood using a micro hematocrit centrifuge (IEC MB Centrifuge, Needham Heights, MA). Blood was centrifuged for 25 min at 700 x g. Plasma was removed and stored at -20°C until analyzed for α-tocopherol (**α-T**), alkaline phosphatase (**AP**), and creatine kinase (**CK**) (all collections), (+)-, and (-)-gossypol isomers (collections 1, 6 and 10). At the end of the experiment animals were slaughtered and portions of liver, heart, and testis were collected and frozen at -20°C until analyzed for α-T, β-carotene (**β-C**), (+)-, (-)-, and total gossypol, Fe, Cu, and Zn. Liver samples were also analyzed for retinol palmitate (**RETP**) and Se. Samples of supplements were collected and analyzed for α-T, RETP, Ca, K, Mg, Mn, P, CP, Fe, Zn, Cu, and Se.

Alpha-tocopherol was analyzed following the procedure described by Njeru et al. (1992) for plasma, and by Njeru et al. (1995) for tissue and feed samples. Tissue and

feed RETP, and β-C were prepared as follows: approximately 1 g of sample (fresh weight) and .1 g of ascorbic acid were homogenized in 10 ml of acetone; 1 ml of the homogenate was deproteinized with 1 mL of ethanol. After adding 2 ml of .9% saline, the mixture was vortexed for 6 min. Samples were double extracted with 3 mL petroleum ether and kept in an ice bath. The petroleum ether extract was dried by evaporation under a stream of N_2 in a 35°C water bath, reconstituted in 750 μL of .1% acetic acid, 29.9% tetrahydrofuran, and 70% iso-octane with .2% added β-hydroxy toluene. The sample was separated in equal amounts in two sealed vials one for RETP and the other vial for β-C analysis. Retinol palmitate was determined by injecting 20 μL of the reconstituted extracted sample (tissue or feed) into the HPLC system. The HPLC systems consisted of a Perkin Elmer 550 terminal (Perkin-Elmer, Analytical Instruments, Norwalk, CT.), an ISS-100 auto sampler (Perkin-Elmer), a Series 4 Liquid chromatograph pump (Perkin-Elmer), and a Lichrosorb Si 60 5μm, 4 mm ID x 250 mm column (Hibar Fertigsaule RT pre-packed column RT 250-4 E, Merck, Darmstadt, Germany). The mobile phase consisted of 70% iso-octane, 29.9% tetrahydrofuran, and .1% acetic acid. The UV detector was an ABI Analytical Spectroflow 757 set at a wavelength of 325 nm and sensitivity of .005. Data were collected by a LCI-100 Laboratory Computing Integrator (Perkin-Elmer). Flow rate was 1 mL/min. The retention time of RETP was 2.47 min. Standards consisted of 10 ng of RETP (Sigma Chemical Co., St. Louis, MO). Analysis of β-C was similar except that the mobile phase was 90% iso-octane, 9.9% tetrahydrofuran, and .1% acetic acid; the wavelength was set at 450 nm, and the retention

time was 3.05 min. Standards consisted of 100 ng β-C (Sigma Chemical Co., St. Louis, MO).

Stimulated lipid peroxidation was performed in heart, testis, and liver samples according to a modification of the procedure of Kornbrust and Mavis (1980). Briefly, approximately 1 g of fresh tissue was homogenized in 9 mL of 1.15% KCl. An aliquot (100 µL) was incubated in a water bath at 37°C for the specified length of time (0, 50, 100, and 200 min) with 500 µL of 8 mM Tris-malate buffer, 200 µL of 5 mM $FeSO_4 \cdot 7H_2O$ and 200 µL of 2 mM ascorbic acid solutions. Peroxidation was terminated by rapid addition of a 2 mL solution containing thiobarbituric acid (.375%) and 15% trichloroacetic acid in .25 N HCl, and boiling in a water bath for 15 min. Samples were then centrifuged for 15 min at 700 x g. The amount of colored product was measured spectrophotometrically at 535 nm. Lipid peroxidation was expressed in terms of nmol malondialdehyde (**MDA**)/mg of protein. Janero and Burghardt (1989) suggested that this test can not be used as anything else other than an empirical indicator of membrane oxidative injury, therefore care should be taken in the interpretation of these results.

Enzymes were determined using kits (CK:Sigma Diagnostic Procedure No. 520; AP: Sigma Diagnostic Procedure No. 104). Minerals were determined according to the procedures described by Rojas et al. (1995) and Whetter and Ullrey (1978). Samples of plasma and tissue for gossypol analyses were shipped in dry ice to Texas A&M University where they were analyzed by HPLC according to the procedures described by Calhoun et al. (1995a), and Kim and Calhoun (1995).

Statistical analyses. Continuous data such as hematocrit, hemoglobin, EOF, lipid peroxidation, plasma CK, AP, (-)-, (+)-, and total gossypol, and α-T were analyzed by repeated measures analysis of variance in a completely randomized design using GLM procedure of SAS (SAS, 1988). The Greenhouse-Geiser Epsilon was used to determine significant levels of the F-test. Analyses of variables containing single or calculated observations where performed by ANOVA (SAS, 1988). Treatment and pen within treatment effects were tested using bull within pen and treatment effect. Pen within treatment effect was removed from the model when it was not significant ($P > .2$). When the pen within treatment effect was kept in the model PROC MIXED (SAS, 1996) was used to calculate the correct standard errors of the least square means for a mixed model. There were two sets of animals with an age difference of approximately 4 wk, therefore age was used as a covariate. Tissue (+)-, (-)-, and total gossypol were analyzed with a nested mixed analysis of covariace model by the PROC MIXED (SAS, 1996), where a 3 x 3 factorial design (three tissues x three isomers) occurs within each animal, where animals (a random effect) were nested within treatments. There were nine measurements within each animal (three gossypol values per each of three tissues) and they were assumed to be equally correlated. When the overall treatment effect was significant ($P < .05$) or tended to be significant ($P < .1$), separation of means was done using Duncan multiple range test for all variables except gossypol tissue concentration for which the Tukey test was used (available in PROC MIXED). Pooled standard errors were reported when all treatments had the same number of replicates, otherwise individual values for each least square mean were given.

Results and Discussion

Free gossypol intake in the animals on GOS and G+4E treatments, calculated from the supplement analyses, ranged from 12.7 to 17 mg FG · kg^{-1} BW · d^{-1} (from 2.2 to 6.5 g FG · $animal^{-1}$ · d^{-1}) during the 10 mo period. On average, supplements had the targeted amount of vitamin E specified in the experimental protocol. Proportions of various components of the supplements are shown in Table 5-1.

Animal performance and blood parameters. Animal ADG, total gain, initial and final weights were not affected by treatment (Table 5-2). Vitamin E has been found to increase ADG in lambs fed a diet containing 20% CSM (Calk et al., 1992). Here the increase in ADG could have been due to a vitamin E effect on intake. Chase et al. (1994) found that bulls fed gossypol containing products (16 g FG · $bull^{-1}$ · d^{-1}) tended to have reduced ($P < .1$) body weight gains; however, this was attributed to a high fat content of the supplements rather than a direct effect of gossypol on performance. Also, a decrease in weight gain may have been caused by the effect of gossypol on digestive enzymes of animals fed CSM or WCS. In vitro digestion of proteins by pepsin and trypsin was reduced when gossypol was added prior to enzymatic digestion (Abou-Donia, 1989). The action of gossypol on the enzymes seems to be related to the binding of gossypol with the ε-amino group of lysine on the protein substrate or to the zymogen that could not be converted to the active enzyme (Abou-Donia, 1989). Supplements used in the present

Table 5-2. Effect of cottonseed meal (CSM) and vitamin E on weight gain and average daily gain (ADG) of dairy bulls[a].

	Supplement[b]			
Item	CON	GOS	G+4E	SE[c]
Initial weight (kg)	163.4	164.3	178.6	4.3
Final weight (kg)	387.8	371.9	402.1	11.9
Weight gain (kg)	224.5	207.6	223.5	10.0
ADG (kg)	1.0	.9	1.0	.1

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

experiment had a high CP concentration which could have increased gossypol fecal excretion and decreased tissue deposition (Abou-Donia, 1976).

Erythrocyte osmotic fragility, as measured in .65 and .55% dilution (Figures 5-1 and 5-2), was greater ($P < .001$) at all collection periods for bulls fed GOS and G+4E supplements than for the CON. Feeding 4,000 IU vitamin E $\cdot h^{-1} \cdot d^{-1}$ did not reduce the increase in EOF caused by gossypol as reported by Velasquez-Pereira et al. (1996c), possibly because the animals in GOS and G+4E had been previously fed 400 mg/kg FG from week 2 to 6 mo of age. Risco et al. (1993) found that Brahman bulls fed 8.2 g FG or 16.6 mg FG $\cdot kg^{-1}$ BW $\cdot d^{-1}$ exhibited elevated EOF compared with controls. These researchers also reported that gossypol caused erythrocyte acanthocytosis in three out of eight bulls fed gossypol, which has been reported in conditions affecting the erythrocyte membrane (Risco et al., 1993). Gray et al. (1993) reported increased EOF in heifers and cows consuming 10 or 20 g of FG $\cdot$ animal$^{-1} \cdot d^{-1}$. Calk et al. (1992) reported that feeding lysine or vitamin E did not protect against the effect of gossypol on EOF in lambs. Gossypol has been found to intercalate into phospholipid bilayers, affecting the structure and dynamics of membranes (Tanphaichitr et al., 1995). This property of gossypol may affect the permeability of the erythrocyte membrane to anions causing the increase in EOF. Although increased EOF has been reported as a clinical indicator of gossypol intoxication in cattle (Calhoun et al., 1990; Lindsey et al., 1980), there are no reports relating this laboratory procedure with clinical or reproductive signs of toxicity.

Feeding gossypol with and without supplemental vitamin E to bulls did not affect hemoglobin concentration (Table 5-3), which was within the normal range throughout the

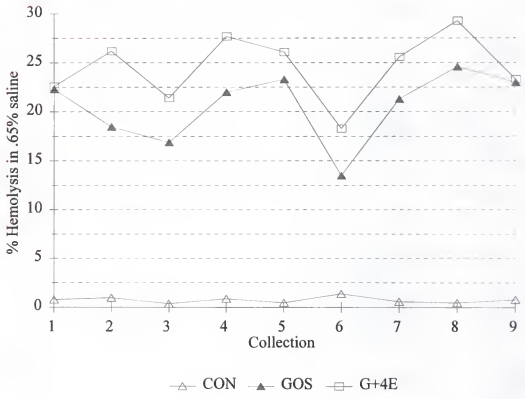

Figure 5-1. Effect of cottonseed meal (CSM) and vitamin E on erythrocyte osmotic fragility in .65% saline. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, 2.5; C2, 2.1; C3, 2.4; C4, 3.1; C5, 2.0; C6, 1.3; C7, 4.5; C8, 1.4; and C9, 1.5.

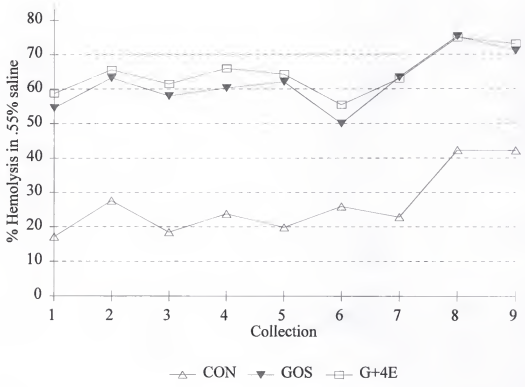

Figure 5-2. Effect of cottonseed meal (CSM) and vitamin E on erythrocyte osmotic fragility in .55% saline. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, 4.3; C2, 4.1; C3, 5.0; C4, 5.7; C5, 4.7; C6, 4.3; C7, 4.4; C8, 4.6; and C9, 4.2.

Table 5-3. Effect of cottonseed meal (CSM) and vitamin E on blood hemoglobin and hematocrit and plasma alkaline phosphatase (AP) and creatine kinase (CK) of dairy bulls[a].

		Collection									
Item	SUP[b]	1	2	3	4	5	6	7	8	9	10
AP (IU/L)	CON	37.2[d]	51.9[d]	46.6[d]	40.6[d]	43.5[d]	40.9[d]	29.2[d]	31.6[d]	33.4[d]	43.5[d]
	GOS	19.9[e]	30.3[e]	24.0[e]	26.5[e]	25.4[e]	25.1[e]	20.7[e]	23.4[d]	18.6[e]	25.1[e]
	G+4E	21.2[e]	32.1[e]	31.1[e]	28.4[e]	28.2[e]	29.9[de]	25.4[de]	30.9[d]	23.8[de]	32.1[e]
SE[c]		3.4	2.9	4.3	2.7	2.7	3.5	2.5	2.9	3.1	2.4
CK (IU/L)	CON	10.3	8.9	9.0	8.9	10.6	9.6	21.5	6.8	10.2	8.0
	GOS	10.4	9.7	11.2	11.5	12.3	11.4	30.8	12.8	19.9	10.1
	G+4E	13.1	9.7	8.8	11.9	13.3	9.9	16.9	13.0	14.1	9.5
SE		.8	.9	.9	1.3	1.2	1.0	5.3	2.4	2.4	1.1
Hemoglobin	CON	14.9	14.3	13.9	13.9	12.8	13.7	12.6	12.7	12.4	13.1
(g/dL)	GOS	13.4	14.1	12.7	12.1	11.6	13.4	11.7	12.2	11.8	12.5
	G+4E	14.0	13.4	12.9	12.7	11.7	13.3	11.7	12.4	12.7	12.1
SE		.6	.6	.5	.4	.4	.4	.4	.4	.4	.3
Hematocrit	CON	37.9[d]	36.4[d]	36.3	36.4[d]	35.6[d]	34.4	36.2[f]	33.6	33.6	34.2[f]
(%)	GOS	33.1[e]	33.9[e]	33.3	33.3[e]	31.4[e]	34.7	33.6[g]	31.9	31.8	32.4[g]
	G+4E	34.9[de]	34.0[e]	34.9	34.4[de]	32.4[e]	35.3	35.4[fg]	33.4	33.4	33.9[fg]
SE		1.2	.8	1.0	.7	.7	1.0	1.0	1.0	.8	.7

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[de]Least square means with different superscripts in the same column differ $P < .05$.

[fg]Least square means with different superscripts in the same column differ $P < .1$.

study. Bulls on the CON treatment had greater ($P < .05$) hematocrit percentage than those on the GOS treatment in collections 1, 2, 4, and 5, and tended to be greater ($P < .1$) for collections 7 and 10 (Table 5-3). There was no difference between the G+4E and GOS or CON treatments in any of the collections except collection 2. Hemoglobin concentration and hematocrit percentage have been reported to be lower in animals receiving gossypol. Lindsey et al. (1980) reported that feeding 24.2 g of FG from solvent extracted CSM to lactating dairy cows decreased hemoglobin concentration and hematocrit percentage. They also reported that the major physiological effects of gossypol in lactating dairy cows were alterations in normal erythrocyte structure, metabolism, and (or) function that affect hemoglobin, EOF, and hematocrit. Furthermore, although Fe and Cu deficiencies can cause a decrease in hemoglobin and hematocrit values, in this experiment Fe and Cu in the supplements (Table 5-1) met the NRC (1989) recommendations and their concentrations in liver were within normal ranges. Iron deficiency has been found to decrease hemoglobin concentration and hematocrit percentage in calves (Ceppi and Blum, 1994), but not to cause an effect on EOF similar to gossypol (Lindsey et al., 1980).

Alkaline phosphatase is a membrane bound enzyme used in the diagnosis of bone and liver disorders and drug exposure. Production of AP is increased in response to primary or secondary hepatocellular disorders. Liver degenerative changes and hemorrhage are signs of gossypol toxicity. Although the AP reference range for ruminants is wide, changes in concentrations of AP would indicate impaired liver function or damage. In our experiment, plasma AP concentration (Table 5-3) decreased

($P < .05$) in bulls fed the GOS and G+4E supplements. Gawai et al. (1995) reported that gossypol depressed liver microsomal enzymes of rats given 5 mg of gossypol intraperitoneally for 5 d. As AP is a membrane bound microsomal enzyme that is produced rather than secreted when hepatic injury occurs, it is possible that gossypol may have decreased the production of this enzyme. Further investigation is needed to determine if gossypol inhibits the production of AP and if concentrations of this enzyme could be used in the diagnosis of gossypol toxicity. Lindsey et al. (1980) found that feeding mature lactating dairy cows 251 to 273 mg of total gossypol (**TG**) $\cdot$ kg^{-1} BW $\cdot$ d^{-1} from WCS and CSM for 14 wk did not cause liver damage.

Treatment did not affect ($P > .1$) plasma CK concentration (Table 5-3). Creatine kinase is an enzyme used in the diagnosis of muscular disorders. Gossypol has been found to cause edema, hypertrophy and dilatation of heart and cardiac muscle degeneration in several species including swine (Abou-Donia, 1989) and cattle (Kerr, 1989). This heart damage is similar to damage caused by Se/vitamin E deficiency. Based on these enzyme concentrations, there was no evidence of muscle damage by feeding gossypol at concentrations provided in this experiment.

Animals fed supplement G+4E had greater ($P < .05$) plasma α-T concentrations than animals fed CON and GOS supplements at all collections (Figure 5-3). Although CON and GOS supplements had the same added vitamin E (30 IU/kg of feed), animals fed GOS had greater ($P < .05$) plasma α-T concentrations than animals receiving CON at all collections except 5 and 6. At this low concentration of supplemental vitamin E,

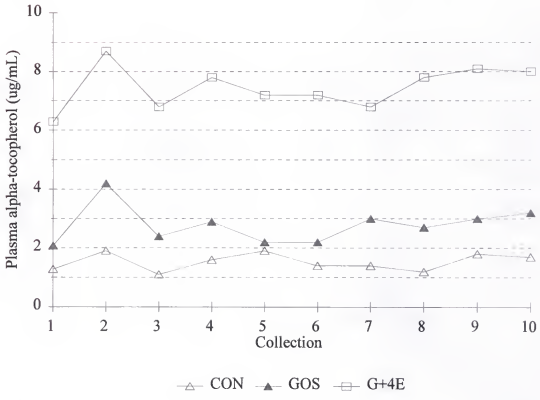

Figure 5-3. Effect of cottonseed meal (CSM) and vitamin E on plasma α-tocopherol concentration of dairy bulls. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each collection (C) were: C1, .3; C2, .2; C3, .3; C4, .2; C5, .3; C6, .4; C7, .4; C8, .3; C9, .4; and C10, .4.

gossypol did not decrease plasma α-T concentration. The increase in α-T concentrations in animals fed GOS could be due to a greater α-T content of CSM based supplements than in SBM based supplements, or to an effect of CSM on absorption of α-T. Since there was no treatment with high vitamin E and no gossypol included in this experiment, it is not possible to speculate on the effect of gossypol on plasma α-T concentration at high levels of vitamin E supplementation. Contrary to our results, Lane and Stuart (1990) reported that serum α-T and β-C were reduced in dairy cows fed high concentrations of gossypol (approximately 40 g). In this study no statistical data were shown and gossypol analysis may not represent the real values. In a more controlled study, Mena et al. (1996) reported that gossypol from CSM, WCS or both (900 to 1800 mg total gossypol/kg) sources did not reduce concentrations of antioxidant vitamins, and that in fact it increased plasma α-tocopherol concentrations.

Willard et al. (1995) found that by d 84 cows given 4 g FG · animal^{-1} · d^{-1} with elevated EOF had lower ($P < .05$) plasma α-T concentrations than cows not supplemented. No supplemental vitamin E was given in the previous experiment. From that experiment, four animals with the highest EOF were chosen from 17 animals receiving 4 g FG · animal^{-1} · d^{-1} in a group fed experiment to determine plasma α-T concentration. It is possible that the animals consuming more supplement (high EOF) with low vitamin E content may have consumed less forage which is an excellent source of vitamin E.

Gossypol enantiomers have been found to have different toxicological and pharmacokinetic effects. Calhoun et al. (1995b) suggested that plasma gossypol

concentration reflects dietary gossypol availability and the proportion of isomers in the source being fed. Furthermore these authors suggested that a plateau was reached after 4 to 6 wk of consumption and remained fairly constant until the diet was changed. Plasma (-)-, (+)- and total gossypol concentrations as affected by dietary supplement are presented in Table 5-4. Plasma (-)-, (+)-, and total gossypol concentrations in bulls fed GOS and G+4E supplements were greater ($P < .05$) than bulls fed CON from the initial sampling to the end of the experiment and were similar ($P > .1$) among the treatments receiving CSM. Plasma total gossypol concentration was lower than the safer upper limit of 5 μg/mL proposed by Calhoun et al. (1995b). Lower plasma total gossypol concentrations were reported by Lindsey et al. (1980) when cows were fed 3.5 or 24.2 g FG · animal^{-1} · d^{-1}. Also Lindsey et al. (1980) did not find differences in plasma gossypol concentrations between cows fed the two levels of FG intake raising a concern of using FG in feed as a measurement of bioavailability. Calhoun et al. (1995b) reported that dairy cows consuming from 27.2 to 33.8 g TG · animal^{-1} · d^{-1} from WCS had average plasma gossypol concentrations of 3.0 μg/mL with a range of 1.2 to 5.8 μg/mL.

Tissue parameters. Tissue concentrations of (+)-, (-)-, and total gossypol are shown in Figure 5-4. No differences ($P > .1$) were found in tissue gossypol concentrations between bulls fed GOS and G+4E supplements and they were greater ($P < .05$) than bulls fed the CON supplement. Liver (+)-gossypol was greater ($P < .01$) than (-)-gossypol concentration in bulls receiving CSM (GOS and G+4E). While heart and testis had greater (-)-gossypol ($P < .05$) than (+)-gossypol on bulls on the same treatments. The order of (+)-gossypol accumulation in tissue was liver > heart and testis

Table 5-4. Effect of cottonseed meal (CSM) and vitamin E on plasma gossypol concentration of dairy bulls (μg/mL)[a]

		Supplement[b]			
Item	Collection	CON	GOS	G+4E	SE[c]
(-) gossypol	1	.00[e]	1.22[d]	1.14[d]	.08
	6	.00[e]	1.40[d]	1.35[d]	.06
	10	.00[e]	1.53[d]	1.69[d]	.11
(+) gossypol	1	.00[e]	1.47[d]	1.28[d]	.09
	6	.00[e]	1.68[d]	1.57[d]	.06
	10	.00[e]	1.85[d]	1.77[d]	.12
Total-gossypol	1	.00[e]	2.69[d]	2.42[d]	.17
	6	.00[e]	3.08[d]	2.91[d]	.12
	10	.00[e]	3.37[d]	3.46[d]	.22

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[de]Least square means with different superscripts in the same row differ $P < .05$.

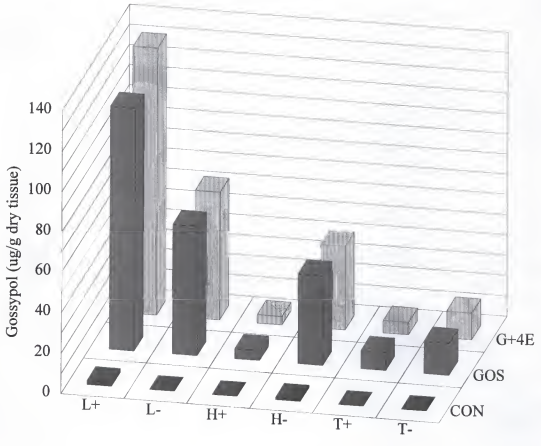

Figure 5-4. Effect of cottonseed meal (CSM) and vitamin E on liver (L), heart (H), and testis (T) (+)-, and (-)-gossypol concentrations. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}; Standard errors for each tissue and isomer were: L+, 7.8; L-, 5.3; H+, .7; H-, 3.5; T+, .6; and T-, 1.11.

($P < .05$), and for (-)-, and total gossypol liver > heart > testis ($P < .01$). Supplementation of vitamin E did not have an effect on (-)-, (+)-, or total gossypol accumulation in tissue.

The fact that gossypol damaged spermatogenic cells in cattle (Chenoweth et al., 1994) and that the testis accumulated less gossypol than the vital organs (i.e. liver and heart), strongly suggests a specific vulnerability and sensitivity of testicular cells to the action of gossypol.

The (-)-gossypol has been shown to be the optically active form that induces fertility impairment in male animals (Wang et al., 1987). The lower accumulation of (-)-gossypol in the liver relative to (+)-gossypol may be related to a greater (-)-gossypol affinity for plasma proteins (Wu and Reidenberg, 1990). The liver is perfused by a fluid similar to plasma which has greater protein concentration than the interstitial fluid found in heart and testis (Joseph et al., 1986). Therefore, (-)-gossypol could be bound to proteins at the time this protein rich fluid perfuses the liver and be redirected to other tissues where it can interact with cellular components that had greater affinity to bind (-)-gossypol than plasma protein. The (+)-gossypol may not be accumulated in tissues other than liver where it seems to be eliminated due to its low specific interaction with cellular components (Wang et al., 1992).

Similar results where (+)-gossypol concentration was greater in liver while (-)-gossypol was greater in heart and muscle were reported in lambs fed 20 to 30 mg FG · kg^{-1} BW· d^{-1} (Kim et al., 1996). In contrast to these results, the proportion of gossypol isomers in liver has been shown to be similar in pigs fed a wide range of gossypol containing diets (Knabe et al., 1995). In rats, similar deposition patterns and a gradual

accumulation of gossypol over time were found by Jensen et al. (1982). The major excretory organ for gossypol appears to be the liver in several species including swine (Abou-Donia and Dieckert, 1974) and rats (Abou-Donia et al., 1970). Abou-Donia (1989) suggested a pathway for the metabolism of gossypol. Gossypol is absorbed from the gastrointestinal tract, mainly the small intestine. It enters the liver via the hepatic artery or through the lymph into the hepatic sinusoid and is taken up by Kupffer cells. In the liver, gossypol is metabolized, conjugated, and excreted with the bile into the duodenum. Some of the gossypol excreted is reabsorbed completing an enterohepatic cycle that may be repeated several times leading to a gradual excretion via feces. The mechanism of excretion seems to require an active secretory process that can be saturated. Gossypol accumulation in liver may be related to its key role in the metabolism of the toxicant.

Liver, heart, and testis concentration of β-C did not differ ($P > .1$) among treatments (Table 5-5). Feeding CSM did not affect tissue deposition of β-C and RETP. However, liver RETP concentration increased ($P < .05$) as vitamin E supplementation increased (Table 5-5). It has been suggested that vitamin E protects retinyl esters in the storage globules of the liver cells, preventing a rapid breakdown of retinyl esters stored in liver (Sondergaard, 1972). Olson (1991) suggested that retinyl ester hydrolase, an enzyme involved in the release of vitamin A from the liver, is regulated by vitamin E status, activated in vitamin E deficiency and inhibited in vitamin E sufficiency. Vitamin E supplementation (G+4E) increased ($P < .05$) α-T concentration in liver, heart, and testis (Table 5-5). Greater ($P < .05$) α-T concentrations in heart and testis were found in

Table 5-5. Effect of cottonseed meal (CSM) and vitamin E on α-tocopherol (α-T), retinol palmitate (RETP), and β-carotene (β-C) concentrations in tissue of dairy bulls (μg/g of fresh tissue)[a].

		Supplement[b]			
Tissue	Item	CON	GOS	G+4E	SE[c]
Liver	α-T	3 .84[e]	6.10[e]	22.58[d]	1.00
	RETP	1.63[e]	1.96[e]	3.70[d]	.56
	β-C	11.51	11.28	11.67	.64
Heart	α-T	6.78[f]	9.10[e]	26.03[d]	.57
	β-C	4.13	4.31	4.56	.20
Testis	α-T	4.76[f]	6.85[e]	16.4[d]	.59
	β-C	2.97	3.11	3.11	.22

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[d]Standard error of the least square means.

[def]Least square means with different superscripts in the same row differ $P < .05$.

bulls fed GOS vs CON supplements at a normal concentration of vitamin E supplementation (30 IU/kg). It has been suggested that a decrease in α-T due to chemical toxicity is found only when the mechanism of cytotoxicity involves lipid peroxidation. However, not all oxidative chemical toxicity involves α-T depletion and lipid peroxidation (Anonymous, 1989).

Some trace minerals (e.g. Fe, Cu, Se, and Zn) have been implicated in gossypol metabolism. Heart failure, seen in gossypol toxicosis, is also one of the clinical signs of Se deficiency. Iron appears to be chelated by gossypol, reducing its bioavailability. Gossypol also chelates Cu and Zn (de Peyster and Wang, 1993). In our experiment, no differences ($P > .1$) were found in heart and testis Cu, Fe, and Zn concentrations among treatments (Table 5-6). However, liver concentrations of Fe and Se were greater ($P < .05$) in bulls fed the GOS than the CON supplements. Accumulation of Fe in liver of animals receiving G+4E was not different ($P > .1$) from bulls fed either the CON or GOS-based diets. Liver Cu was greater ($P < .05$) in the G+4E than CON animals, but similar to GOS, however, it was within the normal range of 200 to 600 μg/g of dry tissue. Contrary to our results, Yu et al. (1981) indicated that gossypol reduced Zn concentration in testis of rats treated with gossypol, and suggested that gossypol chelates Zn and this process may be related to gossypol antispermatogenic effects. Skutches et al. (1973) suggested that gossypol chelates Fe in the liver of pigs, and reduces Fe availability for hemoglobin synthesis. Abou-Donia (1989) indicated that the initial effect of gossypol in swine may be to chelate Fe, thereby interfering with its utilization.

Table 5-6. Effect of cottonseed meal (CSM) and vitamin E on micromineral concentration of liver, heart, and testis of dairy bulls (mg/kg dry matter)[a]

		Supplement[b]			
Tissue	Mineral	CON	GOS	G+4E	SE[c]
Liver	Cu	241.8[e]	353.3[de]	450.1[d]	47.6
	Fe	120.9[e]	215.3[d]	181.8[de]	22.0
	Se	.5[e]	.9[d]	1.1[d]	.1
	Zn	109.6	139.4	111.1	11.3
Heart	Cu	15.2	23.9	14.7	7.0
	Fe	188.9	182.0	161.2	21.8
	Zn	61.0	92.9	66.3	12.6
Testis	Cu	6.9	12.4	9.2	2.6
	Fe	75.1	73.8	74.9	3.5
	Zn	71.4	73.1	76.1	2.5

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[de]Least square means with different superscripts in the same row differ $P < .05$.

In our experiment, although Fe was accumulated in the liver, there was no indication of Fe being unavailable for the synthesis of hemoglobin possibly due to greater than requirement dietary concentrations. Selenium may be involved in the metabolism of gossypol since feeding CSM to bulls increased its accumulation in the liver.

Gossypol has been reported to inhibit free radical damage to lipid membranes in some studies (Janero and Burghardt, 1988) and to stimulate it in others (de Peyster et al., 1984). Bender et al. (1988) suggested that oxidative injury caused by the generation of reactive oxygen species may be the cause of gossypol toxicity. In the previous study, they found a decrease in the concentration of antioxidants in testis of rats fed gossypol acetic acid. Our data on in vitro stimulated lipid peroxidation in liver homogenates (Figure 5-5) indicated that peroxidation of lipids in GOS and G+4E supplemented bulls was lower ($P < .05$) than those supplemented with CON at all times. Heart homogenate lipid peroxidation (Figure 5-6) was lower ($P < .05$) in G+4E than CON supplemented bulls at 50, 100 and 200 min, and than GOS at 100 and 200 min of incubation. Bulls supplemented with GOS had lower ($P < .05$) heart lipid peroxidation than CON at 50 and 100 min of incubation. In the testis (Figure 5-7), G+4E supplemented bulls had lower ($P < .05$) lipid peroxidation than CON at all times (except 0) and lower ($P < .05$) than GOS fed animals at 100 and 200 min of incubation. Consumption of CSM (gossypol) did not increase in vitro lipid peroxidation above those in the CON treatment in any of the tissues tested. The difference in the degree of protection against lipid peroxidation of the GOS treatment may have been due to different patterns of accumulation of gossypol (liver>heart>testis). Sheriff et al. (1986) found similar results on the ability of gossypol

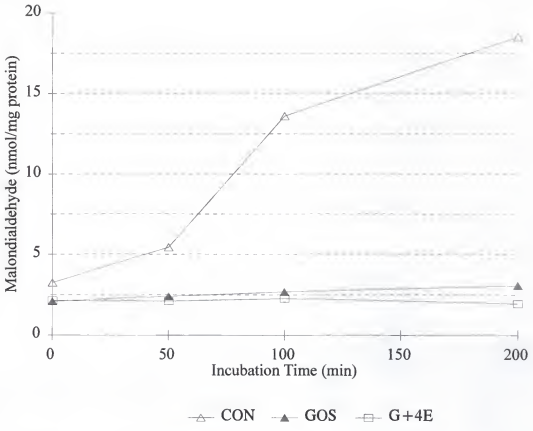

Figure 5-5. Effect of cottonseed meal (CSM) and vitamin E on in vitro stimulated lipid peroxidation of liver homogenates. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each time (T) were: T0, .15; T50, .36; T100, .81; and T200, .80.

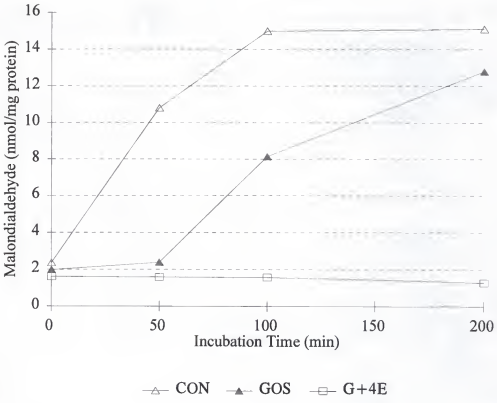

Figure 5-6. Effect of cottonseed meal (CSM) and vitamin E on in vitro stimulated lipid peroxidation of heart homogenates. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each time (T) were: T0, .11; T50, .23; T100, 1.05; and T200, .80.

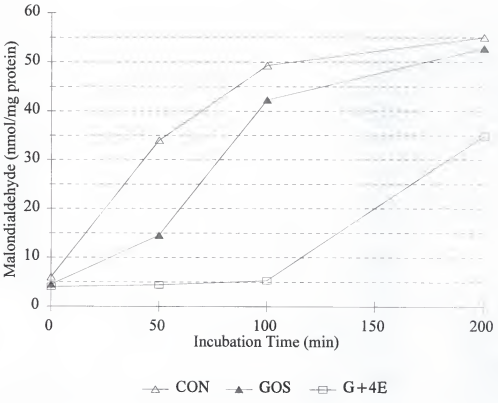

Figure 5-7. Effect of cottonseed meal (CSM) and vitamin E on in vitro stimulated lipid peroxidation of testis homogenates. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each time (T) were: T0, .65; T50, 3.75; T100, 4.10; and T200, 4.10.

to reduce lipid peroxidation in human spermatozoa and erythrocytes. Janero and Burghardt (1988) found that gossypol effectively prevents O_2^- dependant Fe-promoted peroxidation of myocardial membrane phospholipids and could attenuate the kinetics and extent of peroxidation. Furthermore, they suggested that the gossypol antioxidant effect is through scavenging lipid radical intermediates and not by removing Fe from the system through gossypol chelation. de Peyster et al. (1984) found that gossypol when incubated with rat liver microsome and human sperm, promotes the formation of O_2^- thus causing oxidative injury. A possible explanation given to explain these conflicting results is that a metabolic product of gossypol (such as redox-cycling quinone) may be responsible for the ability of gossypol to generate oxygen radicals and cause the side effects associated with gossypol treatment (Janero and Burghardt, 1988; de Peyster et al., 1984). Also, gossypol may promote the formation of reactive oxygen species especially the production of hydrogen peroxide (de Peyster et al., 1984) that could lead to a partial depletion of glutathione as seen in culture rat hepatocytes treated with gossypol (Barhoumi and Burghardt, 1996). Depletion of glutathione may not be accompanied with reduction in α-T concentrations (Anonymous, 1989).

Implications

Cottonseed meal fed to dairy bulls at a concentration provided in this experiment (14 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}) was not detrimental to growth of animals. Tissue gossypol concentration was not affected by vitamin E supplementation nor did gossypol

consumption decrease vitamin E concentration in plasma and (or) tissue. In the testis, concentration of (-) gossypol, the isomer related to antifertility in males, was found in greater concentration than (+)-gossypol.

CHAPTER 6
LONG TERM EFFECTS OF FEEDING COTTONSEED MEAL AND VITAMIN E TO DAIRY BULLS. II. REPRODUCTIVE PARAMETERS

Introduction

Although whole cottonseed (**WCS**), and cottonseed meal (**CSM**) are important sources of protein for ruminants, they contain the toxic polyphenolic pigment gossypol. Gossypol has been found to adversely affect liver function, erythrocyte oxygen carrying or releasing capacity, respiration rate, feed intake and production and reproductive capacity (Lindsey et al., 1980; Calhoun et al., 1990; Gray et al., 1990). Although ruminants seem to have a large capacity to detoxify gossypol, toxicity resulting from consumption of this compound has been observed. Gossypol intake by mature ruminants may overwhelm ruminal detoxification and become absorbed at potentially toxic concentrations (Randel et al., 1992).

In ruminants, gossypol has adversely affected sperm production associated with a damage to the spermatogenic epithelium, leading to reduced germinal cell layers (Arshami and Ruttle, 1988; Chase et al., 1989). Chase et al. (1989) reported delay in age to puberty in Brahman bulls fed 60 mg free gossypol (**FG**) $\cdot$ kg^{-1} BW $\cdot$ d^{-1}, but not when fed 6 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}. After 5 wk of feeding gossypol the percentage of normal spermatozoa was lower in bulls fed 8.2 g FG than in control bulls (Risco et al., 1993).

Bender et al. (1988) found that several antioxidants were reduced by feeding rats high concentrations of gossypol. In dairy cattle, Lane and Stuart (1990) found that feeding high amounts of gossypol decreased plasma α-tocopherol (**α-T**) concentrations indicating a possible relationship between gossypol toxicity and vitamin E.

The present study was designed to evaluate the long term feeding of cottonseed meal and the use of vitamin E to counteract potential gossypol effects on reproductive development of dairy bulls.

Materials and Methods

Animals, diets and management. In a previous phase of the experiment (Chapter 4), the effect of feeding CSM (400 mg FG/kg of diet DM) and supplemental vitamin E on calf performance from 2 wk to six mo of age was tested using four diets based on 1) soybean meal (**SBM**) + 30 IU vitamin E/kg, 2) CSM + 30 IU vitamin E/kg, 3) CSM supplemented with 700 IU vitamin E/kg of diet DM, and 4) CSM supplemented with 1400 IU vitamin E/kg of diet DM. A second phase of this experiment reported herein utilized eight bulls for each of the following dietary treatments (Table 6-1): 1) **CON** a supplement based on SBM + corn + 30 IU vitamin E/kg, 2) **GOS** a supplement based on CSM + corn + 30 IU vitamin E/kg, and 3) **G+4E** a supplement based on CSM + corn + 4,000 IU vitamin E · $bull^{-1}$ · d^{-1}. Supplements GOS and G+4E were formulated to supply 14 mg FG · kg^{-1} BW · d^{-1}. All supplements were isocaloric and isonitrogenous, and satisfied animal requirements for all other nutrients (NRC, 1989). Animals were selected

Table 6-1. Composition of dietary supplements[a]

	CON		GOS		G+4E	
Item	Initial	Final	Initial	Final	Initial	Final
Offered (kg/d)[b]	2.60	5.60	2.70	6.00	2.70	6.00
DM (%)	88.00	88.00	88.00	88.00	88.00	88.00
Ingredient[b]						
SBM (%)	59.0	71.0	----	----	----	----
CSM (%)	----	----	67.00	80.00	67.00	80.00
Corn (%)	38.00	27.50	30.00	18.50	30.00	18.50
Limestone (%)	1.00	.50	1.00	.50	1.00	.50
Minerals (%)	2.00	1.00	2.00	1.00	2.00	1.00
Vit.E (IU/kg)	30.00	30.00	30.00	30.00	1481.50	666.60
Analyses						
(+)-gossypol (%)[c]	.00	.00	.33	.40	.32	.40
(-)-gossypol (%)[c]	.00	.00	.79	.98	.77	.97
Free gossypol (%)[d]	.00	.00	.08	.11	.08	.11
Total gossypol (%)[d]	.00	.00	1.06	1.16	1.06	1.14
Average values[e]						
(+)-gossypol (%)	.00 ± .00		.32 ± .03		.32 ± .03	
(-)-gossypol (%)	.00 ± .00		.79 ± .08		.79 ± .08	
Free gossypol (%)	.00 ± .00		.08 ± .01		.08 ± .01	
Total gossypol (%)	.00 ± .00		1.02 ± .07		1.03 ± .07	
Vitamin A (IU/kg)[f]	2076.61		2759.10		2759.10	
Vitamin E (IU/kg)[b]	38.84 ± 11.55		44.73 ± 13.11		741.42 ± 182.90	
CP (%)[g]	38.59 ± 4.31		37.89 ± 2.03		37.09 ± 2.43	
Ca (%)[g]	.47 ± .17		.48 ± .13		.42 ± .12	
K (%)[g]	1.41 ± .44		1.42 ± .10		1.42 ± .36	
Mg (%)[g]	.22 ± .06		.57 ± .06		.55 ± .10	
P (%)[g]	.71 ± .08		1.12 ± .05		1.11 ± .08	
Cu (mg/kg)[g]	16.67 ± 7.65		15.97 ± 2.60		14.86 ± 3.17	
Zn (mg/kg)[g]	69.33 ± 28.39		73.29 ± 11.77		70.52 ± 13.85	
Mn (mg/kg)[g]	52.61 ± 26.95		36.22 ± 13.51		31.05 ± 13.55	
Fe (mg/kg)[g]	182.59 ± 91.51		161.71 ± 76.54		139.03 ± 40.00	
Se (mg/kg)[g]	.21 ± .11		.20 ± .04		.17 ± .04	

[a]CON = soybean meal (SBM) + corn + 30 IU vitamin E/kg; GOS = cottonseed meal (CSM) + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[b]As fed basis.

[c] As fed. HPLC procedure (Calhoun et al. (1995a), and Kim and Calhoun (1995)). Texas A&M.

[d]As fed. AOACS procedure. Texas A&M.

[e]Mean of 8 mixing ± SD.

[f] As fed. A composited sample from all mixing dates. An IU/kg = Retinol acetate (μg/kg) * 2.91

[g]DM basis.

to represent their previous diets. The bulls were housed in 12 pens, two animals per pen and four pens per treatment, from 6 to 15 mo of age. The diets consisted of the treatment supplement and a free choice low quality hay (vitamin E concentration of <9.0 IU/kg). Supplements were recalculated every month in order to provide 14 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}. The protocol for bull procedures had been approved by the University Animal Use Committee.

Testicular measurements and semen evaluation. Beginning at approximately 12 mo of age and continuing at weekly intervals for nine wk, a breeding soundness evaluation (**BSE**) program was carried out on each bull as described by Chenoweth and Ball, 1980). Semen was analyzed for sperm concentration and motility. Age at puberty was determined when bulls provided an ejaculate containing 50 million sperm with at least 10% showing progressive motility (Wolf et al., 1965). Once sperm production was established, semen was assessed for morphological variation using counts of 200 spermatozoa stained with eosin-nigrosin and wet preparations examined with phase contrast microscopy (Chenoweth et al., 1994). Evaluation consisted of percentage live and normal spermatozoa, and percentage of spermatozoa with primary (proximal droplet, abnormal acrosome, coiled tail, abnormal head, and abnormal midpiece), and secondary (distal cytoplasmic droplet, kinked tail, and detached head) abnormalities. A small amount of semen was added to buffered isotonic formal saline and this preparation was examined for midpiece abnormalities using differential-phase (**DIC**) microscopy employing a Zeiss Axioskop microscope (1000x). In the final semen collection, an aliquot of semen was collected from four bulls per treatment, centrifuged and the seminal

plasma separated and stored for later gossypol analysis following the procedure of Calhoun et al. (1995a).

Before the end of the experiment each bull was administered 100 μg of GnRH intramuscularly. Blood samples were collected 30 min prior to, just before and at 30, 60, 120, 180, 240, and 300 min after GnRH challenge. Plasma was separated by centrifugation and stored at - 20°C for later analysis of testosterone (**T**) by radioimmunoassay (Total testosterone Coat-A-Count® kit from Diagnostic Product Corporation). The intra-assay and inter-assay coefficients of variation were 6.5 and 9.2% respectively, the total binding was 38% and the non specific binding was 1%. Basal concentration of T was calculated as the average of - 30 and 0 min concentrations, maximal concentration was the highest concentration measured after GnRH challenge, and the area under the curve was calculated from 0 to 300 min using the trapezoidal rule (Murdoch and Dunn, 1982).

Libido test. Prior to the beginning and at the end of the BSE, bulls were evaluated for sex-drive according to the technique described by Chenoweth (1983). Briefly, two nonpregnant females of similar size to the bulls were placed in service crates located approximately 5 m apart in a pen. Two bulls were admitted to the pen and observed for 10 min. This test was repeated the following day with, in most cases, different cohorts and restrained females from the previous day. The following sexually-related activities were recorded and timed: investigation, flehmen, licking, chin resting, incomplete mount, complete mount, service and sexual inactivity. The following variables were estimated: number of minutes during the test when sexual inactivity occurred (**NDX**), time to first

mount (**TOM**) and service (**TOS**), number of mounts (**NOM**), and services (**NOS**). Bulls were assigned a score for libido (**LSC**) based on sexual behavior and serving capacity, where 0 represented no sexual interest and 10 represented two services followed by renewed interest. The score then increased by 1 for each subsequent service (Chenoweth et al., 1996).

At the end of the experiment, animals were sacrificed, and the following variables measured: left and right testicular, and epididymal weight, left and right testicular circumference, length, width, and depth. Paired testicular volume (**V**) was calculated as the sum of the volume of the right and the left testicle using the following equation: $V = \pi r^2 h$, where r = (width + depth)/4 and h = length (Fields et al., 1979). Mid-parenchymal portions of the right testicle were excised for sperm production estimation, histopathological and electron microscopy (**EM**) examination.

The portions of testicle for histopathology were fixed in Bouins fixative, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Sections of 5 μ thickness were cut and stained with haematoxylin-eosine and Fe haematoxylin.

The portions for EM were fixed in cold 2% glutaraldehyde in .2 M sodium cacodylate buffer (pH 7.2-7.4) at the slaughter house. At the laboratory testicular tissues were cut in pieces of 1 to 2 mm in size and left in the fixative for an additional hour. They were then washed in buffer (10 min x 6), postfixed in 1% osmium tetroxide for 1 h, washed for 30 min (3 x 10 min), dehydrated through an ethanol-acetone series (25, 50, 75, 100% ethanol, 100% Acetone), and embedded in Spurr's resin. Semen samples from the last collection (5 mL) from each bull were added to tubes containing 1 mL of fixative

as above to stabilize the tissue. In the laboratory the samples were spun down and the pellets resuspended in fixative for 1 h. Pellets were washed in buffer, postfixed in 1% osmium tetroxide (1 h), washed, dehydrated and embedded as were the testes. Thin sections were cut on a RMC MT-6000 XL ultramicrotome, stained with 5% uranyl acetate and lead citrate, then viewed on a Zeiss 10 transmission electron microscope.

Estimates of sperm production were made by counting elongated spermatids and sperm in homogenized tissue as described by Chenoweth et al. (1994). Daily sperm production per g of parenchyma (**DSPG**) and daily sperm production (**DSP**) were estimated using an hematocytometer and the following formula (Chenoweth et al., 1994): DSPG = AX(B+Y)/5.32Y and DSP = DSPG (.99Z) where A = hematocytometer constant, X = hematocytometer count, B = dilution factor, Y = parenchyma sample weight, 5.32 = time divisor for Bos taurus (Amann et al., 1974) and Z = testis parenchyma weight.

Statistical analysis. Data were analyzed by least squares analysis of variance using the GLM procedure of SAS (1988). Testicular and semen characteristics were analyzed as a completely randomized design. Treatment and pen within treatment effects were tested using bull within pen and treatment effect. Pen within treatment effect was removed from the model when it was not significant ($P > .2$). When the pen within treatment effect was kept in the model PROC MIXED (SAS, 1996) was used to calculate the correct standard errors of the least square means for a mixed model. There were two sets of animals with an age difference of 4 wk, therefore age was used as covariate. Due to variability in the numbers of collections per animal, the weight statement of the GLM

procedure of SAS was used for semen characteristics. When the overall treatment effect was significant ($P < .05$) or tended to be significant ($P < .1$), separation of means was done using Duncan multiple range test. Pooled standard errors were reported when all treatments had the same number of replicates, otherwise individual values for each least square mean were given.

Age at puberty was analyzed on the arcsin conversion of the proportion of animals that reached puberty at each collection by treatment. A regression analysis was performed and the 95% confidence intervals of the slope and intercept were compared.

Results and Discussion

Calculated FG intake per animal in this trial ranged from 2.3 g at the beginning of the experiment to 5.3 g at the end. Free gossypol concentrations of approximately 14 mg $\cdot$ kg^{-1} BW $\cdot$ d^{-1} have been found to cause some reproductive problems in the male bovine (Chase et al., 1994; Risco et al., 1993). Data on performance, blood, and tissue responses were discussed in Chapter 5.

Testicular measurements and semen evaluation. Sperm morphology was evaluated using classifications previously described (Chenoweth et al., 1994; Chenoweth et al., 1993). Average percentage of normal and live sperm were lower ($P < .05$) in bulls supplemented with GOS than in bulls fed G+4E and CON (Table 6-2). Risco et al. (1993) found lower percentage of normal sperm in the semen of bulls fed 16.6 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} and defects of sperm midpieces accounted for most of the abnormalities.

Table 6-2. Effect of cottonseed meal (CSM) and vitamin E on semen characteristics of dairy bulls[a]

	Supplement[b]					
Item	CON	SE[c]	GOS	SE	G+4E	SE
No. of bulls	8		6		8	
Motility (%)	63.7[h]	6.9	40.4[i]	9.4	71.0[h]	6.1
Live (%)	42.0[f]	6.0	22.3[g]	6.2	63.7[f]	5.4
Normal (%)	68.3[f]	6.7	29.7[g]	7.0	55.1[f]	6.0
Primary abnormalities[d] (%)	24.4[g]	6.0	59.1[f]	6.0	38.4[g]	5.2
Secondary abnormalities[d] (%)	7.8	2.0	11.1	2.0	6.3	1.8
Abnormal (DIC)[e] (%)	4.1[g]	1.4	12.2[f]	1.4	4.8[g]	1.1

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]Primary = proximal droplet, abnormal acrosome, coiled tail, abnormal head, and abnormal midpiece. Secondary = distal cytoplasmic droplet, kinked tail, and detached head.

[e]Midpiece abnormalities evaluated in isotonic formal saline using differential phase microscopy (**DIC**).

[fg]Means in a row with different superscripts differ $P < .05$.

[hi]Means in a row with different superscripts differ $P < .1$.

Chase et al. (1994) did not find differences in sperm abnormalities when bulls were fed approximately 6 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} from CSM or 60 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} from WCS. Sperm morphology is affected only when greater gossypol concentrations than those used by Chase et al. (1994) are fed and in the form of CSM, since feeding high gossypol diets in the form of WCS has yielded inconsistent results (Cusack and Perry, 1995; Smith et al., 1991; Arshami and Ruttle, 1988). Cusack and Perry (1995) reported that bulls fed 20.4 to 50.8 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} from WCS did not show any sign of reproductive impairment. Similarly Smith et al. (1991) reported that feeding high concentrations of gossypol (64 to 75 FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}) from WCS did not affect sperm characteristics or cause testicular degeneration. In contrast with these two experiments, Arshami and Ruttle (1988) found that WCS, CSM and cottonseed hulls fed to bulls caused testicular histological changes, indicating a detrimental effect on the spermatogenic tissue and associated cells. Several mechanisms have been proposed to explain these inconsistent results. 1) Cusack and Perry (1995) suggested that the feeding of WCS with gossypol chelating agents such as Ca, Na, protein, and trace minerals may have affected the availability of gossypol in his and Smith et al. (1991) studies. 2) Different concentrations of total gossypol and isomers are found in different species of cotton and even within the same species grown in different environments, and 3) Calhoun (1995) suggested that treatment of the WCS (i.e. ammoniation) can modify the pattern of gossypol absorption. Therefore, differences in feeding practices and source of feed may account for different effects among experiments.

The reduction of morphologically normal sperm was accompanied by a reduction ($P < .1$) on the percentage of motile sperm from bulls fed the GOS supplement (Table 6-2). Feeding vitamin E (G+4E) counteracted the negative effect of CSM on the percentage of normal, live, and motile sperm. Chenoweth et al. (1994) found that the feeding of relatively high concentrations of FG to bulls did not adversely affect percentage of live sperm despite a significant reduction in the number of morphologically normal sperm. Although the concentration of FG fed by Chenoweth et al. (1994) was similar to the concentration fed in this experiment, the duration of the trial (10 wk vs 16 mo) may have resulted in a more pronounced effect of gossypol on sperm, since they also observed some differences in sperm motility between the control and the gossypol-treated bulls at the end of the experiment. Reduction in sperm motility has been reported in several animal species treated with gossypol (Randel et al., 1992; Wang et al., 1988). Chenoweth et al. (1994) suggested that motility may be impaired by structural damage to the sperm midpiece in animals treated with gossypol.

Primary abnormalities were found to be greater ($P < .05$) in bulls fed the GOS supplement than in any other treatment, while secondary abnormalities were not affected ($P > .1$) by treatment (Table 6-2). Primary abnormalities have not been affected in bulls fed CSM or WCS in previous studies (Chase et al, 1994; Jimenez et al., 1989). All of the primary defects considered in this experiment are found in the classification of Blom (1973) as major morphological defects which have been correlated with impaired fertility. Such sperm defects also have been associated with disturbed spermatogenesis (Barth and Oko, 1989).

Percentage of abnormal midpieces, as evaluated with differential phase microscopy was greater ($P < .05$) in GOS fed bulls than in the other two treatments (Table 6-2). Although supplementation affected midpiece abnormalities, the percentage of abnormal midpieces was lower than those found by Chenoweth et al. (1994). In this study, the defect in the midpiece (segmental aplasia) was not consistent as reported by Chenoweth et al. (1994) in Brahman bulls or by Oko and Hrudka (1982) in gossypol-treated rats. However, electron microscopy from a bull sperm in the GOS treatment in this study revealed gaps in the mitochondrial helix of the midpiece (Figure 6-1) as reported in rats (Oko and Hrudka, 1982). This sperm defect has been associated with malformations of the mitochondrial sheath which are induced in late spermatogenesis (Chenoweth et al., 1994). These gaps in the mitochondria helix seem to be caused specifically by gossypol and they are observed from late-stage spermatids through epididymal spermatozoa. Also, Randel et al. (1992) suggested that this defect may be responsible for the observed sperm motility reduction in animals treated with gossypol. Electron microscopy of the testis revealed that extensive mitochondrial damage had occurred in the testis of bulls supplemented with GOS (Figure 6-2), presented as a loss of cristae and extensive vacuolization. Similar damage has been seen in tumor cells cultured with (-)-gossypol (Benz et al., 1990) and in the testis of gossypol treated rats (20 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}) (Oko and Hrudka, 1982). Histological examination of the testes revealed that 6 of 8 bulls supplemented with GOS had irregular and degenerative germ cell layers that also showed vacuolation and reduced spermatid numbers. Vitamin E supplemented

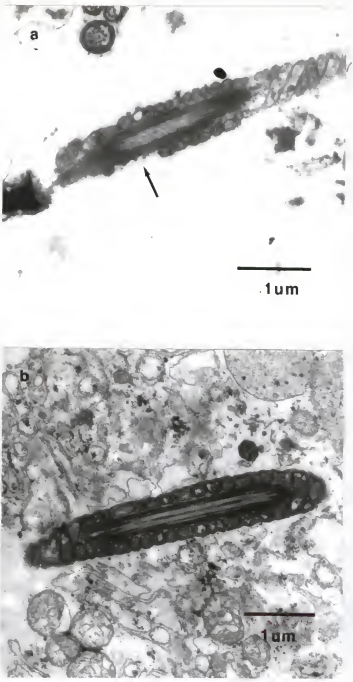

Figure 6-1. Electron micrographs of bull sperm a) supplemented with cottonseed meal (GOS) revealing gaps in the mitochondria helix of the midpiece (magnification 19,430), and b) supplemented with soybean meal (CON) (magnification 18,750).

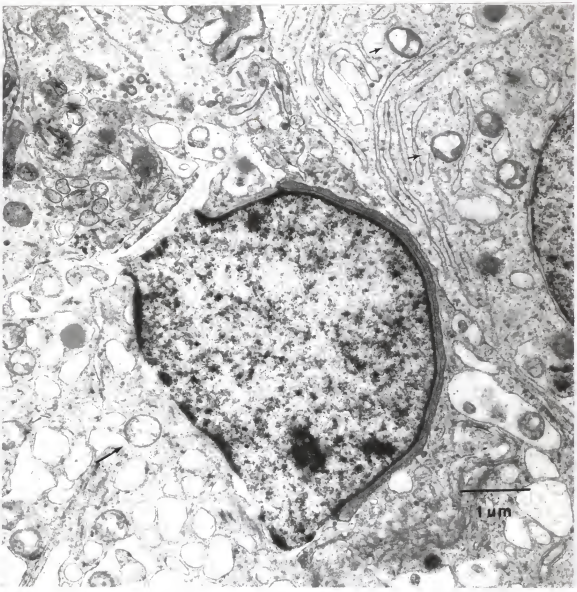

Figure 6-2. Electron micrograph of the testicle of a bull supplemented with cottonseed meal (GOS) revealing extensive vacuolization of mitochondria and loss of cristae. Magnification 18,200.

bulls (G+4E) had less histological damage, with only one animal presenting vacuolization and reduced spermatid numbers.

Several hypotheses have been proposed to explain the detrimental effect of gossypol on male reproduction. 1) Gossypol inhibited the mitochondrial respiratory chain in testicles (Reyes et al., 1988) which could impair sperm energy metabolism; 2) Gossypol binds to proteins and amino acids, therefore potentially affecting synthesis of proteins and their incorporation into spermatocytes and spermatids. Among others, histidine has been found to be involved in the metabolism of gossypol (Javed and Waqar, 1995); 3) Gossypol can alter the properties of lipid membranes which can modify protein function and structure (Cuellar and Ramirez, 1993); 4) Structural integrity of many sperm structures depend on S-S cross linked polypeptides. Disturbance of this bond may be associated with damage to the midpiece mitochondria (Baccetti et al., 1986). Mann and Mann (1981) reported that motility and metabolism of the sperm depends on interchange reactions between S-H and S-S groups. Gossypol may prevent the oxidation of S-H groups by chelating minerals or proteins responsible for this reaction (Chenoweth, P. J. Personal communication); and 5) gossypol has been found to inhibit the generation of free radicals in some studies (Janero and Burghardt, 1988) and to stimulate it in others (de Peyster et al., 1984). Recently, Barhoumi and Burghardt (1996) reported that gossypol promoted the formation of reactive oxygen species and the depletion of glutathione in rat hepatocytes. Bender et al. (1988) reported a reduction in antioxidants in the testes of rats fed gossypol. Damage due to lipid peroxidation in human sperm has been associated

with loss of sperm motility, inactivation of enzymes, and loss of membrane integrity (Aitken and Fisher, 1994).

Daily sperm production per g of parenchyma (10^6/g testicular parenchyma) was reduced ($P < .05$) in animals supplemented with GOS (Table 6-3). The same trend was observed for DSP. Vitamin E supplementation (G+4E) counteracted ($P < .05$) the reduction in sperm production caused by gossypol in CSM. In bulls, gossypol caused damage to the spermatogenic epithelium which reduced the number of germinal cell layers without reducing tubule diameter (Chase et al., 1990). Chenoweth et al. (1994) found lower DSPG and DSP in bulls fed gossypol than in bulls receiving a diet without gossypol. The depression of spermatogenesis is a known effect of gossypol on male reproduction. Randel et al. (1992) suggested that a decrease in sperm production in ruminants fed gossypol could be caused by damage to germ cells contained within the germinal epithelium. Damage to Sertoli cells also may be involved with decreased spermatogenesis.

In this experiment (Chapter 5, Table 5-6), gossypol did not affect testicular concentrations of Cu, Fe, and Zn which were fed within required concentrations. However, it does not imply that they were not chelated by gossypol and therefore unavailable for normal metabolism.

In this study, feeding vitamin E to bulls receiving 14 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1} increased the percentage of normal sperm, percentage motility, DSPG, and DSP and decreased the percentage of primary and midpiece (DIC) abnormalities. Vitamin E may have reduced the negative effect of gossypol on these parameters by reducing the damage

Table 6-3. Effect of cottonseed meal (CSM) and vitamin E on sperm production of dairy bulls[a]

	Supplement[b]			
Item	CON	GOS	G+4E	SE
DSPG[d] ($x10^6$/g)	14.6[f]	10.2[g]	17.6[f]	1.0
DSP[e] ($x10^9$)	3.2[f]	2.2[g]	4.1[f]	.3

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · $animal^{-1}$ · d^{-1}.

[c]Standard error of the least square means.

[d]Daily sperm production per g of parenchyma.

[e]Daily sperm production.

[fg]Means in a row with different superscripts differ $P < .05$.

of reactive species of oxygen or by sparing the effect of enzymes involved in the antioxidant system of the testicle. The effect of vitamin E in protecting the sperm membrane against lipid peroxidation may be more important in late spermiogenesis when sperm discard most of their cytoplasm leading to lowered concentrations of cytoplasmic defensive enzymes (Aitken and Fisher, 1994). The effect of gossypol on the spermatogenic epithelium may not be related to lipid peroxidation since in vitro testicular lipid peroxidation was lower in animals fed CSM than CON, and feeding gossypol did not reduce α-T concentration in testicles (Chapter 5). However, depletion of glutathione as well as direct structural damage to the mitochondrial membrane could be caused by gossypol. We could not explain the fact that only bulls in the GOS treatment in this experiment had detectable concentrations of gossypol in seminal plasma (from .23 to 4.32 μg/mL) but this may be related to the observed beneficial effect of vitamin E supplementation on reproductive parameters of bulls fed gossypol-containing feedstuffs.

Table 6-4 shows the effect of the treatments on testicular characteristics of bulls. Paired testicular and epididymal weights, paired testicular volume and scrotal circumference were not affected by supplementation ($P > .1$). However, animals supplemented with G+4E showed the highest and those fed GOS the lowest testicular weights and volumes. Chenoweth et al. (1994) found a similar trend where gossypol fed bulls had the lowest testicular volumes and weights, but failed to find statistical differences. These results are consistent with studies which did not find any effect of gossypol on scrotal circumference and testicular weights (Chase et al., 1994; Chenoweth et al., 1994; Jimenez et al., 1989). The apparent damage of gossypol to spermatogenic

Table 6-4. Effect of cottonseed meal (CSM) and vitamin E on testicular characteristics of dairy bulls[a]

	Supplement[b]			
Item	CON	GOS	G+4E	SE[c]
Paired testicle weight (g)	494.9	490.0	563.5	37.0
Paired testicle volume (cm^3)	706.8	657.4	747.8	45.0
Paired epididymal weight (g)	48.3	41.8	50.9	3.1
Scrotal circunference (cm)	32.4	31.2	31.4	.8

[a]Least square means.
[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · $animal^{-1}$ · d^{-1}.
[c]Standard error of the least square means.

epithelium has not been associated with reduction of scrotal circumference in the bovine. This could be the result of a reduction in layers of the spermatogenic epithelium without corresponding changes in tubule diameter (Chenoweth et al., 1994).

Percentage of animals that reached puberty (production of 50 X 10^6 sperm with at least 10% motility) is shown in Figure 6-3. During the experimental period, four bulls supplemented with GOS did not produce enough semen to be classified as pubertal. Vitamin E feeding decreased ($P < .05$) and CSM feeding without supplemental vitamin E increased ($P < .05$) age at sexual maturity in this study.

Results of the libido tests are presented in Table 6-5. Bulls fed GOS exhibited more sexual inactivity ($P < .05$) at the first test than bulls in other treatments. Vitamin E supplementation to bulls receiving gossypol (G+4E) improved NOM in the first test and TOS in the second test. There was a trend of gossypol (GOS) to decrease and vitamin E (G+4E) to improve LSC. The lower results of GOS group first test may indicate a relative lack of sexual maturity which agrees with sperm production data. At time of first test (12 mo of age), none of GOS, two of CON, and six of G+4E bulls had reached puberty as per experimental protocol (ejaculate with 50 x 10^6 sperm and at least 10% motility). The effect of vitamin E supplementation on some measurements of sexual behavior may be related to its antioxidant properties and its accumulation in tissues with high steroidogenic activity and thus high reactive oxygen species production such as testicular tissue. Taylor et al. (1991) reported that feeding gossypol to male rats (from 5 to 20 mg gossypol $\cdot$ kg^{-1} BW $\cdot$ d^{-1}) for 11 wks provoked a dose-response decline in sexual motivation. Even at a low dosage (5 mg gossypol $\cdot$ kg^{-1} BW $\cdot$ d^{-1}), males eventually

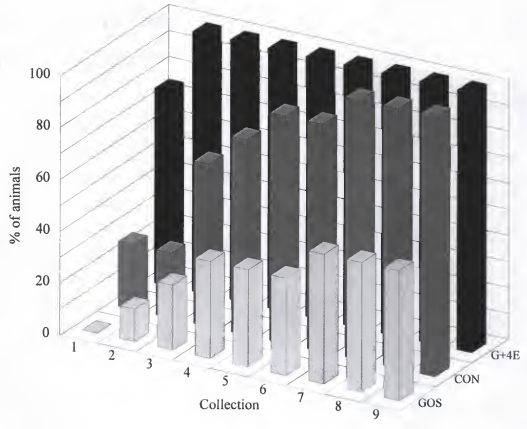

Figure 6-3. Effect of cottonseed meal (CSM) and vitamin E on the percentage of bulls that reached puberty at each semen collection. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

Table 6-5. Effect of cottonseed meal (CSM) and vitamin E on sex drive performance of dairy bulls during two libido tests[a]

		Supplement[b]					
Item[c]	Test	CON	SE[d]	GOS	SE	G+4E	SE
LSC	1	7.9	1.2	6.8	1.0	9.1	1.1
	2	10.4	.7	8.9	.5	10.0	.6
NOM	1	5.7[ef]	1.2	3.2[f]	1.1	6.3[e]	1.2
	2	9.4	1.3	9.5	.9	7.9	1.1
NOS	1	1.4	.5	1.4	.5	1.9	.5
	2	2.4	.4	1.7	.4	2.3	.4
TOM (sec)	1	156.1	60.0	224.5	57.0	135.0	57.0
	2	38.0[e]	7.0	29.0[ef]	5.0	21.0[f]	5.0
TOS (sec)	1	222.8	94.2	231.6	69.2	205.7	69.2
	2	154.4[ef]	71.4	212.8[e]	52.4	69.2[f]	59.8
NDX (min)	1	1.2[h]	.8	3.9[g]	.7	1.7[h]	.8
	2	.1	.2	.1	.1	.2	.2

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]LSC = libido score; NOM = number of mounts; NOS = number of services; TOM = time to first mount; TOS = time to first service; NDX= sexual inaction time (min). Ten minutes allowed per test.

[d]Standard error of the least square means.

[ef]Means in a row with different superscripts differ $P < .1$.

[gh]Means in a row with different superscripts differ $P < .05$.

showed signs of losing interest in sexually receptive females over weeks of administration. Authors suggested that the likely mechanism for the behavioral changes is a gradual suppression of serum testosterone. de Peyster and Srebnik (1988) found that rats administered 10 mg gossypol $\cdot$ kg^{-1} BW $\cdot$ d^{-1} subcutaneously every 5 d had reduced serum testosterone concentrations and decreased accessory organ weights. Possible explanations for these results were that gossypol may cause a direct suppression of steroidogenic enzymes, alteration of cAMP second-messenger function, or interference with cell membrane properties which could result in a disruption of normal receptors in the interstitial cell membrane (de Peyster and Srebnik, 1988).

Chase et al. (1994) reported that bulls fed gossypol (6 or 60 mg FG $\cdot$ kg^{-1} BW $\cdot$ d^{-1}) from CSM and WCS did not show treatment differences in the basal mean or maximal, number of pulses or total T released during 6 h of blood sampling. Likewise, in this experiment, neither the maximal GnRH-induced concentration of T nor the area under the GnRH-induced T curve differed ($P > .1$) among treatments (Table 6-6 and Figure 6-4). There was a trend for gossypol to reduce and vitamin E to increase T production after GnRH challenge. Lin et al. (1980) reported that rat Leydig cells cultured with 10^{-5} and 10^{-7} M of gossypol had reduced LH stimulated production of T. In canine testicular interstitial cells, Mushtaq et al. (1996) reported that gossypol inhibited hCG-stimulated T production and suggested that gossypol may interfere with hCG binding to its receptor and(or) subsequent signal transduction or hCG-dependent enzymatic activity.

Vitamin E effect on the metabolism of gossypol has not yet been investigated. However, vitamin E seemed to confer protection against the detrimental effects of

Table 6-6. Effect of cottonseed meal (CSM) and vitamin E on testosterone concentration in plasma of bulls after a GnRH challenge[a]

	Supplement[b]			
Measurement	CON	GOS	G+4E	SE[c]
Basal (ng/mL)	.64	.47	.64	.15
Maximal[d] (ng/mL)	9.74	8.42	10.60	.90
AUC[e]	2176.77	1933.14	2457.64	213.50

[a]Least square means.

[b]CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}.

[c]Standard error of the least square means.

[d]GnRH induced.

[e]Area under the GnRH induced testosterone curve (ng · mL^{-1} · min^{-1})

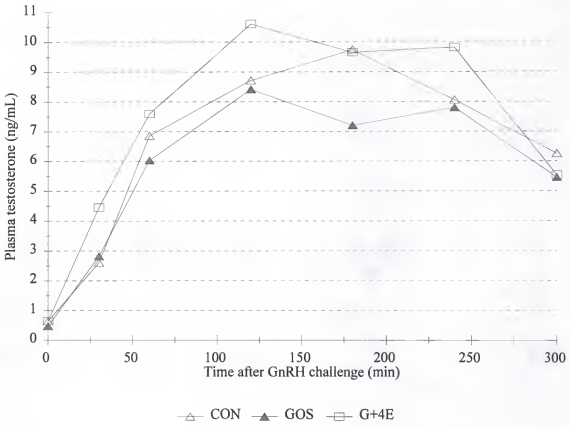

Figure 6-4. Effect of cottonseed meal (CSM) and vitamin E on plasma testosterone concentration after a GnRH challenge. Supplements were: CON = soybean meal + corn + 30 IU vitamin E/kg; GOS = CSM + corn + 30 IU vitamin E/kg; G+4E = CSM + corn + 4,000 IU vitamin E · animal^{-1} · d^{-1}. Standard errors for each time (T) were: T0, .15; T30, .64; T60, .73; T120, .92; T180, .99; T240, 1.08; and T300, 1.00.

gossypol on sperm production and quality. The possible mechanism involved may be related to protection against reactive oxygen species produced by gossypol, or by sparing the effect of other antioxidants affected directly by gossypol (i.e. GSH), or by direct physical interaction of gossypol or its metabolites with α-T in lipid membranes. The latter supposition is supported by the fact that no gossypol was present in seminal plasma of G+4E animals.

Implications

Cottonseed meal fed to Holstein bulls at a concentration to provide 14 mg FG · kg^{-1} BW · d^{-1} from 6 mo to 16 mo of age resulted in increased sperm abnormalities, decreased sperm production, and adversely affected some aspects of sexual behavior. Vitamin E feeding at a concentration of 4,000 IU · h^{-1} · d^{-1} reversed the negative effects of gossypol on reproductive measurements.

CHAPTER 7
SUMMARY AND CONCLUSIONS

By-products of the cotton fiber and cottonseed oil industry such as whole cottonseed and cottonseed meal (**CSM**) are important sources of economical protein and energy in livestock rations. Gossypol [(2,2'-binaphthalene)-8,8'-dicarboxaldehyde-1,1'6,6',7,7'-hexahydroxy-5,5'-diisopropyl-3,3'-dimethyl] is a yellow polyphenolic pigment found in cottonseed. This pigment is recognized to be toxic to nonruminants and at high enough concentrations, to ruminants, limiting the use of whole cottonseed and its by-products as feed to livestock.

There are few data showing the effect of supplemental vitamin E in counteracting gossypol toxicity. At the University of Florida, a series of experiments were undertaken to evaluate the value of supplemental vitamin E on the physiological response to CSM (gossypol) fed to ruminants and young ruminants prior to rumen development.

Experiment 1 was conducted for 112 d with yearling beef heifers to evaluate the effects of feeding CSM with different concentrations of vitamin E on hematological and tissue components. Thirty-two yearling beef heifers (n=8) were assigned randomly to four dietary treatments: 1) **CON** a supplement based on soybean meal (**SBM**) + corn + 30 IU vitamin E/kg; 2) **GOS** a supplement based on CSM + corn + 30 IU vitamin E/kg; 3) **G+2E** a supplement based on CSM + corn + 2,000 IU vitamin E animal^{-1} · d^{-1}; and 4)

G+4E a supplement based on CSM + corn + 4,000 IU vitamin E animal^{-1} · d^{-1}. Cottonseed meal was fed to provide 4 g of free gossypol (**FG**) animal^{-1} · d^{-1}. Blood samples were collected at the start of the experiment and every two wk thereafter up to 16 wk. There was a time x treatment interaction ($P < .01$) for plasma **α-tocopherol** (**α-T**) concentration; however, feeding gossypol did not reduce plasma **α-T**. Weight gain, retinol palmitate, retinol, **β-carotene** (**β-C**), hemoglobin and hematocrit were not affected by treatment. Erythrocyte osmotic fragility (**EOF**) was increased ($P < .05$) in gossypol fed animals; however, vitamin E supplementation lowered EOF. Heifers fed supplements GOS, G+2E, and G+4E had greater ($P < .01$) (-), (+), and total gossypol than CON from collection 2 (after two wk of feeding the supplements) up to the end of the experiment. There was a treatment effect ($P < .05$) on vitamin E and gossypol concentrations in different tissues, while not significant ($P > .05$) for trace minerals (Cu, Zn, Fe, and Se). Vitamin E concentration in tissue increased with increased dietary supplementation. In heart and neck muscle (-)-gossypol was greater ($P < .05$) than (+)-gossypol, while the reverse was true for liver. Gossypol decreased in vitro lipid peroxidation of liver homogenates and did not have any effect on vitamin concentration of tissue. Gossypol pattern of disposition in tissue was liver > heart > muscle.

In experiment 2, newborn male Holstein calves were used to test the effect of 400 mg FG/kg of feed DM and to determine if vitamin E supplementation could counteract gossypol toxicity. The experiment was divided in two phases: 1) individual feeding, and 2) group feeding. The treatments were as follow: **CON**: SBM based starter; **GOS**: CSM based starter; **G+2E**: CSM based starter + 2,000 IU vitamin E · calf^{-1} · d^{-1}; and **G+4E**:

CSM based starter + 4,000 IU vitamin E · $calf^{-1}$ · d^{-1}. Vitamin E (G+4E) improved ($P < .05$) weight gain and feed intake over CON in most periods. Plasma gossypol concentrations were greater ($P < .05$) in calves receiving CSM than in CON calves; however, there was no difference among those calves which received CSM. Hemoglobin and hematocrit were decreased ($P < .05$) in GOS fed calves, whereas vitamin E supplementation counteracted ($P < .05$) this effect. Plasma **α**-T concentration was not affected ($P > .1$) by gossypol intake and followed the supplementation pattern. During the experimental period, 10 calves died, 6 from the GOS and 2 each from the G+2E and G+4E treatments. Here, necropsy findings from some calves were compatible with gossypol toxicity. Histopathological examination revealed centrilobular necrosis in the liver and atrophy and vacuolation of cardiocytes.

Experiment 3 was conducted to determine the effect of long term feeding of CSM on hematological, tissue and reproductive parameters of Holstein bulls. Twenty four Holstein bulls, approximately 6 mo of age were placed on the following dietary treatments: 1) **CON** a supplement based on SBM + corn + 30 IU vitamin E/kg , 2) **GOS** a supplement based on CSM + corn + 30 IU vitamin E/kg, and 3) **G+4E** a supplement based on CSM + corn + 4,000 IU vitamin E · $bull^{-1}$ · d^{-1}. Cottonseed meal based diets were formulated to supply 14 mg FG · kg^{-1} BW · d^{-1}. These animals had been in experiment 2 which evaluated the effect of feeding the same type of diets but from one wk to 6 mo of age. Blood and weight were collected every mo for a total of 10 collections. Animal ADG, total gain, and final weights were not affected by treatment. Erythrocyte osmotic fragility was greater ($P < .01$) in bulls fed GOS and G+4E compared

to the CON treatment. Feeding 4,000 IU vitamin E · $bull^{-1}$ · d^{-1} did not reduce the increase in EOF caused by gossypol as in experiment 1. The CON supplemented bulls had greater ($P < .05$) hematocrit percentage than the bulls fed GOS in collections 1, 2, 4, and 5, and tended to be greater ($P < .1$) in collections 7 and 10. Animals receiving G+4E had greater ($P < .05$) plasma α-T concentration than animals in the CON and GOS treatments in all collections, and animals in GOS treatment had greater ($P < .05$) plasma α-T concentration than animals in CON in all collections except 5 and 6. Plasma (-)-, (+)-, and total gossypol concentrations in bulls fed GOS and G+4E were greater ($P < .05$) than CON fed bulls from the initial sampling to the end of the experiment, and were similar ($P > .1$) among treatments receiving CSM. Percentage motility, normal, and live sperm, and daily sperm production were lower ($P < .1$) in bulls fed GOS than in the other two treatments. Percentage of primary abnormalities and abnormal midpieces were greater ($P < .05$) in the GOS supplemented bulls than in the other two treatments. At 12 and 16 mo of age bulls were given two assessments for sex-drive traits. Bulls receiving gossypol (GOS) exhibited less sexual activity ($P < .05$) at the first test than bulls in other treatments. Vitamin E supplementation to bulls receiving gossypol improved the number of mounts in the first test and time to first service in the second test. There was a trend for gossypol to decrease and vitamin E to improve libido score. The results of GOS first test may indicate a lack of sexual maturity of bulls fed the GOS supplement which agrees with sperm production data. At time of first test (12 mo of age) none of GOS, two of CON, and six of G+4E bulls had reached puberty as per experimental protocol. Vitamin E supplementation did not affect plasma or tissue gossypol concentration. Cottonseed

meal seemed to aid in the absorption and deposition of α-T in animals fed a normal concentration of vitamin E. Gossypol pattern of disposition in tissue was liver > heart > testis. In vitro lipid peroxidation of tissue indicates that gossypol acts as an antioxidant in lipid peroxidation systems and its role as an antioxidant may be dose or tissue dependant. Vitamin E supplementation reduced lipid peroxidation in heart and testis below the level of the other two treatments. Feeding CSM to dairy bulls did not affect growth, nor did it affect vitamin E status. Gossypol negatively affected some reproductive measurements of Holstein bulls; however vitamin E supplementation counteracted the detrimental effect of gossypol.

In conclusion, in experiment 1, gossypol fed at 4 g FG · animal^{-1} · d^{-1} at low concentration of vitamin E supplementation (30 mg/kg of feed) did not decrease concentrations of antioxidant vitamins, including α-T, vitamin A and β-C or have any detrimental effect on performance of beef heifers. In experiment 2, CSM fed at a concentration to provide 400 mg FG/kg of diet DM resulted in death of 10 calves with necropsy findings similar to those reported for fatally intoxicated preruminants. Vitamin E supplementation at high concentrations increased feed intake and weight gain and may have conferred some protection upon calves against gossypol toxicity. In experiment 3, CSM fed to dairy bulls at a concentration provided in this experiment (14 mg FG · kg^{-1} BW · d^{-1}) was not detrimental to growth of animals. Tissue gossypol concentration was not affected by vitamin E supplementation, nor did gossypol consumption decreased vitamin E concentration in plasma and (or) tissue. In the testis, concentration of (-) gossypol, the isomer related to antifertility in males, was found in greater concentration

than (+)-gossypol. Gossypol increased sperm abnormalities, decreased sperm production, and adversely affected some aspects of sexual behavior. Vitamin E feeding at a concentration of 4,000 IU $\cdot h^{-1} \cdot d^{-1}$ reversed the negative effect of gossypol on selected reproductive measurements.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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