Characterizing Compensatory Effects of Silymarin on Gossypol Toxicosis in Lines of Chickens Divergently Selected for Humoral Immune Response

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ABSTRACT

Feed costs are approximately 70% of total production cost for poultry producers. Poultry diets in the United States generally consist of 2 grains: corn and soybean meal. In recent years, the cost of these grains has dramatically increased. Due to these price increases, producers seek alternative feeds that provide adequate nutrition, and are also more affordable than "traditional" grains. Cottonseed meal is one alternative that is both affordable and an excellent source of crude protein. However, cottonseed meal contains gossypol, a pigment toxic to chickens.

This study had two main objectives. The first objective was to determine if silymarin, an extract from milk thistle, could offset or prevent gossypol toxicosis. The second objective was to determine if divergent selection for humoral immune response would have an impact on the ability of the chicken to cope with gossypol toxicosis. Two preliminary studies were conducted. One determined basal activities of liver detoxification enzymes at various ages. The other determined concentrations of gossypol and silymarin that should be added to the diet to elicit a response. The information gathered from the second preliminary study was used to conduct the final experiment.

In the final experiment, chickens from each of 2 lines selected for humoral immunity were exposed to diets containing gossypol, silymarin, gossypol and silymarin, and a control. Humoral immunity had no impact on the ability of the chicken to cope with gossypol toxicosis. Silymarin did not alleviate gossypol toxicosis. Future studies will focus on using a lower gossypol concentration in the diet.

Key words: chicken, gossypol, silymarin, toxicosis

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DEDICATION

To my parents, Phil and Gloria Blevins.

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LIST OF ABBREVIATIONS

4-MU	4-methylumbelliferone
7-BQ	7-benzyloxyquinoline
ANOVA	analysis of variance
AST	aspartate aminotransferase
bu	bushel
BW	body weight
C	control
CP	crude protein
CSM	cottonseed meal
CYP450	cytochromes P450
CYP450 3A4	cytochrome P450 3A4
d	day
df	degrees of freedom
DM	dry matter
G	gossypol
g	Gram
g	gravity
GLM	general linear model
GGT	gamma glutamyltransferase
G-S	gossypol and silymarin combined
GSH	glutathione
GST	glutathione-S-transferase
h	hour
HA	high antibody
HG	high gossypol
HS	high silymarin
i.v.	intravenous
LA	low antibody
LG	low gossypol
LS	low silymarin
m	meter
M	molar
ME	metabolizable energy
min	minute
mo	month
NADH	reduced nicotinamide adenine dinucleotide
nm	nanometers
QR	quinone reductase
ROS	reactive oxygen species
S	silymarin
SBM	soybean meal
SEM	standard error of the mean
SRBC	sheep red blood cells
wk	week

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INTRODUCTION

Corn prices have increased substantially in recent years. Stillman et al. (2009) reported that feed prices doubled from 2006 to 2008. Corn prices averaged about $5/bu in 2008 compared to 3 years ago when corn averaged just over $1/bu (Stillman et al., 2009). This escalation in corn price is, in large part, a result of increased corn-based ethanol production. Poultry diets in the United States are generally a mixture of corn and soybean meal with the majority of that mixture being corn. Feed costs are approximately 70% of total costs of poultry production. Therefore, poultry companies have resorted to shutting down processing plants as well as terminating less profitable products in an attempt to deal with rising feed cost of feed (Thornton, 2008).

Cottonseed meal (**CSM**) is a practical alternative feed. It is a good source of protein (Nagalakshmi et al., 2007; Rahman et al., 2001). Although CSM could not replace corn due to its inadequate energy content, it is a sensible substitute for soybean meal (**SBM**). Cottonseed meal is similar to soybean meal as a protein source (45% CP vs. 49% CP) (Bath et al., 1995). As of January 20, 2009, CSM prices averaged $0.26/kg ($235/ton); while SBM prices averaged $0.35/kg ($319/ton) (USDA Market News Service, 2009). This could reduce the amount of SBM fed and thus reduce total feed cost. However, feeding CSM to chickens has proven difficult largely due to gossypol, a pigment produced by cotton.

Gossypol in chicken diets is associated with growth depression, lameness, decreased egg size and hatchability, and discoloration of yolk and albumen (Nagalakshmi et al., 2007). Also, chickens vary genetically in their ability to handle the gossypol toxin (Lordelo et al., 2007). Although several strategies to reduce gossypol content in CSM have been tested, most have had limited success.

An alternative strategy would be to develop a feed additive that protects the chicken from harmful effects of gossypol rather than remove gossypol from feed. Gossypol accumulates in the chicken's liver (Lordelo et al., 2004), the main detoxification organ in the body. Incorporation in the diet of a compound that induces liver enzyme activity could help rid the body of gossypol, thereby reducing or even eliminating its toxic effects.

Chapter 1: Review of Literature

Liver and Its Enzymes

The liver is arguably the most important organ in the body. It performs over 500 functions essential to the maintenance of homeostasis including bile production, plasma protein production, conversion of glucose to glycogen for storage, and regulation of blood clotting. One of the most important functions of the liver is the ability to detoxify harmful or toxic substances. The liver can convert dangerous toxicants into benign metabolites which can be excreted through the bile and/or urine.

Liver metabolism of toxins occurs in two phases (Ioannides, 2002). Briefly, phase I metabolism is a chemical reaction involving the addition of a functional group (e.g., hydroxyl group, carboxyl group) to a chemical. Addition of this functional group causes the chemical to become more reactive with other compounds so that it may become more polar. This is accomplished by phase II metabolism. During phase II, the chemically-modified toxicant is conjugated with endogenous substrates such as glucuronic acid and sulfate (Ioannides, 2002). Addition of these endogenous substrates results in highly polar compounds, which can easily be excreted through the bile and urine.

Enzymes that will be analyzed in this study are described below. They were chosen because of their vital role in detoxification. Furthermore, the chemical structures of gossypol and silymarin necessitate that some, if not all of these, pathways could be utilized.

Phase I Enzymes

Cytochromes P450. The discovery of cytochromes P450 (**CYP450**) was first reported in 1958. At first, it was thought only to be a single enzyme; however, further research proved that CYP450 are a superfamily of heme proteins that can contribute either to the detoxification or the activation of a toxicant (Vaz, 2001; Lewis, 2003). They are found in most, if not all, living organisms from bacteria to mammals. Cytochrome P450 3A4 (**CYP 3A4**) is the main cytochrome responsible for drug and exogenous chemical metabolism (Anzenbacher and Anzenbacherova, 2001). Thus, the cytochrome of main focus in this study will be CYP3A4.

Cytochrome P450 3A4 oxidizes its substrate, producing a monooxygenated product and a molecule of water (Anzenbacher and Anzenbacherova, 2001). This produces a highly reactive intermediate molecule that can proceed to phase II metabolism for further detoxification (Lorr

and Bloom, 1986). There are a wide variety of CYP3A4 substrates including many compounds commonly introduced into the body such as acetaminophen (Anzenbacher and Anzenbacherova, 2001). Cytochromes may produce harmful free radicals, which can oxidize components of the cell and compromise cell integrity. Thus, the CYP450 pathway can be harmful or helpful to the body depending on the substrate in question (Vignati et al., 2005).

Because intermediates of phase I metabolism can proceed to phase II metabolism, a study of phase I enzymes alone is inadequate. Furthermore, considerable genetic variation is associated with cytrochrome activity. Vignati et al. (2005) reported that over 60% of the variation in CYP3A activity was associated with genetic factors, suggesting that in addition to study of enzyme activity (i.e., phenotypic), genotypic evaluation should also be considered.

Phase II Enzymes

Glutathione-S-transferase. One of the most important phase II metabolic pathways is glutathione conjugation. Some toxicants after undergoing phase I metabolism become more electrophilic or electron rich. This allows the toxicant to be easily attacked by the nucleophilic (electron deficient) glutathione. Specifically, the sulfhydryl group of glutathione is conjugated to the toxicant, providing the polarity needed for excretion (Ioannides, 2002). In a process that many scientists consider phase III liver metabolism, the glutathione conjugate is converted into a mercapturic acid derivative and excreted from the body (Thurman et al., 1994). These derivatives are usually excreted in the bile because of their high molecular weight (Ioannides, 2002).

Glutathione is an endogenous peptide. Cells of the liver and other organs contain high concentrations of glutathione. Glutathione conjugation can occur spontaneously, but occurs more quickly when catalyzed by the enzyme glutathione-S-transferase (**GST**). This enzyme is found predominantly in the cytosol of hepatocytes, but can also be found, to a lesser degree, in the endoplasmic reticulum (Ioannides, 2002). Glutathione-S-transferases constitute about 10% of the soluble protein in the liver (Rana and Taketa, 1997).

Quinone Reductase. Quinones are highly electrophilic metabolites of molecules such as benzenes, phenols, and other aromatics. Quinones are also dietary constituents of plants. Quinones are harmful because they form semiquinones during metabolism. Semiquinones are

reactive oxygen species (**ROS**) and can cause considerable oxidative damage to the body (Talalay and Dinkova-Kostova, 2004).

As a cytosolic liver enzyme, quinone reductase (**QR**) catalyzes the reduction of quinones to hydroquinones. This prevents the production of semiquinones and thereby protects the body from oxidative damage that can be caused by quinones. Hydroquinones that are formed are then subject to glucuronidation. Although QR differs from traditional phase II enzymes in that no endogenous substrate is conjugated to the xenobiotic, it is categorized as such.

Diagnosis of Chemically-Induced Liver Injury

Both acute and chronic exposure to toxicants causes morphologic changes to liver tissue including lipid accumulation, cellular necrosis, and, possibly, neoplasia (Plaa and Charbonneau, 2008). Diagnosis of these conditions requires microscopic observation of tissue. Unfortunately, this requires killing the chicken and thus is not helpful for monitoring liver health. However, there are less destructive procedures that can be used. When liver cells are damaged, they release their contents into the blood stream. These contents include hepatic enzymes. Elevated levels of these enzymes in plasma are indicative of liver damage (Plaa and Charbonneau, 2008).

Aspartate Aminotransferase. Aspartate aminotransferase (**AST**) catalyzes the conversion of oxaloacetate to alpha-ketoglutarate in the TCA cycle and is found in liver as well as other tissues such as muscle (Plaa and Charbonneau, 2008; Sutherland et al., 2008). Aspartate aminotransferase is a sensitive yet, non-specific indicator, of liver damage because is also detects damage in other tissues (Harr, 2000; Plaa and Charbonneau, 2008). In human medicine, AST was used controversially as an indicator of coronary occlusion; however, this technique is now dated. Furthermore, AST in avian species is usually measured along with creatine kinase to distinguish between liver and muscle damage. This would seem to discount AST as an appropriate indicator of liver health; however, this technique has been successfully used in avian species. Also, enzymes such as alanine aminotransferase that are specific to mammalian liver damage are not found in birds (Harr, 2002). Therefore, AST is a good choice for detecting liver damage in chickens.

Gamma Glutamyltransferase. Gamma glutamyltransferase (**GGT**) is an enzyme located in hepatocyte membranes as well as cells in the bile duct (Plaa and Charbonneau, 2008). This enzyme is involved in degradation of glutathione by catalyzing hydrolysis of the gamma

glutamyl bond between glutamate and cysteine (Pompella, 2006). Because of its location, elevated plasma GGT is an indicator of biliary disease. Harr (2002) reported that plasma GGT is more likely to be elevated in birds than in mammals during cholestasis or biliary epithelial disorders. Therefore, GGT is a useful tool to determine if toxicants affect bile ducts of chickens. Furthermore, rats experience elevated GGT activities in response to gossypol treatment (Deoras, 1997)

Gossypol

Gossypol is a polyphenolic, yellow pigment found predominantly in the lysigenous glands of cotton plants (Kenar, 2006; Nagalakshmi et al., 2007). On account of its highly toxic nature, gossypol is hypothesized to be part of the plant's defense mechanism against fungi and insects. Research reported by Phogat et al. (2000) suggests that increasing gossypol content of cotton could decrease bollworm incidence and damage. At the end of the 19th century, gossypol was proposed as a useful dye material, but soon after was implicated in cottonseed meal toxicity (Kenar, 2006). Cottonseed meal is the second most valuable by-product of the cotton industry. Approximately 990 pounds of cottonseed are produced per acre of cotton grown. This is milled and sold as livestock feed. In particular, it serves as a cheap protein supplement (Rahman et al., 2001).

Cottonseed meal is toxic to many species, in particular young ruminants and monogastric animals (Skutches et al., 1974; Matondi et al., 2007). Gossypol is particularly harmful to chickens, which when fed a cottonseed meal diet can compromise their well being. One of the most notable complications is yolk discoloration attributed to the interaction between yolk iron molecules and gossypol. Other problems include growth depression, decreased egg size and production, and lameness (Panigrahi et al., 1987; Nagalakshmi et al., 2007).

The contraceptive properties of gossypol have been known for decades. In fact, gossypol is such a powerful contraceptive that many countries have investigated its use in human medicine as a vasectomy alternative (Waites et al., 1998). Mohan et al. (1989) reported that roosters fed gossypol experience reduced semen volume and concentration. They also report reduced activity of enzymes essential to normal testicular and sperm function. Kalla and Chadha (1990) also reported reduced fertility in roosters fed gossypol.

In mammalian species (including humans), gossypol is considered to be a cardiac toxin, causing labored breathing, jugular vein distention, and finally heart failure due to congestion and venous stasis (Smith, 1957; Skutches et al., 1974; Kovacie, 2003). However, there is a dearth of evidence that gossypol is a cardiac toxin in chickens. Research instead points to gossypol as a hepatic and reproductive toxin in chickens. Lordelo et al. (2004) found that hens fed a diet containing cottonseed meal accumulated gossypol in the liver. Effects on chicken reproduction were described previously.

Because limited toxicity has been reported in other organ systems, and the tendency for gossypol to accumulate in the liver of chickens, it is not unreasonable to hypothesize that liver enzyme function will be negatively impacted in chickens exposed. It is also not unreasonable to hypothesize that chickens fed a diet containing gossypol in conjunction with a hepatoprotective compound would be less likely to experience a toxicosis.

Effects of Gossypol on Liver Enzymes. Sugiyama et al. (1984) reported that gossypol binds to GST B and prevents GST B binding of the enzyme with normal substrates such as lithocholic acid. Furthermore, Lee et al. (1982) reported that this inhibition was competitive when in the presence of 1-chloro-2, 4-dinitrobenzene, a known GST substrate. Because this is competitive inhibition, gossypol is not changed by the enzyme, but only prevents normal GST substrates from reacting. Therefore, GST activity should be inversely proportional to the amount of gossypol in the body.

Gossypol has been shown to both inhibit and induce CYP450. The bollworm, *Helicoverpa armigera*, when fed a diet containing gossypol experiences an induction in gene expression of the CP450 gene, CYP6AE14 and subsequent protein synthesis. Furthermore, suppression of this gene results in decreased bollworm tolerance of gossypol (Mao et al., 2007). However, Ma and Back (1984) explicitly state that gossypol inhibits CYP450 activity in the rat. Therefore, effects of gossypol on CYP450 activity and gene expression could either be species dependent or subfamily dependent.

Silymarin

Silymarin is an extract from the fruit and seeds of milk thistle (*Silybum marianum*) (Tedesco et al., 2004). It is a complex of four flavonoligans: silibinin, isosilybin, silydianin, and silycristin (Schiavone et al., 2007). Silibinin is considered the most biologically active of the four

flavonoligans and constitutes approximately 80% of the total weight of standardized extracts of silymarin (Detaille et al., 2008).

For at least the past two centuries, milk thistle extracts have been used as a form of self-medication for disease (Schrieber et al., 2008), and remain popular as a safe and effective liver protectant medication. Currently, silibinin, under the trade name Legalon, is being successfully used to improve glycemic control for type 2 diabetes (Detaille et al., 2008). Silymarin is an effective medicine because it has the ability to scavenge for free radicals and has potent antioxidant properties (Comelli et al., 2007; Wu et al., 2007). Rats given acetaminophen and then treated with silymarin experienced improved glutathione concentration and reduced oxidative stress (Post-White et al., 2007).

Effects of Silymarin on Liver Enzymes. Silymarin is primarily metabolized into sulfate and glucuronide conjugates by phase II liver enzymes (Wen et al., 2007). This suggests that silymarin could possibly induce phase II enzyme activity, which would in turn provide protection against toxicants. However, little is known about the ability of silymarin to actually induce phase II enzyme expression (Kiruthiga et al., 2007).

Silibinin inhibits phase I pathways in the liver (Post-White et al., 2007). Tedesco et al. (2004) reported that silibinin could inhibit the cytochrome P450 pathway. The importance of this lies in the ability of CYP450 to both detoxify toxins and make a toxin more harmful by producing ROS. Inhibition of this pathway prevents the formation of free radicals as found by Kiruthiga et al. (2007) when they treated hemolysates with benzo-*a*-pyrene, a known carcinogen, followed by silymarin. They concluded that silymarin inhibits CYP450 and prevents reduction in phase II enzymes such as GST (Kiruthiga et al., 2007).

Although silibinin seems to be the most active of the four flavonoligans, the entire silymarin complex is a better antioxidant than silibinin alone (Comelli et al., 2007). This suggests that all four flavonoligans play a role in the hepatoprotective qualities of silymarin (Comelli et al., 2007). Silymarin also protects other cells in the body. However, when absorbed, silymarin is found in highest concentrations in liver and bile (Comelli et al., 2007).

When considering the question of chickens and exposure to gossypol, silymarin has the potential to provide protection against the negative side effects of gossypol. As previously mentioned, gossypol is toxic to the liver in chickens, whereas silymarin is used to treat liver disease. Therefore, silymarin could potentially act as a protecting agent against gossypol toxicity.

Silymarin has been used successfully to treat chickens suffering from aflatoxicosis (Tedesco et al., 2004). Chickens suffering from aflatoxicosis and then treated with silymarin showed less pathologic changes in liver tissue than those with no treatment. Silymarin also increased body weight gain and feed intake in these chickens because of the decreased effects of aflatoxicosis (Tedesco et al., 2004).

Genetically Divergent Chickens

Response to gossypol can vary among chickens of differing genetic backgrounds (Lordelo et al., 2007). Therefore, to study the potential effect of various genotypes on detoxification of gossypol, it is important to use chickens with known genetic differences. Populations of chickens used were developed at Virginia Polytechnic Institute and State University. For 35 generations, lines of White Leghorn chickens have been selected from a common founder population for their ability to produce antibodies against a single i.v. injection of sheep red blood cells (**SRBC**). Two lines have been developed: one that produces a high antibody response (**HA**) against SRBC and another that produces a low antibody response (**LA**) against SRBC (Kuehn et al., 2006).

Mice fed gossypol at a dose of 50 or 75 mg/kg per d had fewer plaque-forming cells in response to SRBC injection and had depressed spleen cell counts. Furthermore, these mice experienced a reduction in humoral immune response (Sein, 1986). One then may hypothesize that an increase in immune system integrity (i.e., HA chickens) may correspond to increased resistance to gossypol. Thus the HA and LA lines represent two genetically diverse populations that may respond differently to a toxicant challenge. Furthermore, because these two lines are genetically distinct, their use may facilitate identifying specific genes associated with detoxification.

HYPOTHESES

1. Phase I and phase II detoxification enzyme activity depends on several factors including sex and age. Basal enzyme activity should change over time. Basal enzyme activity should also be different between sexes (Chapter 2).
2. Gossypol is a toxin produced by cotton plants and found in cottonseed meal. Gossypol is highly toxic to chickens. Therefore, feeding gossypol to chickens should result in decreased feed consumption and body weight gain. Gossypol also has a negative impact on the liver. Therefore, feeding gossypol to chickens should cause a subsequent reduction in liver enzyme activity, reducing the ability of the liver to detoxify gossypol (Chapters 3 and 4).
3. Silymarin is a hepatoprotective agent. Although silymarin should not have an impact on feed intake or body weight gain, feeding silymarin to chickens should cause an increase in phase II liver enzymes and a possible decrease in phase I enzymes such as cytochrome P450 3A4 (Chapter 3). Therefore, feeding silymarin in conjunction with gossypol should cause a reduction in the negative side effects of gossypol such as reduced weight gain and feed intake (Chapter 4).
4. It is probable that chickens with a superior immune system will be less affected by gossypol. Therefore, when gossypol is fed to a population of chickens divergently selected for immune response to SRBC, the chickens selected for high antibody response should gain more weight and have higher feed efficiency than chickens selected for low antibody response (Chapter 4).

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Chapter 2: Basal Phase I and Phase II Liver Enzyme Activity in Lines of White Leghorn Chickens Selected for Humoral Immune Response

ABSTRACT Phase I and phase II liver enzymes are essential to xenobiotic metabolism. Expression of these enzymes is dependent upon factors such as age and sex. The objective of this study was to determine basal liver enzyme levels in male and female White Leghorn chickens to provide reference values for future studies. Chickens from two lines, divergently selected for 35 generation for high antibody (**HA**) and low antibody (**LA**) immune response, were used. They were fed a corn-soybean meal diet. Six male and 6 female chickens from each line were killed at each of the following ages 4, 8, 12, and 20 wk. At 16 wk, 4 HA males, 6 LA males, 5 HA females, and 8 LA females were killed rather than 6. Livers were collected and used for enzyme analyses. Liver tissue was analyzed for quinone reductase, glutathione-S-transferase, and cytochrome P450 3A4 activity. All data were analyzed using ANOVA. There were no appreciable differences in enzyme activity level between high and low antibody chickens at any age. At 12 wk, quinone reductase and glutathione-S-transferase activities were higher in males than in females ($P< 0.001$), but there was no apparent trend across ages due to sex. Across sexes, cytochrome P450 3A4 activity was substantially greater in 4 and 8 wk than in 12, 16, and 20 wk chickens ($P< 0.001$). This study provides insight into enzyme activities of liver enzymes; however, except for cytochrome P450 3A4, no trends across ages were observed. Furthermore, neither sex nor selection line affected basal enzyme activity.

Key words chicken, humoral immunity, liver enzyme activity

INTRODUCTION

Enzymes produced by the liver are essential for xenobiotic and pharmaceutical metabolism. For instance, glutathione-S-transferase (**GST**) and quinone reductase (**QR**) catalyze reactions that make xenobiotics more polar so that they can be easily excreted in bile and urine (Ioannides, 2002). Enzymes of the cytochrome P450 3A family are responsible for 45 to 60% of pharmaceutical metabolism (Burk and Wojnowski, 2004). Gene expression and enzymatic activity of metabolic enzymes are heavily dependent upon species, sex, age, genotype and environment (Waxman and Holloway, 2009; Wauthier et al., 2007). Changes in enzyme gene expression and activity during development determine drug efficacy as well as toxicity of compounds. Therefore, it is important to understand how enzyme activity changes over time,

and differs between species and genotypes, to understand how individuals cope with toxicants over their lifetimes.

Cytochrome P450 3A4 (**CYP450 3A4**) expression is nearly devoid in the human fetus. It is not until approximately 3 yr of age that cytochrome P450 3A7 expression ceases and CYP450 3A4 becomes the dominant cytochrome for the remainder of the individual's life (Hines, 2007). Unlike humans, male mice express appreciable levels of CYP450 3A4 before birth; these levels then increase rapidly after birth. Attenuation of CYP450 3A4 expression does not begin until 45 d of age (Hart et al., 2009). Furthermore, there is considerable variability within age groups in CYP450 3A4 enzyme expression due to genotype. Some authors report times of "hyper-variability" in enzyme expression where variability between individuals of the same age is heightened (Hines, 2007). This is usually attributed to structural polymorphisms (Hines and McCarver, 2002). For example, individuals who are homozygous for the QR gene, NQ01, completely lack QR enzyme activity whereas enzyme activity varies among heterozygous individuals (Talalay and Dinkova-Kostova, 2004).

Enzyme activity varies in other species as well. Coulet et al. (1996) reported that GST activity did not change in chickens between 3 and 9 wk of age. However, at 12 wk GST activity increased by 60%. Maurice et al. (1991) found in Barred Plymouth Rock chickens that males had approximately 15% more hepatic GST activity than females. Chicks have been reported to have detectable cytochrome levels as early as day 7 of incubation. These levels increased dramatically at day of hatch and then plateaued 3 days post-hatch (Lorr and Bloom, 1987).

The objective of this study is to characterize sex, age, and line differences in GST, QR, and CYP450 3A4 activity between 2 lines of White Leghorn chickens divergently selected for humoral immune response. This information will provide baseline enzyme activity levels that can be used for comparison in future studies.

MATERIALS AND METHODS

Animals and Tissue Collection

Selection lines of White Leghorn chickens have been developed at Virginia Tech. These lines have been divergently selected for 35 generations based on antibody response to a 0.1 ml intravenous injection of a 0.25% solution of sheep red blood cells 5 d post injection. Through

this selection two lines have been developed: a low antibody (**LA**) and a high antibody (**HA**) response line (Gross and Siegel, 1980; Martin and Dunnington, 1990).

In the current experiment, 4 and 8 wk old chickens from these lines were housed in 3.7 x 1.6 m floor pens and allowed free access to water and a mash diet (corn-soybean meal: 20% CP; 2685 kcal ME/kg on a DM basis). They were exposed to light 24 h/d. Twelve, 16, and 20 wk old chickens were housed in 5.2 x 4.6 m floor pens and allowed free access to water and a corn-soybean meal diet (on a DM basis, 20% CP and 2685 kcal/kg ME). They were kept under 14 h light and 10 h dark.

Six male and 6 female chickens from both lines were killed by cervical dislocation at 4, 8, 12, and 20 wk of age. In the design, the chickens used were to be from a single hatch. However, due to a poor initial hatch, such was not possible. Four and 8 wk males and females, and 12 and 20 wk females, hatched March 20, 2007. Twelve and 20 wk males hatched August 13, 2007, and 16 wk males and females hatched July 6, 2007. At 16 wk, 4 HA males, 6 LA males, 5 HA females, and 8 LA females were killed rather than 6. Following cervical dislocation, a section of the left lobe of the liver was removed, chopped into small pieces using a razor blade and split into three samples. The samples were double-wrapped in aluminum foil and frozen in liquid nitrogen. Samples were stored at -80˚C.

The Virginia Tech Institutional Animal Care and Use Committee approved all housing conditions and experimental procedures.

Enzyme activity assays

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Sample Preparation. Liver samples were weighed and a 25% homogenate was prepared in 0.1 *M* phosphate buffer (pH 7.4 with 1.15% KCl) using a Polytron blender (Brinkman Instruments, Westbury, NY). Homogenates were centrifuged for 10 min at 15,000 X *g* at 5°C. Supernatant was re-centrifuged for 60 min at 50,000 X *g* at 5°C. The supernatant, cytosol, was transferred and stored at -80°C until assayed. The pelleted portion, containing the microsomes, was resuspended in an equal volume of 10m*M* Tris-acetate buffer (pH 7.4, 0.1 M EDTA, 20% glycerol) and stored at -80^{o}C. Enzyme activity and protein assays were performed in 96-well microtiter plates. Quinone reductase and GST activities were measured in liver cytosol fractions

and CYP450 3A4 activity was measured in microsomal fractions. For each enzyme assay and the protein assay, samples from each chicken were analyzed in triplicate.

Quinone Reductase. Quinone reductase activity was assayed using protocols described by Prochaska and Santamaria (1988). Briefly, quinone reductase uses NADPH to reduce menadione, which then reduces thiazolyl blue tetrazolium to blue formazan. Quinone reductase activity is indirectly determined by measuring the amount of NADPH oxidized (nmoles) per minute per mg protein. Liver cytosol was diluted 1:8 with 0.1*M* phosphate buffer (pH 7.4, 1.15% KCl). Fifty µl of diluted cytosol and 200 µl of the reaction mixture were added in triplicate to a 96-well microplate. The reaction was allowed to progress for 5 minutes; it was stopped with a dicoumarol solution (0.3 m*M* dicoumarol in 1.5% dimethyl sulfoxide in 5 mM potassium phosphate buffer at pH 7.4). The plate was then read using a microplate spectrophotometer (Molecular Devices, Corp., Sunnyvale, CA) at an absorbance of 610 nm. The concentration of enzyme was determined with the Beer-Lambert law using an extinction coefficient of 11,300 M/cm and path length of 0.57 cm. Enzyme activity (nmoles NAPDH oxidized/min/mg protein) was then calculated.

Glutathione-S-transferase. The GST assay used a 20 m*M* solution of 1-chloro-2,4-dinitrobenzene as a substrate and a 20 m*M* solution of glutathione as a cofactor. Liver cytosol was diluted 1:32 with 0.1*M* phosphate buffer (pH 7.4, 1.15% KCl). Each well contained 40 µl of diluted cytosol, 220 µl of 0.1 *M* of sodium phosphate buffer (pH 6.5) warmed to 30^{o}C, 20 µl of the 1-chloro-2,4-dinitrobenze solution, and 20 µl of the glutathione solution. The plate was read at 340 nm using a microplate spectrophotometer (Molecular Devices, Corp., Sunnyvale, CA) for 5 min with readings taken at 15 s intervals. An extinction coefficient of 9.6 m*M*/cm and a light path length of 0.57 cm were used to calculate enzyme concentration. Enzyme activity was expressed in µmole glutathione conjugated/min per mg protein (Mannervik and Jemth, 1999; Kaplowitz et al., 1975; Habig et al., 1974).

Cytochrome P450 3A4. Cytochrome P450, specifically the 3A4 family, activity was assessed using a fluorescent spectrophotometry method, which was derived from Crespi et al. (1997). The substrate used for this assay was a stock solution of 40 m*M* 7-benzyloxyquinoline (7-BQ) dissolved in acetonitrile. A working solution of 400 µM 7-BQ was made by diluting the stock solution with a 50 mM phosphate buffer (pH 7.4). This working solution was further diluted with buffer to establish a standard curve ranging from 0-40 µ*M* concentrations of 7-BQ.

One hundred µl of the respective standards or 100 µl of the 80 µ*M* 7-BQ substrate was added to each of the microplate wells. The plate was incubated at 37^{o}C for 15 min.

The microsomal fraction of each sample was diluted 1:4 with 10 m*M* Tris-acetate buffer (pH 7.4, 0.1 mM EDTA, 20% glycerol). Following the 15 min incubation, 20 µl of each diluted microsomal sample was added to the plate in triplicate. Eighty µl of the NADP generating system, as described by Crespi et al. (1997), was added to each well. The reaction was allowed to progress for 30 min at 37^{o}C. The reaction was stopped with 75 µl per well of a solution of 80% acetonitrile and 20% 0.5M Tris buffer (pH 9). The plate was read at an excitation and emission wavelength of 410 nm and 538 nm, respectively (Spectra Max M5, Molecular Devices, Corp., Sunnyvale, CA). Enzyme activity was determined via interpolation from the standard curve. Activity was reported as µmoles of 7-BQ oxidized/ min per mg protein.

Protein. All enzyme activities are reported on a per mg protein basis. Cytosolic as well as microsomal proteins were determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). This is a protein-dye binding method, which is based upon the Bradford method (Bradford, 1976). Bovine serum albumin was used to create a standard curve.

Statistical Analysis

All analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary, NC). Enzyme measurements were analyzed using the GLM procedure. The following model was fitted

$$Y_{ijkl} = \mu + S_i + A_j + L_k + (SA)_{ij} + (SL)_{ik} + (AL)_{jk} + e_{ijkl}$$

where Y_{ijk} was the enzyme activity for a chicken of sex S (i = 1 or 2, for male and female, respectively) and age A (j = 1, …, 5, for 4, 8, 12, 16 and 20 wk, respectively) from selection line L (k= 1 or 2, for HA or LA, respectively), and e_{ijkl} was the residual. Type III sums of squares were used to form test statistics due to imbalance in chicken numbers across ages. The 3-way interaction of sex, age and line was included in the initial statistical model; it was excluded since it did not define significant variation in the activity of any enzyme assessed.

Significant age by sex interactions were detected for enzyme activity levels. Least squares means for sexes were compared within an age using Student's t-test.

RESULTS

There was no sex by line interaction in the activity level for any of the enzymes considered.

Quinone Reductase

For QR, there was an age by sex interaction ($P = 0.003$) (Table 2.1). Differences between sexes were in general small, except at wk 12. At 12 wk, QR activity was approximately 1.4 times greater in females than males ($P< 0.001$). At 20 wk, QR activity in females was 0.8 that in males, a smaller yet significant difference ($P< 0.05$) (Figure 2.1).

There was also an age by line interaction for QR ($P = 0.009$). Between 4 wk and 16 wk, QR activity in HA chickens was between 1.1 and 1.2 times that in LA chickens (Figure 2.2). At 20 wk, this relationship between lines reversed, with QR activity in HA chickens 0.94 times that in LA chickens. At no age was the difference between lines significant (Table 2.1).

Glutathione-S-transferase

There was an age by sex interaction for GST activity ($P = 0.003$). Males and females had comparable GST activity at 4, 8, 16, and 20 wk. The interaction reflected GST activity in females being 1.3 times that of males at 12 wk ($P < 0.001$) (Table 2.2). There were no differences between HA and LA chickens ($P = 0.204$).

Cytochrome P450 3A4

For CYP450 3A4 activity, an interaction between age and sex ($P < 0.001$) was also detected. Cytochrome P450 3A4 activity was similar between males and females at 4 and 8 wk, ranging between 3.14 and 3.49 μmoles 7-BQ oxidized/min per mg protein (Table 2.3). At 12 wk, males experienced a sharp decline in activity as compared to females (across lines, on average, to 1.19 7-BQ oxidized/min per mg protein; $P < 0.001$; Figure 2.3). At wk 16, their activity levels decreased further (across lines, on average, to 0.733 7-BQ oxidized/min per mg protein). In females, CYP450 3A4 activity dropped sharply at 16 wk to a level comparable with males. After 16 wk, there was no further decline in either sex.

There were no appreciable differences in activity between HA and LA males and females at any age. However, starting at wk 12, CYP450 3A4 activity declined in a similar fashion as that observed among sexes and ages.

DISCUSSION

Cytochrome P450 3A4 activity was highest in the younger chickens (i.e., 4 and 8 wk), while activity declined in the older chickens (.i.e., 12 and 16 wk). Glutathione-S-transferase and

QR activity was relatively constant across ages, with the exception of 12 wk. For all three enzymes, activity at 12 wk decreased in males as compared to females. This may reflect a 'true' sex difference. However, males and females at 12 wk were from different hatches. Therefore, it is possible that this interaction reflected an unforeseen hatch effect.

Maurice et al. (1991) found distinct sex differences in hepatic GST activity among Barred Plymouth Rock and Rhode Island Red chickens. In their study, male chickens had significantly higher activity than females. However, here, males and females had similar activity levels. The oldest chickens in the current experiment were 20 wk old. Chickens used by Maurice et al. (1991) were 30 wk old. Results reported elsewhere (Lorr and Bloom, 1987; Hines, 2007; Hart et al., 2009), and observed for CYP450 3A4 in this study, indicate that age influences enzyme activity. By 30 wk of age, male chickens in this study may have greater GST activity than females. Furthermore, Maurice et al. (1991) evaluated different breeds of chickens, and Barred Plymouth Rocks had significantly higher GST activity than Rhode Island Reds (Maurice et al., 1991). Breed differences in enzyme activity level at different ages may therefore exist.

Differences in enzyme activity among ages have been observed for GST activity. Jang et al. (2001) reported hepatic GST activity gradually increased with age in Wistar rats. Such was not the case in the current study, as there was no unambiguous pattern in GST activity across ages. That finding agrees with Jung et al. (1996). They found no clear pattern in GST activity in 1, 2, 3, 5, and 12 mo old rats. Similarly, there was no clear pattern in male and female chickens in the current study. Perhaps, as Jung et al. (1996) concluded, there is a more complicated network of interactions involved in enzyme activity than simply age.

Cytochrome P450 3A4 activity was high in young chickens but then decreased substantially with age. Hart et al. (2009) found that CYP 3A4 gene expression increased rapidly in young mice, and then began to decrease in 20 d old mice continuing until 45 d. Furthermore, Coulet et al. (1996) found that whereas CYP450 3A4 protein expression increased in male broilers from 3 to 9 wk of age, by at 12 wk protein expression decreased. Those results are consistent with this study, where CYP450 3A4 activity in males declined appreciably at 12 wk. It is possible that CYP450 3A4 activity is greater in younger chickens because it is the more dominant cytochrome at the time. In humans, cytochrome P450 3A7 is the dominant cytochrome in young children, but as they age this activity wanes and CYP450 3A4 becomes the

dominant cytochrome (Hines, 2007). One can hypothesize that CYP450 3A4 activity decreases over time in chickens as another cytochrome system increases.

There is very little information on patterns of QR activity across ages. Quinone reductase activity seems to be heavily influenced by genotype (Talalay and Dinkova- Kostova, 2004). The gene, NQ01, is foremost involved in QR expression. An epidemiological study in China found that industrial workers with a mutant NQ01 allele were more likely to develop hemotoxicity due to benzene exposure than those with normal NQ01 alleles (Nebert et al., 2002). Furthermore, NQ01 genotype affected breast cancer survival rates (Fagerholm et al., 2008). Five year survival rate of women homozygous for the NQ01 mutant allele, NQ01*2, was 47% compared to 67% in women with a normal NQ01 genotype. The NQ01*2 genotype is associated with reduced QR activity. It can be hypothesized that chickens with lower QR activity had different variants of the NQ01 genotype.

CONCLUSIONS

Cytochrome P450 3A4 activity was the only enzyme considered that had a clear trend over time: younger chickens had higher activity levels than older chickens. Younger individuals may need such heightened CYP450 3A4 activity to cope with environmental challenges early in life before other physiologically processes mature.

Neither sex nor selection line clearly affected enzyme activity level. The age by sex interaction observed for all three enzyme systems was largely due to differences in activity levels between sexes at 12 wk. However, chickens of the two sexes were from separate hatches approximately 5 mo apart. Such was not intended in the design of the experiment, but an unfortunate consequence of a poor hatch. Although the management and housing conditions were the same for both hatches, there is a possibility hatch effects contributed to this interaction. If the experiment was repeated, it would be useful for the chickens defining all ages, sexes and lines to be derived from a single hatch, and with the entire design duplicated across hatches.

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Table 2.1 Least squares means for quinone reductase (QR) activity across ages by sex and line [high antibody (HA) and low antibody (LA) response lines]

	QR Activity (nmoles NADH oxidized/min per mg protein)			
Age (wk)	Male	Female	HA	LA
4	2.51[a]	2.86[a]	2.90[a]	2.46[a]
8	2.46[a]	2.59[a]	2.74[a]	2.31[a]
12	1.85[a]	2.62[b]	2.33[a]	2.14[a]
16	2.82[a]	2.86[a]	2.46[a]	2.05[a]
20	2.52[a]	2.03[b]	2.21[a]	2.34[a]
Maximum SEM[1]	0.17	0.16	0.19	0.16

[1] Error df = 102

[ab]Means in the same row within sex or within line with different superscripts are different ($P < 0.05$)

Table 2.2 Least squares means for glutathione-S-transferase (GST) activity across ages by sex and line [high antibody (HA) and low antibody (LA) response lines]

	GST Activity (μmoles GSH[1] conjugated/min per mg protein)			
Age (wk)	Male	Female	HA	LA
4	0.088[a]	0.083[a]	0.092[a]	0.079[b]
8	0.083[a]	0.089[a]	0.085[a]	0.087[a]
12	0.068[a]	0.091[b]	0.078[a]	0.081[a]
16	0.066[a]	0.065[a]	0.067[a]	0.064[a]
20	0.083[a]	0.088[a]	0.087[a]	0.084[a]
Maximum SEM[2]	0.004	0.004	0.004	0.004

[1]Glutathione
[2] Error df = 101
[ab]Means in the same row within sex or within line with different superscripts are different ($P < 0.05$)

Table 2.3 Least squares means for cytochrome P450 3A4 (CYP450 3A4) activity across ages by sex and line [high antibody (HA) and low antibody (LA) response lines]

	CYP450 3A4 Activity (µmoles7-BQ[1] oxidized/min per mg protein)			
Age (wk)	Male	Female	HA	LA
4	3.15[a]	3.36[a]	3.44[a]	3.07[a]
8	3.49[a]	3.18[a]	3.27[a]	3.40[a]
12	1.19[a]	2.66[b]	1.90[a]	1.96[a]
16	0.73[a]	0.76[a]	0.77[a]	0.72[a]
20	0.67[a]	1.16[b]	0.87[a]	0.96[a]
Maximum SEM[2]	0.24	0.20	0.24	0.20

[1]Benzyloxyquinoline
[2] Error df = 97
[ab]Means in the same row within sex or within line with different superscripts are different ($P < 0.05$)

Figure 2.1 Quinone reductase activity across lines for male and female chickens. There was an age by sex interaction (P=0.003) largely due to the difference at 12 wk.

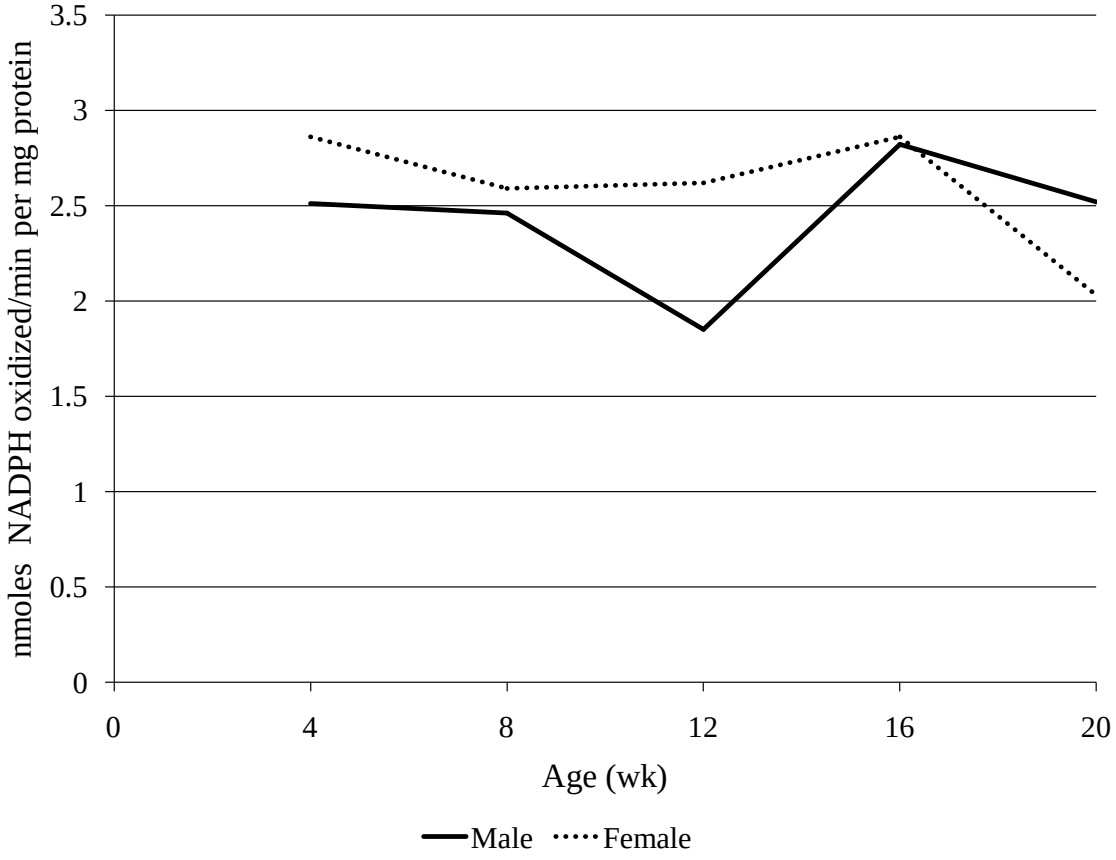


Figure 2.2 Quinone reductase activity across sexes for high antibody (HA) and low antibody (LA) response lines. There was an age by line interaction ($P = 0.009$).

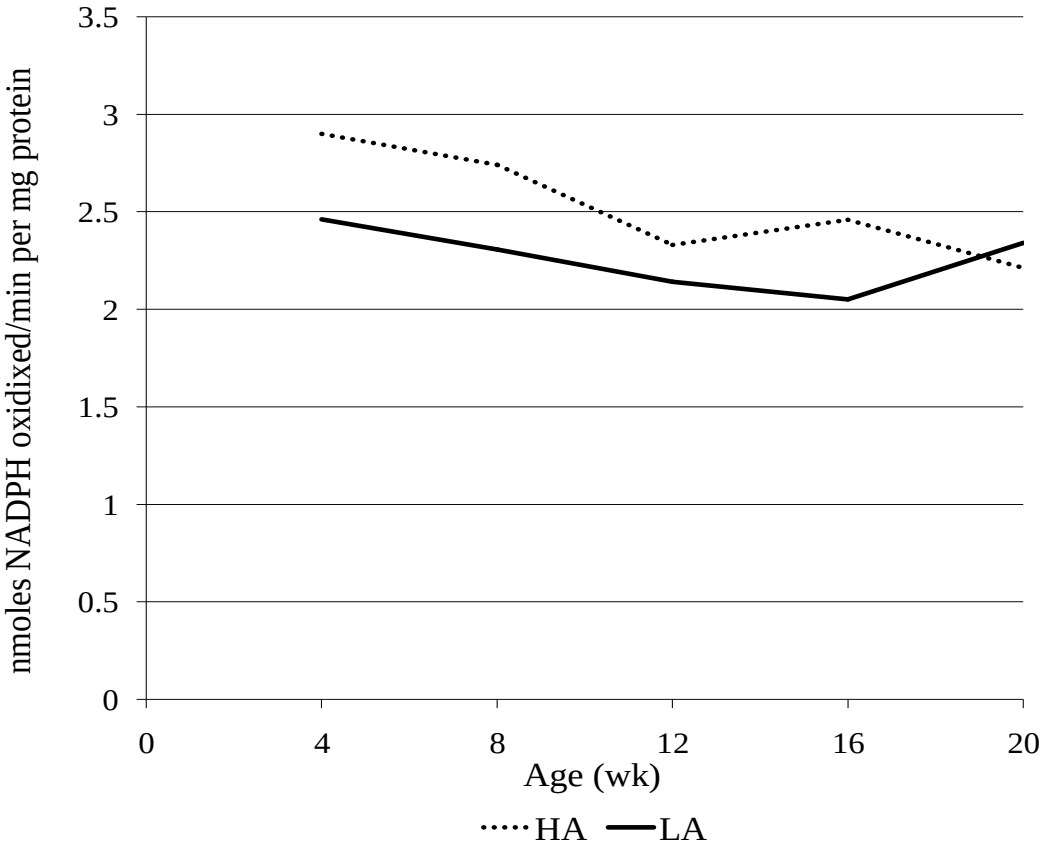
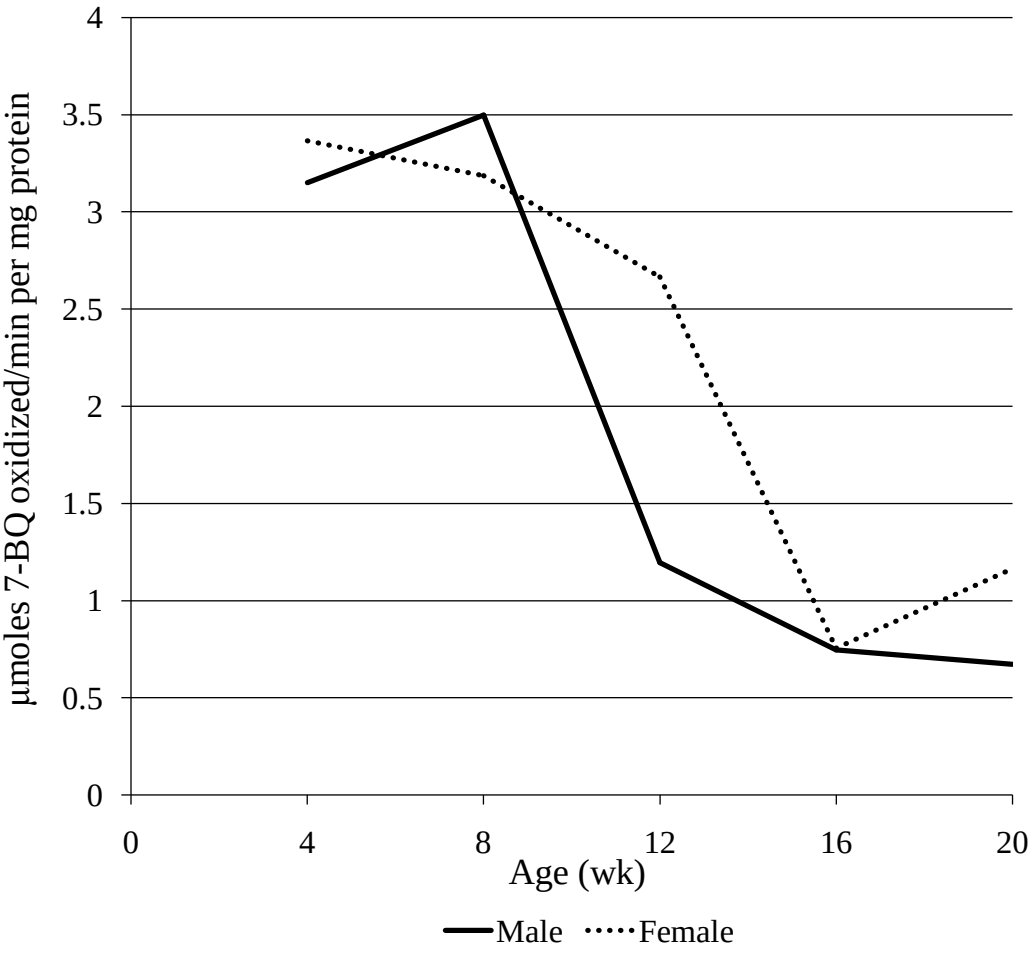


Figure 2.3 Cytochrome P450 3A4 activity across lines for male and female chickens. There was an age by sex interaction ($P < 0.001$) due to differences at 12 wk.



CHAPTER 3: Evaluation of a Toxic Concentration of Gossypol and a Beneficial Concentration of Silymarin in White Leghorn Chickens Divergently Selected for Humoral Immune Response

ABSTRACT Gossypol is a hepatic toxin to chickens. However, the dose at which toxicosis occurs may differ depending on age, sex and genetic stock. Silymarin, a milk thistle extract, is beneficial to the liver and potentially helpful in preventing gossypol toxicosis. The objective of this study was to determine at what dose gossypol would exhibit a toxic response on a layer type chicken and also to determine at what dose silymarin would prove to be beneficial. Sixty female and 30 male White Leghorn chickens from lines selected for humoral immune response were used. Three birds from a sex and line were randomly assigned to a cage and fed a corn-soybean meal (control) diet for 10 d. Cages were randomly assigned to one of 5 diets: high gossypol (1000 ppm), low gossypol (500 ppm), high silymarin (1000 ppm), low silymarin (500 ppm), or control. Two male and 4 female cages were assigned to each treatment. The compounds were added and mixed into the control diet which was in mash form. Weight and feed intake data were collected for 4 wk. Male chickens were bled each wk to determine iron, hematocrit and hemoglobin levels. Chickens were then killed and their livers collected to determine enzyme activity. Weekly measures were analyzed using repeated measures and regression methodologies. Liver enzyme data were analyzed using ANOVA. For males, BW gain ceased at wk 2 in chickens fed high gossypol; by wk 4, they had lower BW than chickens on other diets ($P = 0.002$). Feed intake in high gossypol males declined continually after wk 1 ($P< 0.001$). High gossypol females behaved similarly. There were no differences among treatments for iron, hemoglobin or hematocrit ($P = 0.610$, $P = 0.946$ and $P = 0.84,5$ respectively). There were also no differences among treatments for liver enzymes for either sex. Gossypol fed at 1000 ppm was toxic whereas 500 ppm was not. Neither dose of silymarin caused any differences in liver enzyme activity, feed intake, or BW gain above the control.

Key words: gossypol, silymarin, chicken, humoral immunity

INTRODUCTION

The famous 16th century scientist, Paracelsus noted that "All substances are poisons; there is none which is not a poison. The right dose differentiates a poison from a remedy (Langman and Kapur, 2006)." This early observation is now one of the cornerstones of

toxicology and is known as the dose-response relationship. The dose-response relationship varies greatly among compounds. For example, arsenic can cause a toxicosis at concentrations as low as 10 μg/L of drinking water (Kapaj et al., 2006), whereas the reference dose for acetaminophen toxicity is 143 mg/kg body weight (Tenenbein, 2004). A good knowledge of the dose at which a toxic response occurs is essential when conducting toxicological experiments. The same can be said for beneficial compounds.

Gossypol is a toxic pigment produced by cotton. In chickens, it is a hepatic toxicant. Chickens that consume diets with a high concentration of cottonseed meal (**CSM**) suffer from BW loss, reduced feed intake, and decreased egg weight (Nagalakshmi et al., 2007). As with other toxic compounds, toxicity depends on the amount of CSM or free gossypol in the diet. Furthermore, the dose at which toxicity occurs varies based on breed and age. Inclusion of CSM at 150 mg/kg can depress body weight and feed intake of broilers. However, it has been reported that chicks will not show signs of toxicosis until CSM is included at 280 mg/kg (Nagalakshmi et al., 2007).

Silymarin is a compound extracted from the seeds of milk thistle (*Silybum marianum*). It is composed of at least four flavonoligans that have strong antioxidant properties (Comelli et al., 2007). It has been studied extensively as a hepatoprotective agent. Thus, silymarin may protect the chicken from gossypol toxicosis. Dose-response issues also apply to silymarin (Post-White et al., 2007).

This study has three objectives. The first objective is to determine a dose of gossypol at which layer type chickens (White Leghorns) will exhibit a toxic response. The second objective is to determine a dose of silymarin that will be safe as well as beneficial to the chicken. These beneficial effects could range from improved hepatocyte plasma membrane integrity to increased phase II liver enzyme activity. The final objective of this study is to identify one or more biological markers that can be used to determine the general health of the chicken in vivo.

MATERIALS AND METHODS

Animals and Experimental Design

Selection lines of White Leghorn chickens have been developed at Virginia Tech. These lines have been divergently selected for 35 generations based on antibody response, as previously described in chapter 2. These lines were a low antibody (**LA**) and a high antibody

(**HA**) response line (Gross and Siegel, 1980; Martin and Dunnington, 1990; Kuehn et al., 2006). Thirty male and 60 female chickens from both lines (90 chickens in total) at approximately 77 d of age were used. The male chickens were balanced for line; among female birds, 15 were HA and 45 were LA. The chickens were housed in 10 cages within each of 3 batteries. Three chickens of the same sex and line (HA or LA) were randomly assigned to a cage, with the provision that males and females were housed in separate batteries.

Prior to placement in cages, chickens were housed in 3.7 x 1.6 m floor pens and allowed free access to water and feed (corn-soybean meal: 20% CP; 2685 kcal ME/kg on a DM basis). They were exposed to light 24 h/d. All chickens were moved into their assigned cage (30x15x16.5cm) 10 d prior to the beginning of the experiment. During this time period, chickens were fed a corn-soybean meal diet (on a DM basis, 2685 kcal/kg ME and 22.1% CP) and allowed to acclimate to their new environment. Thereafter, dietary treatments were applied. Throughout the experiment, birds were offered free access to water and feed and exposed to light 24 h daily.

The Virginia Tech Institutional Animal Care and Use Committee approved all housing and experimental procedures.

Treatments

There were 4 treatments and a control in this experiment. The treatments were high gossypol (**HG**), low gossypol (**LG**), high silymarin (**HS**), and low silymarin (**LS**). Gossypol was purchased from TimTec, LLC (Newark, DE) and silymarin was purchased from SigmaAldrich (St. Louis, MO). Chickens assigned to HG and HS treatments were fed the corn-soybean meal diet containing 0.1% gossypol and silymarin, respectively. Chickens assigned to the LG and LS treatments were fed the corn-soybean meal diet containing 0.05% gossypol and silymarin, respectively. Chickens assigned to be controls (**C**) received the corn-soybean meal diet alone.

Within males, a cage in a line was randomly assigned to a treatment or the control diet. Thus each treatment and the control were assigned to 2 cages. Within females, each treatment and the control diet was assigned randomly to 4 cages. All chickens were given their assigned treatments for 4 wk.

Feed was mixed each Friday. Given the small weight of gossypol and silymarin added to the meal, the compounds were first thoroughly mixed in approximately 50 g of feed. This blend

was then combined with the appropriate amount of meal to achieve the desired concentration, and mixed for 10 min in an industrial Hobart mixer. Both silymarin and gossypol are light sensitive; therefore, the diets were stored in plastic buckets with lids and placed inside a black plastic bag.

Feed Analysis

Samples from all 4 treatment diets and the control diet were analyzed for DM, CP, crude fat, crude fiber and amino acid content (Cumberland Valley Analytical Services, Hagerstown, MD).

Weight and Feed Intake

On Monday of each wk the chickens were weighed individually. Each cage was allocated enough feed presumed to be sufficient to last a wk. The weight of the feed offered was recorded, with any residual feed from the previous wk weighed and discarded. For some cages, additional feed needed to be added during the wk. When done, its weight was recorded.

Blood Collection and Processing

Following the acclimation period, each Monday blood was collected from all male chickens. Approximately 1.5 ml of blood was collected via venous puncture of the wing vein using a 25 gauge needle and a 3 ml syringe. Blood collection was alternated between the right and left wing to minimize stress on the chicken and to allow the wings to heal between bleedings. Blood was then deposited in a 4 ml lithium heparin Vacutainer tube (BD Vacutainer, Franklin Lakes, NJ). Blood was placed on ice immediately following collection. On the same day, blood was drawn out of the Vacutainer tubes with capillary tubes to measure hematocrits. Samples were then centrifuged at 4000x*g* for 10 min and plasma was collected and stored at -20˚C for iron and hemoglobin analyses.

Two capillary tubes of whole blood were spun for 4 min to determine hematocrit values. Plasma iron levels were determined using a QuantiChrom Iron Assay Kit (BioAssay Systems, Hayward, CA). Plasma hemoglobin levels were determined using a QuantiChrom Hemoglobin Assay Kit (BioAssay Systems, Hayward, CA).

Tissue Collection

After 4 wk on treatment, all chickens were killed via cervical dislocation. A section of the left lobe of the liver was removed and sub-divided into three smaller samples. Each sample was double-wrapped in aluminum foil and frozen in liquid nitrogen, and then stored at -80˚C until analyzed for enzyme activity.

Enzyme Assays

All tissue preparation, enzyme activity, and protein determination methods have been previously described in detail in chapter 2. Briefly, each liver sample was prepared as a 25% homogenate in 0.1 M phosphate buffer (pH 7.4 with 1.15% KCl). Quinone reductase (**QR**) activity was assayed using protocols described by Prochaska and Santamaria (1988). Quinone reductase activity is indirectly determined by measuring the amount of NADPH oxidized (nmoles) per minute at 610 nm using a microplate spectrophotometer per mg protein. Glutathione-s-transferase (**GST**) was measured as the amount of glutathione conjugated to the substrate 1-chloro-2,4-dinitrobenzene at 340 nm (Habig et al., 1974; Kaplowitz et al., 1975; Mannervik and Jemth, 1999). Cytochrome P450 3A4 (**CYP3A4**) activity was determined by fluorescent spectrophotometry method (Crespi, 1997). Protein concentration was determined using the BioRad method (Bio-Rad Laboratories, Hercules, CA).

Statistical Analyses

All analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary, NC). In males, each line was represented equally although only one cage from each line was assigned to a diet. In females, 4 cages were assigned to each diet; however, most chickens were LA. Given the extent of imbalance in numbers between sexes (10 male and 30 female cages), data on males and females were analyzed separately. Since delineating line differences was not the focus of this experiment, and given the predominance of LA chickens, line was considered random.

Males. Cage within line and diet was the experimental unit. Measurements collected on individual male birds were averaged by cage. For consistency, feed intake was expressed as the weekly intake of an individual bird. Because the same chickens within a cage were measured each wk over the length of the experiment, a repeated measures analysis using the MIXED procedure was conducted. The linear mixed model was fitted for measurements collected on a weekly basis (i.e., BW, feed intake, hematocrit):

$$Y_{ijkl} = \mu + W_i + L_j + D_k + (WD)_{ik} + e_{(ijk)l} \quad [1]$$

where Y_{ijkl} was the response variable recorded each wk W (i = 1,...,5) from line L (j = 1 or 2, for HA or LA, respectively) randomly assigned to diet D (k = 1, ..., 5, for HG, LG, HS, LS, or C, respectively) by cage (l = 1 or 2), and μ was the overall mean. Week and diet were fitted as fixed effects, along with their interaction (WD). Line was fitted as a random effect. Since the time interval between successive measurements was constant (a single wk), an auto-regressive covariance structure among weeks with homogenous variances was assumed. Differences among diets were tested across all levels of time (wk).

Additionally, a linear mixed model using the MIXED procedure was fitted:

$$Y_{ijkl} = L_j + \boldsymbol{\beta} x_i(D_k) + e^A_{(jk)l} + e^B_{(ijk)l} \quad [2]$$

where the vector $\boldsymbol{\beta}$ was regression coefficients (intercept, and linear and quadratic terms) for the covariate wk (x). In order to model differences in the values of these coefficients by diet, interaction of diet with these coefficients was fitted. The random terms were line, cage nested within the line by diet ($e^A_{(jk)l}$), and residual ($e^B_{(ijk)l}$). The error term used to test the significance of the regression coefficients in conjunction with diet was $e^A_{(jk)l}$.

Liver enzyme measurements were analyzed using ANOVA. The model fitted was:

$$Y_{jkl} = \mu + L_j + D_k + e_{(jk)l} \quad [3]$$

where Y_{jkl} was the average enzyme activity level in cage l within a line j and diet k, and $e_{(jk)l}$ was the residual.

Females. Measurements collected on individual female birds were also averaged by cage. Similar to males, models [2] and [3] were fitted to the relevant data. Since blood samples were collected exclusively on males, hematocrit was not assessed in females.

In females, there were 4 cages (l = 1, . . .,4) assigned to each treatment. Therefore, cage nested within line by diet ($e^A_{(jk)l}$) was a more appropriate error term for testing main effects with the repeated measures model. Model [1] was modified as:

$$Y_{ijkl} = \mu + W_i + L_j + D_k + (WD)_{ik} + e^A_{(jk)l} + e^B_{(ijk)l} \quad [4]$$

where $e^B_{(ijk)l}$ was the residual.

RESULTS

Feed Analysis

All diets were similar in DM and nutrient content. Most importantly, there were no differences in lysine, methionine, or cysteine content (Table 3.1).

Body Weight and Feed Intake

Males. On average, chickens fed all 4 treatments and the control gained weight during the first 2 wk of treatment (Figure 3.1; Table 3.2). However, at wk 2 weight gain in chickens fed the HG diet ceased; by wk 4 they began losing weight. Based on the repeated measures analysis (model [1]), at wk 4 these chickens had lower BW than chickens fed the other treatments (P = 0.020).

With the regression analysis (model [2]), the slope of the regression of BW on time differed for chickens fed HG as compared to chickens on the other treatments (P< 0.001). Because BW gain in HG fed chickens slowed, and then BW decreased, including a quadratic term in the model improved the fit of the regression for this treatment (P < 0.001). In chickens fed the other diets, BW gain remained constant over time. These results are consistent with those from the repeated measures analysis, namely that BW was adversely affected by the HG diet.

Feed intake of chickens fed the HS, LS and C diets increased from the beginning of the experiment until wk 2 (Figure 3.2; Table 3.3). At wk 3, their feed intake stabilized. Feed intake for chickens fed LG reached its maximum at wk 2, and decreased slightly by wk 4. Feed intake for chickens fed HG increased the first wk and continually decreased thereafter. Based on the repeated measures analysis, HG fed chickens consumed less feed than chickens on all other diets at wk 2 (P = 0.009). This difference increased thereafter (P< 0.001). At wk 4, chickens on the low gossypol diet ate slightly less than those on C, HS, and LS, although the difference was not significant.

Slope of the regression for feed intake on time was different between HG fed chickens and those consuming other diets (P< 0.001). Because of the plateau in feed consumption, including a quadratic term improved fit (P<0.001). The value of the quadratic regression coefficient was different in HG fed chickens compared to the other diets (P< 0.001). These

findings corroborate those from the repeated measures analyses: HG fed chickens consumed less feed on average than chickens on other diets.

Females. Similar to male chickens, female chickens fed LG, HS, LS and C diets gained weight during the entire experimental period (Figure 3.3; Table 3.4). Body weight gain for chickens fed HG ceased at wk 2, and these birds began losing weight by wk 4. Based on repeated measures analysis (model [4]), BW in HG fed chickens was different from that on all other diets at wk 3 ($P = 0.004$). This difference in BW was greater by wk 4 ($P< 0.001$).

Slope of the regression (model [2]) for BW on time was different for HG than to remaining diets ($P < 0.001$). Because HG chickens stopped gaining weight, and started losing BW, including the quadratic term improved fit for this treatment ($P = 0.015$).

Feed intake increased over the first 3 wk, and then became stable at wk 4, in chickens fed LG, HS, LS, and C diets (Figure 3.4; Table 3.5). In chickens fed HG, feed intake increased the first wk, and continually declined thereafter. Based on repeated measures analysis, feed intake was different in HG fed chickens as compared to all other diets at wk 2 ($P = 0.005$). This difference increased thereafter ($P < 0.001$).

Regression analysis corroborated these results. Slope of the regression of feed intake on time was different for HG chickens than those fed other diets ($P<0.001$). Including the quadratic term improved model fit because of the plateau in feed intake on all diets ($P< 0.001$). With the decrease in food intake in HG fed chickens, the value of the coefficient of the quadratic term differed ($P<0.001$).

Clinical Chemistry

Based on repeated measures analyses (model [1]), there were no differences among diets in hemoglobin, hematocrit, or iron values ($P = 0.610$, $P = 0.946$, and $P = 0.845$ respectively) in the male chickens (Table 3.6). Regression analyses suggested the values of these measurements changed over time, although hemoglobin, hematocrit, and iron values did not differ among diets ($P = 0.590$, $P = 0.095$, and $P = 0.205$).

Liver Enzymes

Males. There were no differences in CYP450 3A4, GST, or QR activity between treatments ($P = 0.87$, $P = 0.57$, and $P = 0.18$, respectively; Table 3.7).

Females. There were no differences among diets for CYP3A4, GST, or QR activity (P = 0.09, P = 0.09, and P = 0.86, respectively; Table 3.7).

DISCUSSION

Reduced BW gain, even weight loss, and reduced feed intake are classic signs of gossypol toxicosis (mohan et al., 1989; henry et al., 2001; nagalakshmi et al., 2007). Chickens fed the HG diet exhibited these signs by the second wk of treatment. Such was not the case for chickens on the LG diet.

Gossypol toxicity is based on the amount of free gossypol available to the chicken (Nagalakshmi et al., 2007; Panigrahi and Morris, 1991). Gossypol within a feed mixture can bind to essential nutrients such as lysine, thereby making such nutrients unavailable to the animal. Henry et al. (2001) reported this may cause reduced BW and subsequent health problems in chickens. Feed analyses (Table 3.1) showed no clear differences in amino acid content among diets.

Hermes et al. (1983) reported that some chickens were tolerant of free gossypol concentrations up to 0.05% of the diet. The LG diet had a total gossypol content of 0.05%. Total gossypol content of the HG diet was 0.10%. Even if some gossypol was bound, this 0.10% concentration in feed was clearly high enough to cause a toxicosis. As a result, reduced BW and feed intake were observed in chickens fed the HG diet but, not the LG diet.

There were no differences in BW or feed intake between HS and LS diets. Furthermore, there were no differences in BW or feed intake between silymarin diets and control. Tedesco et al. (2004) reported that silymarin improved BW gain and feed intake in chickens suffering from aflatoxicosis. However, there was no indication from this study that silymarin would cause improved BW gain or feed intake in a healthy chicken.

Quinone reductase and GST are important phase II enzymes. Gossypol accumulates in the liver of the chicken (Lordelo et al., 2004). Thus, the activity of liver enzymes may be altered by gossypol storage. Furthermore, gossypol binds to GST preventing it from binding to normal substrates, causing a reduction in GST activity (Lee et al., 1982; Sugiyama et al., 1984). Also, QR is responsible for the reduction of dietary quinones that arise from metabolism of phenols, aromatics, and polycyclic aromatic compounds (Talalay and Dinkova-Kostova, 2004). Gossypol is a prime candidate for QR because it is a polyphenolic compound.

Nevertheless, no differences in either GST or QR activity among diets were observed. Chickens may have been fed the HG diet long enough to affect weight and feed intake, but not long enough to alter liver activity. Furthermore, exposure to gossypol depended on the chicken ingesting feed. Feed intake dramatically decreased toward the end of the experiment. Therefore, chickens were not exposed to the same level of gossypol as at the beginning of the experiment; this may have allowed recovery of hepatic enzyme activity. As observed in humans, hepatocytes have the ability to regenerate (Zaret and Grompe, 2009). Additionally, silymarin did not induce either GST or QR activity. Silymarin is well characterized as an antioxidant, but its capacity to induce liver enzyme activity is less certain (Comelli et al., 2007; Wu et al., 2007; Kiruthiga et al., 2007). On account of the time of enzyme activity measurement and the doses of silymarin used, silymarin was not a phase ii enzyme inducer in this study.

Cytochrome P450 3A4 is an important phase I enzyme in xenobiotic metabolism. Several studies have shown that gossypol and silymarin both inhibit CYP450 activity (Kiruthiga et al., 2007; Ma and Back, 1984). An in vitro study indicated that CYP450 3A4 inhibition depended on silymarin concentration and time of exposure (Sridar et al., 2003). Cytochrome P450 3A4 was not inhibited by either gossypol or silymarin in the present study. Chickens possibly were not exposed to sufficient concentrations of gossypol or silymarin to cause an inhibition in enzyme activity. Such may particularly have been the case with gossypol, where feed consumption on HG decreased over time, which reduced exposure.

Hematocrit, iron, and hemoglobin are all physiological markers of health. Gossypol is associated with erythrocyte fragility and reduced hematocrits (Adeyemo, 2008; Matondi et al., 2007; Henry et al., 2001). Furthermore, gossypol binds to iron making it unavailable to the animal (Panigrahi and Morris, 1991). However, in this study, gossypol did not affect hematocrit, iron, or hemoglobin levels.

CONCLUSIONS

Gossypol added to the diet at a level of 0.05% did not cause a toxicosis in male or female White Leghorn chickens, although it did at a level of 0.10%. In future toxicology studies involving gossypol, including the compound in the diet at the higher level seems prudent to

ensure that a toxic response is exhibited. Hematocrit, iron, and hemoglobin were not reliable choices for non-invasive indicators of general health in this study. Therefore, it may be more advantageous to use serum markers specific to liver health such as aspartate aminotransferase and gamma glutamyltransferase.

One dosage of silymarin was not superior to the other based on the indices measured. Such might not be the case when silymarin is used to alleviate gossypol toxicosis. Inclusion of silymarin at the higher dosage in the diet may therefore be more advantageous. However, this pilot study was not conclusive in determining a beneficial dose of silymarin in an unchallenged chicken. Nevertheless, both doses of silymarin were proven safe.

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Table 3.1 Chemical composition of treatments and control diets

Diet	DM (%)	DM Basis (%)				W/W%[1]		
		Crude Protein	Crude Fat	Crude Fiber	Ash	Cysteine	Lysine	Methionine
High Gossypol	88.6	20.5	9.4	5.2	5.4	0.35	1.11	0.30
Low Gossypol	88.8	21.4	3.0	4.6	5.7	0.34	1.08	0.30
High Silymarin	88.7	20.3	3.1	4.2	5.4	0.38	1.09	0.31
Low Silymarin	88.4	20.0	3.0	4.7	5.6	0.35	1.17	0.31
Control	88.1	22.1	2.8	4.3	6.0	0.38	1.24	0.32

[1] W/W% = g/100 g of sample

Table 3.2 Least squares means for body weight of male chickens by diet and wk

	BW (g) by wk[1]				
Diet[1]	0	1	2	3	4
High Gossypol	684.1[a]	802.0[a]	863.3[a]	880.2[a]	844.0[a]
Low Gossypol	705.0[a]	826.3[a]	913.8[a]	998.8[a]	1077.5[b]
High Silymarin	748.0[a]	869.8[a]	948.0[a]	1049.2[a]	1159.7[b]
Low Silymarin	642.5[a]	759.5[a]	869.8[a]	961.0[a]	1072.0[b]
Control	704.0[a]	821.5[a]	919.3[a]	1017.2[a]	1126.0[b]

[1] SEM equals 56.9 g, error df = 5
[ab]Means in the same column with different superscripts are different ($P < 0.05$)

Table 3.3 Least squares means feed intake of male chickens by diet and wk

	Feed intake (g/wk) by wk[1]				
Diet[1]	0	1	2	3	4
High Gossypol	404.6[a]	436.2[a]	417.8[a]	382.2[a]	243.4[a]
Low Gossypol	412.7[a]	452.3[a]	494.4[b]	492.6[b]	426.5[b]
High Silymarin	426.5[a]	470.0[a]	528.4[b]	544.2[b]	547.6[b]
Low Silymarin	356.5[a]	419.5[a]	515.0[b]	516.0[b]	526.5[b]
Control	413.4[a]	435.3[a]	524.2[b]	528.0[b]	499.3[b]

[1] SEM = 27.7 g/wk, error df = 10
[ab]Means in the same column with different superscripts are different ($P < 0.01$)

Table 3.4 Least squares means for body weight of female chickens by diet and wk

	BW (g) by wk[1]					
Diet	0	1	2	3	4	SEM
High Gossypol	562.9[a]	643.3[a]	683.1[a]	703.6[a]	656.7[a]	28.4
Low Gossypol	577.6[a]	662.2[a]	732.7[a]	793.2[b]	848.0[b]	27.8
High Silymarin	609.9[a]	690.1[a]	752.3[a]	816.8[b]	874.4[b]	30.1
Low Silymarin	585.3[a]	667.9[a]	718.9[a]	797.8[b]	856.3[b]	28.4
Control	557.3[a]	632.8[a]	697.2[a]	764.9[b]	817.4[b]	28.4

[1] Maximum SEM = 31.5 g for a wk, with error df = 18
[ab]Means in the same column with different superscripts are different ($P < 0.01$)

Table 3.5 Least squares means for feed intake of female chickens by diet and wk

	Feed Intake (g/wk) by wk[1]					
Diet	0	1	2	3	4	SEM[2]
High Gossypol	308.9[a]	342.9[a]	311.3[a]	294.5[a]	229.8[a]	15.3
Low Gossypol	322.2[a]	345.7[a]	377.4[b]	375.3[b]	369.4[b]	15.0
High Silymarin	336.9[a]	364.0[a]	373.1[b]	396.6[b]	393.6[b]	16.4
Low Silymarin	323.7[a]	352.4[a]	357.0[b]	393.4[b]	382.1[b]	15.3
Control	314.0[a]	337.3[a]	352.6[b]	382.6[b]	381.6[b]	15.3

[1] Maximum SEM =16.4 g/wk for a wk, with error df = 18
[2] Error df = 18
[ab]Means in the same column with different superscripts are different ($P < 0.01$)

Table 3.6 Least squares means for iron, hemoglobin, and hematocrit of male chickens by diet and wk

	Iron (μg/dL)[1]					Hemoglobin (g/dL)[2]					Hematocrit (%)[3]				
Diet	0	1	2	3	4	0	1	2	3	4	0	1	2	3	4
High Gossypol	493.3[a]	356.1[a]	648.1[a]	276.2[a]	219.3[a]	14.68[a]	11.84[a]	14.14[a]	9.99[a]	10.64[a]	26.0[a]	31.6[a]	31.3[a]	33.6[a]	34.5[a]
Low Gossypol	424.1[a]	384.8[a]	699.1[a]	255.2[a]	280.8[a]	13.92[a]	13.26[a]	14.74[a]	10.25[a]	11.14[a]	31.3[a]	29.8[a]	30.3[a]	32.1[a]	32.8[a]
High Silymarin	546.8[a]	460.8[a]	609.1[a]	160.6[a]	207.8[a]	11.55[a]	11.54[a]	11.97[a]	11.10[a]	11.08[a]	30.2[a]	30.8[a]	30.3[a]	32.3[a]	32.6[a]
Low Silymarin	488.8[a]	411.1[a]	248.9[a]	248.9[a]	269.3[a]	11.87[a]	12.32[a]	14.16[a]	11.15[a]	10.61[a]	29.6[a]	30.5[a]	31.5[a]	34.3[a]	32.3[a]
Control	324.3[a]	434.2[a]	407.5[a]	311.0[a]	184.2[a]	10.50[a]	12.30[a]	11.85[a]	10.49[a]	10.72[a]	29.3[a]	30.7[a]	31.2[a]	30.8[a]	32.5[a]

[1] SEM = 107.4 μg/dL, error df = 17; [2] SEM = 1206 mg/dL, error df = 16; [3] SEM = 2.70, error df = 19
[ab]Means in the same column with different superscripts are different ($P < 0.05$)

Table 3.7 Least squares means for cytochrome P450 3A4 (CYP450 3A4), glutathione-S-transferase (GST), and quinone reductase (QR) activity in males and females by diet

	CYP450 3A4 (μmol 7-BQ[1] oxidized/min per mg protein)		GST (mmol GSH[2] conjugated/min per mg protein)		QR (nmoles NADH oxidized/min per mg protein)	
Diet	Male	Female	Male	Female	Male	Female
High Gossypol	3.21[a]	2.92[a]	0.100[a]	0.091[a]	1.36[a]	1.36[a]
Low Gossypol	2.20[a]	1.79[a]	0.080[a]	0.088[a]	1.18[a]	1.23[a]
High Silymarin	2.91[a]	2.08[a]	0.107[a]	0.086[a]	1.67[a]	1.32[a]
Low Silymarin	2.71[a]	2.04[a]	0.091[a]	0.089[a]	1.15[a]	1.42[a]
Control	2.59[a]	2.75[a]	0.094[a]	0.092[a]	1.32[a]	1.37[a]
SEM[3]	0.69	0.31	0.01	0.002	0.13	0.14

[1] 7-benzyloxyquinoline
[2] Glutathione
[3] Male Error df = 5; Female Error df = 15
[ab] Means in the same column with different superscripts are different ($P < 0.01$)

Figure 3.1 Average body weight (g) of male chickens fed high gossypol (HG), low gossypol(LG), high silymarin (HS), low silymarin (LS), and control (C) diets. Chickens fed HG had significantly lower body weight at wk 4 (P = 0.02) compared to other diets.

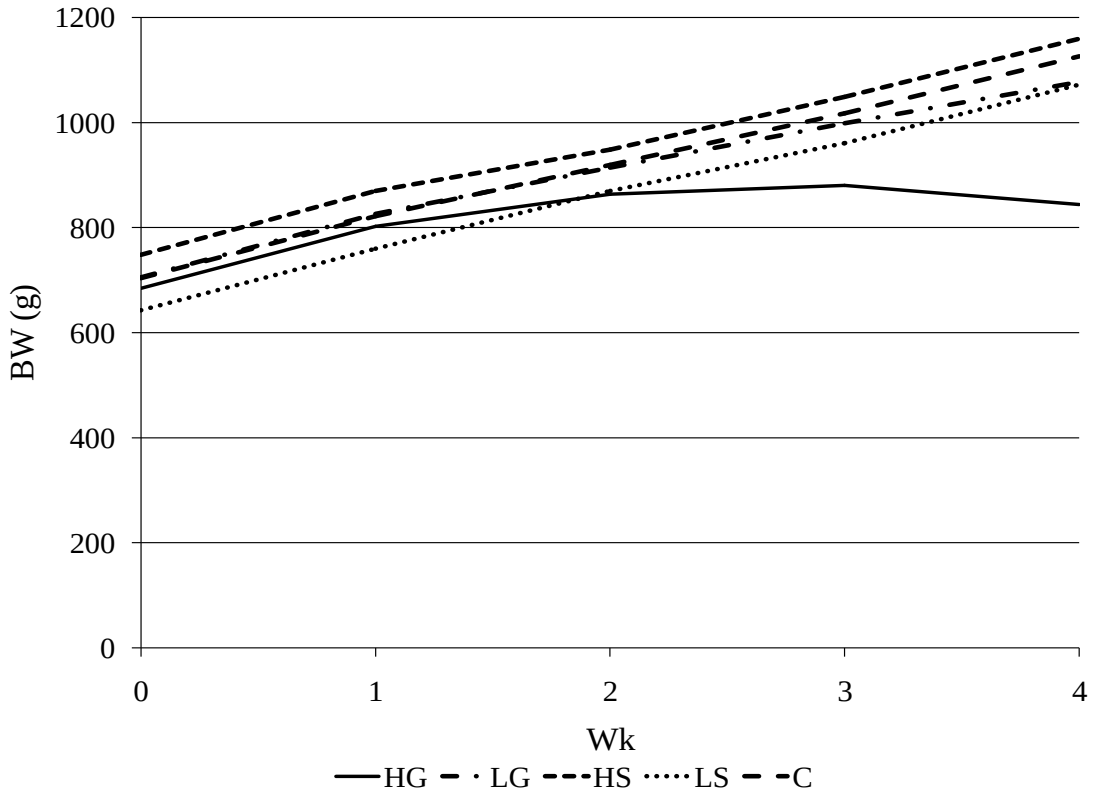


Figure 3.2 Average weekly feed intake (g/wk) of male chickens fed high gossypol (HG), low gossypol (LG), high silymarin (HS), low silymarin (LS), and control (C) diets. Chickens fed HG consumed less feed at wk 2 ($P = 0.001$). This difference increased throughout the experiment ($P < 0.001$).

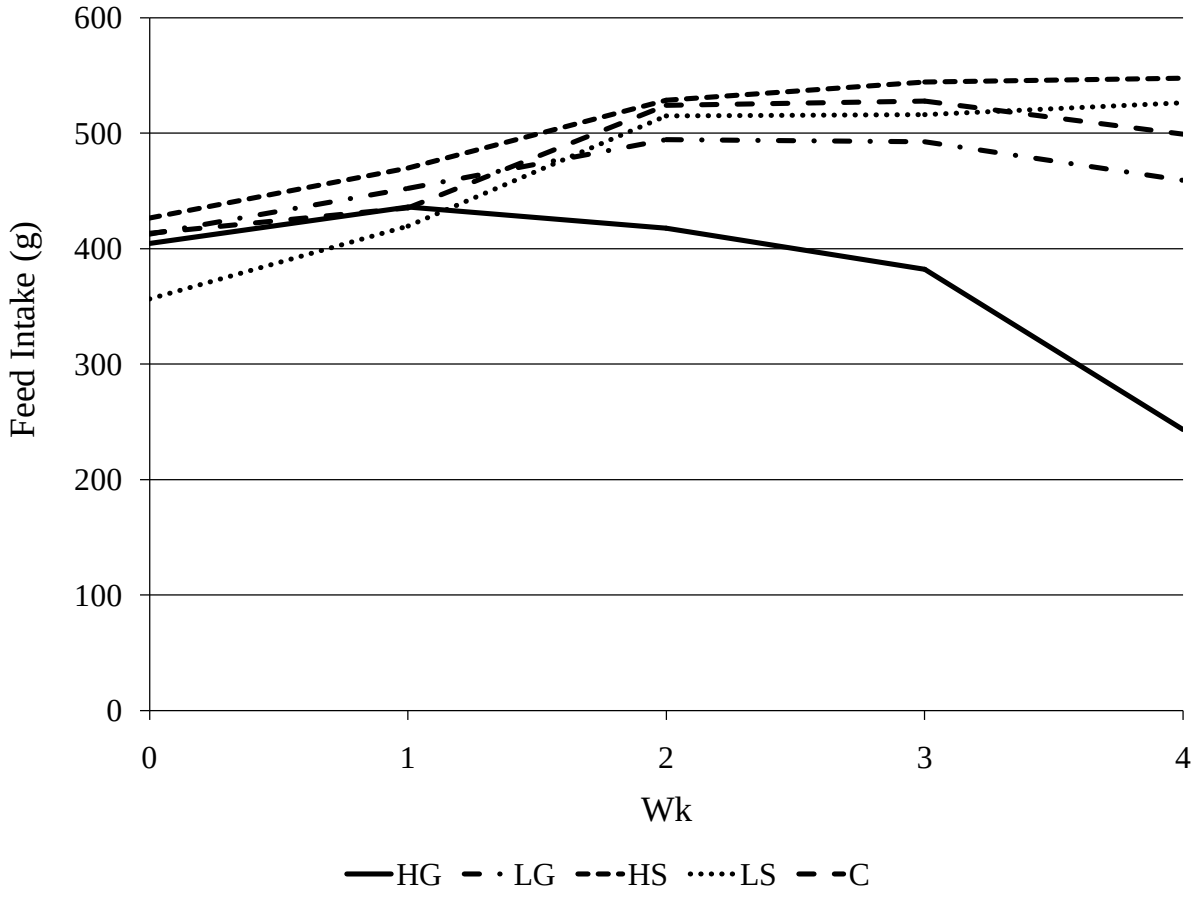


Figure 3.3 Average body weight (g) of female chickens fed high gossypol (HG), low gossypol (LG), high silymarin (HS), low silymarin (LS), and control (C) diets. Chickens fed HG had lower body weight than chickens on other diets at wk 3 ($P = 0.004$). This difference increased by wk 4 ($P < 0.001$).

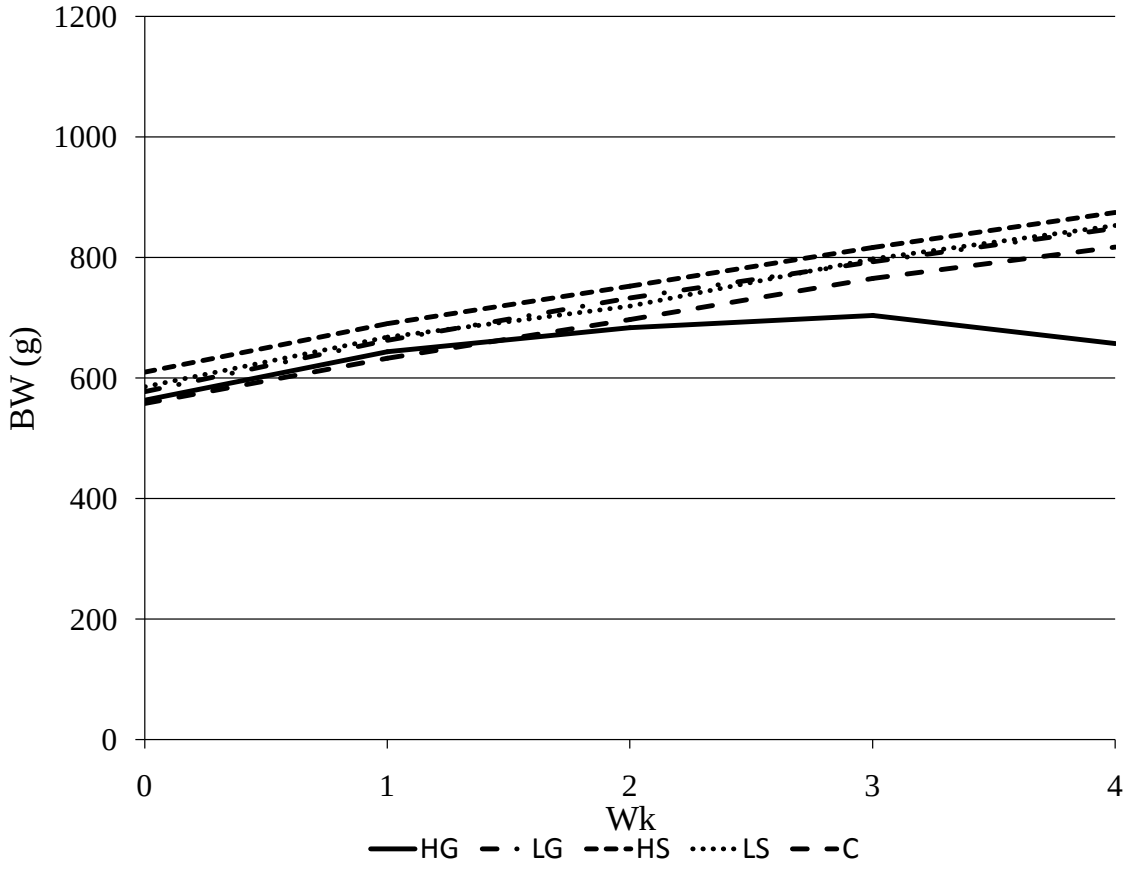
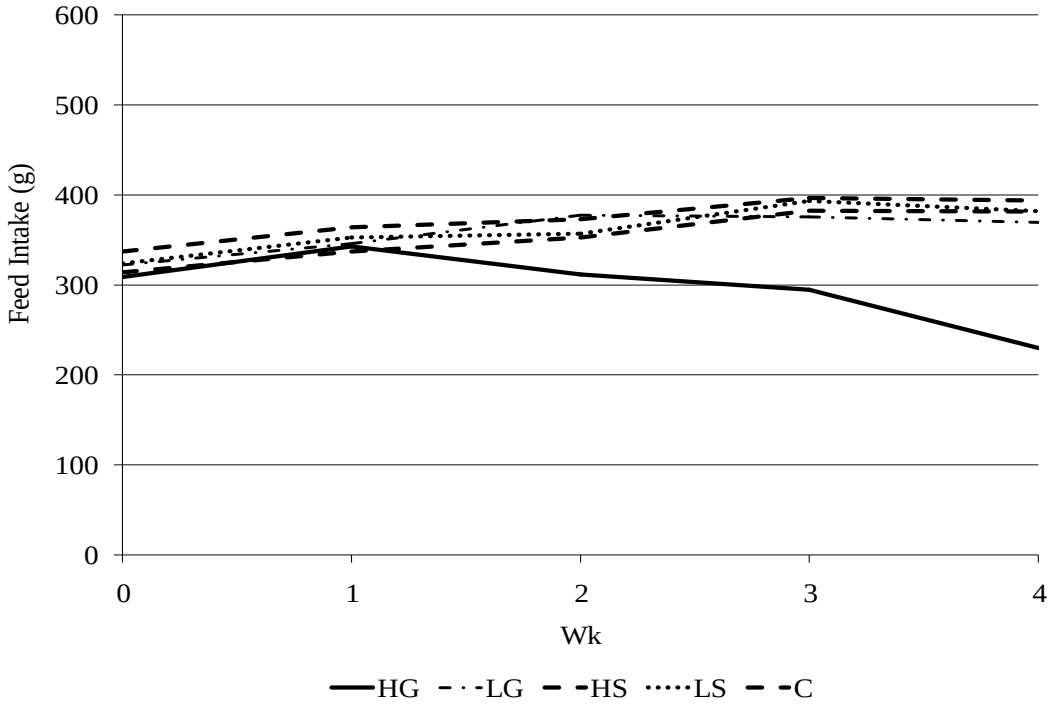


Figure 3.4 Average weekly feed intake (g/wk) for a female chicken fed high gossypol (HG), low gossypol (LG), high silymarin (HS), low silymarin (LS), and control (C) diets. Chickens fed HG had lower feed intake than chickens on other diets at wk 2 ($P = 0.005$). The difference increased continually throughout the experiment ($P < 0.001$).



CHAPTER 4: Use of Silymarin to Treat Gossypol Toxicosis in Lines of White Leghorn Chickens Divergently Selected for Humoral Immune Response

ABSTRACT Gossypol, a pigment of cotton, is a hepatic toxin for chickens. Consumption of cottonseed meal is thus problematic which reduces the used of it as an alternative feed. Silymarin is an extract from milk thistle. Since silymarin has hepatoprotective qualities, it could potentially serve as a feed additive to offset the toxicity of gossypol. The objective of this study was to determine if silymarin could counteract gossypol toxicosis. One hundred and forty-four male chickens from lines divergently selected for humoral immunity were used. Three individuals from each line were randomly assigned to a cage, and fed a corn-soybean meal (control) diet for 14 d. Six cages per line were then randomly assigned one of four dietary treatments (1000 ppm gossypol, 1000 ppm silymarin, or 1000 ppm both gossypol and silymarin or the control diet). Weight and feed intake data were collected for 3 wk, with chickens bled weekly to collect plasma and determine hematocrits. Chickens were killed, and livers collected with tissue prepared for histology and enzymatic activity analyses. Weekly measures were analyzed with repeated measures and regression methodologies. Plasma and liver enzyme activity, and histological measures, were analyzed using ANOVA. Chickens assigned to the gossypol and gossypol-silymarin diets stopped gaining weight at wk 2 ($P < 0.001$) and lost weight by wk 3 ($P < 0.001$). Gamma glutamyltransferase was also elevated in these chickens at wk 2; activities increased further by wk 3 ($P < 0.001$). Histological examination of liver slices indicated they also had substantial lipidosis ($P < 0.001$). Furthermore, quinone reductase activity was increased in gossypol and gossypol-silymarin treated birds compared to control and silymarin treated birds ($P < 0.001$). Silymarin did not alleviate any clinical signs of gossypol toxicosis. Lower gossypol concentrations in the feed may be more responsive to amelioration with silymarin as a feed additive.

Key words: chicken, gossypol, silymarin, quinone reductase, gamma glutamyltransferase

INTRODUCTION

Corn prices have increased substantially over the past few years (Stillman et al., 2009), with poultry producers seeking alternative feeds. One potential feed alternative is cottonseed meal (**CSM**). Cottonseed meal lacks adequate amounts of energy to be used as a substitute for corn. However, CSM is a good source of protein, comparable to soybean meal (**SBM**)

(Nagalakshmi et al., 2007; Rahman et al., 2001; Bath et al., 1995) and could be used to replace SBM to offset the cost of corn. As of January 20, 2009, CSM prices averaged $0.26/kg ($235/ton) whereas SBM prices averaged $0.35/kg ($319/ton) (USDA Market News Service, 2009). Unfortunately, CSM contains the toxin gossypol, a pigment produced by the cotton plant (Kenar, 2006). Gossypol in chicken diets is associated with growth depression, lameness, decreased egg size and hatchability, and yolk and albumen discoloration (Nagalakshmi et al., 2007).

In mammalian species (including humans), gossypol is considered a cardiac toxin, causing labored breathing, jugular vein distension, and finally heart failure due to congestion and venous stasis (Smith, 1957; Skutches et al., 1974; Kovacie, 2003). However, there is a dearth of evidence that gossypol is a cardiac toxin in chickens. Instead, it acts as a hepatic toxin. Lordelo et al. (2004) reported that hens fed a diet containing cottonseed meal accumulated gossypol in the liver.

Silymarin, an extract from the seeds of milk thistle (*Silybum marianum*) is a complex of 4 flavonligans that have strong antioxidant and free radical scavenging properties (Tedesco et al., 2004; Comelli et al., 2007; Schiavone et al., 2007; Wu et al., 2007). For at least the past 2 centuries, milk thistle extracts have been used to treat disease (Schrieber et al., 2008), and silymarin, in particular, remains highly regarded as a safe and effective medication. Currently, milk thistle, under the trade name Legalon, is being used to improve glycemic control for type 2 diabetes (Detaille et al., 2008). Milk thistle extracts have also been used to successfully treat hepatitis patients (Bares et al., 2008; Ferenci et al., 2008). Finally, silymarin has been used to control aflatoxicosis in chickens (Tedesco et al., 2004). Silymarin is therefore a potential treatment for gossypol toxicosis and could be used as a feed additive to allow CSM to be fed to chickens without negative side effects.

This study had two objectives. The first objective was to determine if silymarin would reduce the effects of gossypol toxicosis in two lines of chickens divergently selected for humoral immune response. Therefore, it followed that the second objective of this study was to determine if chickens selected for superior humoral immune response would respond differently to gossypol than those selected for inferior immune response.

MATERIALS AND METHODS

Animals and Experimental Design

Two lines of White Leghorn chickens have been developed at Virginia Tech by divergent selection for 35 generations based on antibody response (see chapter 2 for further details). These lines include a low antibody (**LA**) and a high antibody (**HA**) response line (Gross and Siegel, 1980; Martin and Dunnington, 1990; Kuehn et al., 2006).

Chicks were hatched in December and housed in Petersime starter batteries with 24 h light exposure and ad libitum access to feed (corn-soybean meal mash diet) and water. At 35 d of age, 72 males from each line (144 chickens in total) were moved from the Petersime batteries (model 2SD 24, Petersime Incubator Co., Gettysburg, OH) to larger Harford batteries (Harford Metal Products, Aberdeen, MD). All chickens were housed in a single room and the batteries contained 12 cages (4 cages per row; 30x15x16.5cm). Five batteries were used. All 12 cages were used in the first 2 batteries, and 8 cages were used in the last 3 batteries (upper two rows). Within line, 3 chickens were randomly assigned to a cage, and cage to a location in a battery. Each battery contained an equal number of HA and LA cages.

All chickens were moved into their assigned cage 14 d prior to the beginning of the experiment (i.e. 35 d of age). During this time period the chickens were fed a corn-soybean meal diet (on a DM basis, 23.5 % CP and 2685 kcal/kg ME) in mash form and allowed to acclimate to the new environment. Throughout the acclimation and experimental periods, all birds were given free access to water and feed and exposed to light 24 h a day.

The Virginia Tech Institutional Animal Care and Use Committee approved all housing and experimental procedures.

Treatments

Four dietary treatments and were randomly assigned to each cage of chickens within battery and line. Cages assigned to the control received the corn-soybean meal diet in mash form. Six cages were assigned to each diet within a line. The diets included gossypol (**G**), silymarin (**S**), and gossypol and silymarin combined (**G-S**), and a control (**C)**. A preliminary study (chapter 3) indicated that gossypol at a level of 0.05% did not cause apparent toxicosis. Therefore, gossypol was added to the control corn-soybean meal at a level of 0.1%, as was silymarin. Cages assigned to G-S received a diet containing both gossypol and silymarin at a level of 0.1%.

The silymarin dose was demonstrated to be safe in the pilot study. Gossypol was purchased from TimTec, LLC (Newark, DE) and silymarin was purchased from Sigma-Aldrich (St. Louis, MO).

Feed was mixed on Friday of each wk. Given the small weight of gossypol and silymarin added to the meal, compounds were first thoroughly mixed in approximately 50 g of feed. This blend was then combined with the appropriate amount of meal to achieve the desired concentration, and mixed for 10 min in an industrial Hobart mixer. Both silymarin and gossypol are light sensitive; therefore, the diets were stored in plastic buckets with lids and then placed inside a black plastic bag.

Weight and Feed Intake

Each Monday morning chickens were individually weighed. Each cage was allocated enough feed presumed sufficient to last a wk. Weight of the feed offered was recorded, with any residual feed from the previous wk weighed and discarded. For some cages, it was necessary to provide additional feed during the wk. When done, its weight was recorded.

Blood Collection and Processing

On Monday morning of each experimental wk, blood was collected from the brachial vein with a 25 gauge needle and 3 ml syringe. Blood collection was alternated between the right and left wing to allow healing between bleedings. Approximately 1.5 ml of blood was collected each wk, placed in a 4 ml Vacutainer tube containing lithium heparin (BD Vacutainer, Franklin Lakes, NJ), and blood was placed on ice to minimize enzyme activity loss. On the same day, 2 capillary tubes of whole blood were drawn from the Vacutainer tubes and centrifuged (model 335, Fisher Scientific, Pittsburgh, PA) for 4 min to determine hematocrit values. Samples were then centrifuged (model 5403, Eppendorf, Westbury, NY) at 4000x*g* for 10 min and plasma was collected and stored at -20˚C for aspartate aminotransferase (**AST**) and gamma-glutamyltransferase (**GGT**) activity determination, and for total plasma protein determination.

Aspartate Aminotransferase. Aspartate aminotransferase activity was measured using a spectrophotometric kit purchased from Biotron Diagnostics (Hemet, CA). Directions included in the kit were for individual cuvet use. These protocols were modified so that the kit could be used with a 96-well microtiter plate. Spectrophotometric absorbances were obtained per kit instructions by mixing 100µl with 200 µl of reagent provided in the kit. All samples were run in duplicate.

Gamma glutamyltransferase. Gamma glutamyltransferase activity was measured using a spectrophometric kit purchased from Pointe Scientific (Canton, MI). Protocols were modified so that the kit could be used with a 96-well microtiter plate. Modifications resulted in the use of 25 µl of plasma with 225 µl of reagent. All samples were run in triplicate.

Plasma Protein. Total plasma protein was determined using the BioRad method (Bio-Rad Laboratories, Hercules, CA). Bovine serum albumen was used to create a standard curve. All plasma samples were diluted 1:100 with deionized water and run in triplicate in a 96-well microtiter plate.

Tissue Collection

After 3 wk of treatment, all chickens were killed via cervical dislocation. A section of the left lobe of the liver was removed and sub-divided into three smaller samples. Each sample was double-wrapped in aluminum foil and frozen in liquid nitrogen. All samples were stored at -80˚C until analyzed for enzyme activity. Two liver slices from each chicken were also collected and stored in 10% buffered formalin for histological analysis.

Enzyme Assays

All tissue preparation, enzyme activity, and protein determination methods have been described in detail elsewhere (chapter 2). Briefly, each liver sample was prepared as a 25% homogenate in 0.1 M phosphate buffer (pH 7.4 with 1.15% KCl). Quinone reductase (**QR**) activity was assayed using protocols described by Prochaska and Santamaria (1988). Quinone reductase activity is indirectly determined by measuring the amount of NADPH oxidized (nmoles) per min per mg protein at 610 nm using a microplate spectrophotometer . Glutathione-S-transferase (**GST**) was measured as the amount of glutathione conjugated to the substrate 1-chloro-2,4-dinitrobenzene at 340 nm (Habig et al., 1974; Kaplowitz et al., 1975; Mannervik and Jemth, 1999). Cytochrome P450 3A4 (**CYP450 3A4**) activity was determined by a fluorescent spectrophotometry method (Crespi, 1997). Protein concentration was determined in microsomes or cytosol using the BioRad method (Bio-Rad Laboratories, Hercules, CA).

Histological Analysis

Liver slices were removed from formalin and dehydrated using ethanol and xylene, and then infiltrated with paraffin. These tissues were embedded in paraffin blocks. The blocks were

sent to Histo-Scientific Research Laboratories (Mt. Jackson, VA) where 5 μm slices were cut from each block and fixed to glass slides. Slides were stained with hematoxylin and eosin. One slide per chicken was prepared. Each slide was reviewed by a board-certified veterinary pathologist and received a score for lipidosis, heterophils, and lymphoid nodules on a 0 (no abnormality) to 3 (extensive abnormality) score (Table 4.1).

Feed Analyses

Samples from all diets were analyzed for DM, CP, crude fat, crude fiber and amino acid content (Cumberland Valley Analytical Services, Hagerstown, MD). Samples from G and G-S diets were analyzed for free gossypol content (Pope Testing Laboratories, Inc., Irving, TX). Results of these analyses are presented in Table 4.2.

Statistical Analyses

All analyses were conducted using SAS 9.1 (SAS Institute Inc., Cary, NC). Cage within line and diet was the experimental unit. Where measurements were collected on individual chickens, these were averaged by cage. For consistency, feed intake for a cage was expressed as the average weekly intake of an individual chicken.

The same chickens within a cage were measured each wk over the length of the experiment. Therefore, a repeated measures analysis using the MIXED procedure was conducted. The linear mixed model fitted was:

$$Y_{ijkl} = \mu + W_i + L_j + D_k + (WL)_{ij} + (WD)_{ik} + (LD)_{jk} + (WLD)_{ijk} + e^{A}_{(jk)l} + e^{B}_{(ijk)l} \quad [1]$$

where Y_{ijkl} was the response variable recorded each wk W (i = 1,..., 4) from line L (j = 1 or 2, for HA or LA, respectively) randomly assigned to diet D (k = 1, ..., 4, for G, S, GS or C, respectively) by cage (l = 1,…,6), and μ was the overall mean. Week, line and diet were fitted as fixed effects, along with their interactions. Random terms were cage nested within the line by diet ($e^{A}_{(jk)l}$), and residual ($e^{B}_{(ijk)l}$). Since the time interval between successive measurements was constant (a single wk), an auto-regressive covariance structure among weeks with homogenous variances was assumed. Differences among lines and diets, singly and in combination, were tested across all levels of time (wk). The error term used to form these test statistics was $e^{A}_{(jk)l}$.

In addition, for those measurements collected weekly (i.e., BW, feed intake, plasma enzymes, hematocrit), a linear mixed model was fitted using the MIXED procedure as:

$$Y_{ijkl} = \boldsymbol{\beta} x_i \left(L_j + D_k + (LD)_{jk} \right) + e^{A}_{(jk)l} + e^{B}_{(ijk)l} \quad [2]$$

where the vector **β** was regression coefficients (intercept, and linear and quadratic terms) for the covariate wk (x). In order to model differences in the values of these coefficients by line and diet and line by diet their interaction with these effects was fitted. The error term used to test the significance of the regression coefficients, in conjunction with line, diet, and the line-diet interaction, was $e^{A}_{(jk)l}$.

Liver enzyme measurements were analyzed using ANOVA. The model fitted was:

$$Y_{jkl} = \mu + L_j + D_k + (LD)_{jk} + e_{(jk)l} \quad [3]$$

where Y_{jkl} was the average enzyme activity level in cage l within a line j and diet k, and $e_{(jk)l}$ was the residual. Comparisons of means for line, diet and their interaction were based on orthogonal contrasts.

Histological measurements were analyzed in two ways. First, scores for each histological measurement (lipidosis, heterophils, and lymphoid nodules) were averaged for each cage and analyzed using ANOVA with model [3]. Secondly, the scores on individual chickens were analyzed using a chi-square test.

RESULTS

Feed Analyses

All diets were similar for DM, CP, crude fat, crude fiber, and ash content. Most importantly, there were no differences in lysine, methionine, or cysteine content (Table 4.2). Furthermore, free gossypol content was similar in the G and G-S diets.

Body Weight and Feed Intake

On average, chickens on all treatments gained weight during the first 2 wk of the experimental period. By wk 2, weight gains in chickens fed G and G-S ceased, and remained unchanged thereafter (Figure 4.1). Based on the repeated measures analysis (model [1]), by wk 3 ($P < 0.001$) these chickens had lower BW than C and S chickens, with the difference increasing thereafter (Table 4.3). The LA chickens gained less weight, on average, than HA chickens ($P < 0.001$) regardless of diet (Table 4.4).

Slope of the regression of BW on wk (model [2]) for G and G-S fed chickens differed from that of C and S chickens ($P < 0.001$), further substantiating that these chickens gained less weight than C and S chickens. Furthermore, BW gains in C and S chickens were relatively constant over time [only the value of the slope differed from zero ($P < 0.001$)]; whereas the BW gains in G and G-S chickens slowed necessitating the fit of a quadratic term in the model ($P < 0.001$).

During the first 2 wk, there were no differences in feed intake among diets ($P = 0.891$). However, during wk 3, based on the repeated measures analyses, chickens fed diet G and G-S consumed less feed (on average, 0.74 times as much; $P = 0.001$) than chickens fed C and S diets (Figure 4.2; Table 4.5). During the last wk of treatment, LA chickens fed G and G-S diets consumed less feed (on average, 0.73 times as much; $P < 0.001$) than LA chickens consuming C and S diets. Also, HA chickens fed diet G and G-S consumed less feed than HA chickens fed C and S (on average 0.75 times as much, $P < 0.001$). However, there were no differences in feed intake between C and S treatments for HA and LA chickens.

With the fit of the regression, there was a difference between G and G-S fed chickens with S and C fed chickens in the shape of their food intake curves; for the pairs of treatments, both the values of their linear and quadratic coefficients differed ($P < 0.001$). Differences in slope reflected chickens fed diets G and G-S eating less than chickens fed S and C diets in general over the length of the experiment. Differences in the quadratic terms coincided with more substantial reduction in feed intake of G and G-S chickens after wk 3, as compared to that of S and C chickens.

Liver Enzymes

There were no differences among diets (model [3]) for GST or CYP450 3A4 activity ($P = 0.126$ and $P = 0.647$, respectively). Quinone reductase activity (Table 4.6) was higher ($P < 0.001$) in chickens fed diet G and G-S than those fed diets S and C. Line had no effect on QR ($P = 0.615$), GST ($P = 0.333$), or CYP450 3A4 activity ($P = 0.078$).

Histological Measurements

There were no line-diet interactions for histological measures. Lipidosis in chickens fed G and G-S diets was approximately 1.7 times that of chickens fed diets S and C ($P < 0.001$). However, there were no histological signs that damage in the G and G-S treated chickens was

irreversible. Diet had no effect on lymphoid nodules ($P = 0.070$) or heterophils ($P = 0.079$). Unlike diet, line had no effect on lipidosis score ($P = 0.413$); although, LA chickens had more (1.69 ± 0.097) heterophils than HA chickens (0.90 ± 0.097) ($P < 0.001$). High antibody line chickens had a greater number (1.45 ± 0.054) of lymphoid nodules compared to LA chickens (1.00 ± 0.054) ($P < 0.001$).

Chi square analysis was also performed on these data. More chickens fed G and G-S diets (n = 44) had category 2 and 3 lipidosis scores than those fed S and C diets (n = 10) ($P < 0.001$). As with the ANOVA, there were no differences in heterophils ($P = 0.336$) or lymphoid nodules ($P = 0.415$) among diets. More LA chickens (n = 35) had category 2 and 3 heterophil scores than to HA chickens (n = 6) ($P < 0.001$) and there were more HA chickens (n = 30) with category 2 and 3 lymphoid nodules scores than LA chickens (n = 2) ($P < 0.001$). However, as found with ANOVA, there were no differences in lipidosis between lines ($P = 0.842$).

Clinical Chemistry

Chickens fed diet G and G-S had higher GGT levels than those fed diets C and S diets beginning at wk 2 (Table 4.7). Based on the repeated measures analysis (model [1]), this difference became substantially larger during wk 3 ($P < 0.001$). Irrespective of diet, GGT level in HA chickens was, on average, 1.2 times that of LA chickens ($P = 0.002$; Table 4.4). However, there was a line-diet-wk interaction beginning at wk 2 ($P = 0.014$), which increased by wk 3 ($P < 0.001$). Thus the difference between lines became larger by wk 3 depending on diet.

Including both the linear and quadratic coefficients in the regression (model [2]) of GGT on wk improved the fit of the statistical model. For chickens fed G and G-S diets, GGT activities increased consistently with wk throughout the experiment (Figure 4.3). The shape of that relationship appeared curvilinear in chickens fed C and S diets and the linear ($P < 0.001$) and the quadratic coefficients ($P = 0.093$) differed among these pairs of treatments. When comparing lines, e values of the intercept ($P < 0.001$) and quadratic coefficient ($P = 0.019$) differed between HA and LA chickens.

Chickens on all treatments had similar AST levels during the first wk of treatment (Table 4.8). During the second wk, chickens fed diets G and G-S had substantially higher (on average, 2.0 times as much) AST levels than those fed diets C and S diets ($P < 0.001$). However, by the

third wk, chickens fed diets G and G-S had substantially lower (on average, 0.27 times as much) AST levels as compared to chickens fed C and S diets ($P < 0.001$).

Similar to GGT, including both the linear and quadratic coefficients in the regression of AST on wk improved the fit of model [2]. For chickens fed C and S diets, AST activities increased consistently with wk throughout the experiment; the relationship for chickens fed G and G-S diets instead was curvilinear. In chickens fed C and S diets, only the values of the slope differed from 0 ($P < 0.001$); however, for chickens fed G and G-S diets, a quadratic term also had to be fitted ($P < 0.001$). Only values of the intercepts for the regression of AST on wk differed in HA and LA chickens ($P = 0.029$).

Diet had no effect on hematocrit values. As shown in Figure 4.4, hematocrit values were reduced as the experiment progressed. Still, across wk, hematocrit values in HA chickens were, on average, 1.1 times that of LA chickens ($P < 0.001$).

There was no relationship between hematocrits and wk among diets (linear term: $P = 0.164$; quadratic term: $P = 0.935$). Intercepts for regression of hematocrit on wk for HA and LA chickens differed ($P < 0.001$; Figure 4.4).

DISCUSSION

Dietary Effects

Reduced BW and feed intake are common signs of gossypol toxicosis (Mohan et al., 1989; Henry et al., 2001; Nagalakshmi et al., 2007). Gossypol binds essential amino acids such as lysine (Henry et al., 2001). Reduced feed intake could reflect insufficiencies in essential nutrients. However, feed analyses indicated that there were no differences in amino acid composition among diets (Table 4.2). It is more likely that reduced feed intake was a consequence of ill health, with the depression in weight gains in chickens fed the gossypol diets reflecting voluntary reductions in feed intake rather than nutrient composition of the diet per se. Addition of silymarin to the diet did not offset the effects of gossypol on BW or feed intake in G-S fed chickens.

Gamma glutamyltransferase and AST are physiological markers of liver health. High plasma GGT has been associated with biliary hyperplasia and bile duct carcinoma (Harr, 2002). Significantly higher GGT values in G and G-S treated chickens implied that gossypol was causing damage to biliary ducts. Furthermore, average plasma GGT concentrations during the

final wk for G and G-S treated chickens were 12.9 and 13.8 U/L, respectively. These values were above the normal range of 0-10 U/L for birds (Harr, 2002).

Plasma AST increased in chickens fed diets G and G-S during the second wk of treatment, which indicated that gossypol was eliciting a toxic response. Surprisingly, AST levels dropped precipitously in these chickens during the last wk of treatment, which coincided with lower feed intake. Reduced feed intake may have resulted in reduced exposure to gossypol allowing liver recovery. There was no histological evidence that permanent liver damage had occurred; therefore, liver recovery may have begun once gossypol exposure was reduced.

The AST values observed in this study do not coincide with those reported elsewhere in the literature (Hrubec et al., 2002). Chickens fed G and G-S diets had AST values 10-fold lower than reported as normal in mammalian species. Although the spectrophotometric kit used to assay AST has been validated in mammalian species, it had not been validated in birds (Biotron Diagnostics; Hemet, CA). Therefore, it is possible that chicken plasma contains matrix constituents not encountered by the kit's manufacturer that interfere with absorbance. Although this means that AST values in this experiment cannot be compared to mammalian values, none-the-less G and G-S treated birds had significantly different values than C and S treated birds.

A silymarin concentration of 600 mg/kg reduced plasma GGT and AST in broilers with aflatoxicosis (Tedesco et al., 2004). Furthermore, silymarin significantly reduced these enzymes in human patients with liver disease (Wellington and Jarvis, 2001). However, in this experiment, plasma AST and GGT levels were not 'improved' (e.g., lower level compared to G treated chickens) in the G-S treated group.

Gossypol causes erythrocyte fragility and has been repeatedly reported to decrease hematocrits (Henry et al., 2001; Matondi et al., 2007; Adeyemo, 2008). No differences in hematocrits were observed among diets in this study, which is contrary to most literature. However, a study conducted in 7 wk old chickens fed gossypol by oral intubation did not significantly influence hematocrit values (Kalla and Chadha, 1990).

Quinone reductase is a phase II cytosolic enzyme that is responsible for the reduction of quinones produced through the metabolism of dietary phenols, aromatics, and polycyclic aromatic compounds (Talalay and Dinkova-Kostova, 2004). Gossypol is a polyphenolic compound, making it a prime substrate for QR. Therefore, it is not surprising that QR activity was increased in chickens on diet G and G-S to compensate for increased quinone production.

Silymarin is hypothesized to be a phase II enzyme inducer (Zhao and Agarwal, 1999). However, QR activity in chickens on diet S was not different from C. Furthermore, QR activity in chickens fed diet G-S was not greater than G fed chickens.

Glutathione-S-transferase is also an important phase II enzyme. Gossypol reportedly binds to GST preventing it from binding with normal substrates. This causes a reduction in GST activity (Lee et al., 1982; Sugiyama et al., 1984). Kiruthiga et al. (2007) reported that silymarin prevented reduction in GST activity. There were no differences in GST activity between G and C treated chickens in this experiment. Therefore, there is no reason to expect that silymarin would have a therapeutic effect on GST activity in G-S treated chickens.

Cytochrome P450 3A4 is an important phase I enzyme in xenobiotic metabolism. Several studies have shown that gossypol and silymarin both inhibit cytochrome P450 activity (Ma and Black, 1984; Johansen and Misra, 1990; Kiruthiga et al., 2007). An in vitro study with gossypol concentrations of 25, 50, 100, and 250 μM indicated that CYP450 3A4 inhibition depended on silymarin concentration and length of exposure (Sridar et al., 2003). Cytochrome P450 3A4 was not inhibited in this experiment. There was no difference in activity among diets. The concentration of silymarin may have been insufficient to elicit such a response. Additionally, Kosina et al. (2005) reported that silymarin did not inhibit CYP450 3A4 activity in human hepatocytes in culture.

Lipidosis scores were higher in chickens fed diet G and G-S than those fed the other diets which are indicative of lipid storage and thus liver malfunction (Plaa and Charbonneau, 2008). Lipidosis is reversible and there were no histological indicators of permanent liver damage. Thus, it can be hypothesized liver health would be improved in G and G-S treated chickens if they were switched to a gossypol free diet. However, if exposed to a high enough dosage of gossypol for a substantial time period, irreversible damage would be possible.

Line Sensitivities to Diet

There was no overwhelming evidence that the HA and LA chickens responded differently to the diets. Low antibody chickens fed diets G and G-S consumed less feed and weighed less than HA chickens. This is contradictory to a toxicology study with these lines, in which LA chickens weighed more than HA chickens (Ubosi et al., 1984). However, this experiment studied aflatoxin, an established immunotoxin (Bondy and Pestka, 2000). Therefore, it is not

unreasonable to assume that chickens selected for immune response would respond differently to an immunotoxin than to gossypol.

In terms of clinical chemistry, LA chickens had higher AST concentrations than HA chickens, although the opposite was the case for GGT concentrations. These results do not suggest a greater sensitivity to gossypol toxicity in one line than the other. Differences in lymphoid nodules and heterophils between HA and LA chickens were independent of diet. Therefore, these hematopoietic differences appear simply due to the long-term selection for antibody response (Gross and Siegel, 1980).

CONCLUSIONS

Silymarin did not alleviate the effects of gossypol toxicosis. None of the criteria used to assess liver health were improved in chickens on the combined gossypol and silymarin diet. Furthermore, silymarin alone did not affect liver function in the way reported previously in the literature. One of the main concerns with silymarin is its bioavailability. There are several reports that silymarin is not very lipid soluble. Thus silymarin often needs to be administered within a phospholipid complex or i.v. (Bares et al., 2008; Ferenci et al., 2008). Silymarin was added directly to the diet without any alterations. Therefore, bioavailability may be a contributing reason silymarin did not elicit the physiological responses previously reported. It is also possible the concentration of gossypol used in the diets was too high, so that no matter the amount of silymarin fed damage would occur. Perhaps, gossypol fed at a level between 0.05 and 0.10% in the diet would cause toxicosis yet still respond to silymarin treatment. Future studies should test various concentrations of gossypol in the diet. In addition, there was no substantial difference in response to gossypol in the HA and LA lines. Long-term selection for humoral immune response appears to offer no advantage in coping with gossypol toxicosis.

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Table 4.1 Scoring systems for histological analyses

Score	Lipidosis	Heterophils	Lymphoid Nodules
0	None	None	None
1	25% of cells with cytoplasm vacuolated	Few in some portal areas	1 per 4x magnification
2	50% of cells with cytoplasm vacuolated	Few in half of portal areas	2-3 per 4x magnification
3	100% of cells with cytoplasm vacuolated	Few in all portal areas	>3 per 4x magnification

Table 4.2 Chemical composition of diets

		DM Basis (%)				W/W%[1]			%
Diet	DM (%)	Crude Protein	Crude Fat	Crude Fiber	Ash	Cysteine	Lysine	Methionine	Free Gossypol
Gossypol	89.0	24.1	3.8	4.5	5.9	0.36	1.19	0.31	0.040
Gossypol + Silymarin	88.9	23.8	3.8	4.9	6.0	0.37	1.21	0.32	0.043
Silymarin	89.1	24.4	3.8	5.7	6.1	0.37	1.20	0.31	----------
Control	89.2	23.5	3.6	5.0	5.7	0.37	1.22	0.32	----------

[1] W/W% = g/100 g of sample

Table 4.3 Least squares means for BW across lines for chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

	BW (g) by wk[1]			
Diet	0	1	2	3
Gossypol	446.4[a]	537.6[a]	595.3[a]	577.1[a]
Gossypol + Silymarin	444.3[a]	527.4[a]	563.0[a]	538.1[a]
Silymarin	439.7[a]	546.9[a]	655.6[a]	773.3[b]
Control	436.3[a]	546.4[a]	657.6[a]	769.6[b]

[1] SEM = 11.7, error df = 51

[ab]Means in the same column with different superscripts are different ($P < 0.001$)

Table 4.4 Least squares means for weekly BW, feed intake, gamma glutamyltransferase (GGT), and aspartate aminotransferase (AST) for high antibody (HA) and low antibody (LA) chickens across diets

	BW (g) by wk[1]				Feed Intake (g) by wk[2]				GGT (U/L) by wk[3]				AST (U/L) by wk[4]			
Line	0	1	2	3	0	1	2	3	0	1	2	3	0	1	2	3
HA	445.5[a]	544.7[a]	624.9[a]	686.9[a]	272.3[a]	340.5[a]	372.5[a]	372.5[a]	9.2[a]	6.0[a]	10.9[a]	11.6[a]	1.2[a]	3.5[a]	6.5[a]	6.5[a]
LA	437.8[a]	534.5[a]	610.8[a]	642.8[b]	278.2[a]	350.2[a]	391.8[a]	381.9[a]	6.2[a]	6.6[a]	9.1[a]	10.0[a]	1.1[a]	6.2[a]	9.4[a]	7.0

[1]Maximum SEM = 8.3, error df = 51; [2]Maximum SEM = 5.4, error df = 73; [3]Maximum SEM = 0.51, error df = 40; [4]Maximum SEM = 1.5, error df = 40

[ab]Means in the same column with different superscripts are different ($P < 0.01$)

Table 4.5 Least squares means for weekly feed intake (per bird) across lines for chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

	Feed intake (g) by wk[1]			
Diet	0	1	2	3
Gossypol	276.1[a]	345.5[a]	378.8[a]	343.3[a]
Gossypol + Silymarin	276.4[a]	347.1[a]	366.4[a]	299.4[a]
Silymarin	273.6[a]	347.3[a]	391.7[a]	437.1[b]
Control	274.9[a]	338.2[a]	391.7[a]	431.8[b]

[1]SEM = 7.6, error df = 73
[ab]Means in the same column with different superscripts are different ($P < 0.001$)

Table 4.6 Least squares means for quinone reductase (QR) activity across lines on chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

Diet	QR Activity[1] (nmoles NADH oxidized/min per mg protein)	GST Activity[2] (mmoles GSH[3] conjugated/min per mg protein)	CYP450 3A4[4] (μmoles 7-BQ[5]/min per mg protein)
Gossypol	3.20[a]	0.017[a]	0.239[a]
Gossypol + Silymarin	3.45[a]	0.015[a]	0.246[a]
Silymarin	2.82[b]	0.016[a]	0.261[a]
Control	2.75[b]	0.014[a]	0.281[a]

[1]SEM = 0.144, error df = 40
[2]SEM = 0.009, error df = 40
[3]Glutathione
[4]SEM = 0.025, error df = 40
[5]Benzyloxyquinoline
[ab]Means in the same column with different superscripts are different ($P < 0.001$)

Table 4.7 Least squares means for gamma glutamyltransferase (GGT) across lines for chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

	GGT (U/L) by wk[1]			
Diet	0	1	2	3
Gossypol	7.6[a]	6.0[a]	11.0[a]	12.9[a]
Gossypol + Silymarin	7.6[a]	6.0[a]	10.3[a]	13.8[a]
Silymarin	7.8[a]	7.0[a]	9.6[a]	7.7[b]
Control	7.8[a]	6.2[a]	9.2[a]	9.1[b]
SEM	0.39	0.72	0.59	0.64

[1] Maximum SEM = 0.72; error df = 40
[ab]Means in the same column with different superscripts are different ($P < 0.01$)

Table 4.8 Least squares means for aspartate aminotransferase (AST) across lines for chickens fed gossypol, gossypol and silymarin, silymarin, and control diets

	AST (U/L) by wk[1]			
Diet	0	1	2	3
Gossypol	1.1[a]	4.0[a]	7.6[a]	3.5[a]
Gossypol + Silymarin	1.3[a]	3.8[a]	13.4[a]	2.4[a]
Silymarin	0.8[a]	6.9[a]	5.1[b]	9.4[b]
Control	1.5[a]	4.6[a]	5.8[b]	11.7[b]
SEM	0.3	0.7	2.1	1.3

[1]Maximum SEM = 2.1; error df = 40
[ab]Means in the same column with different superscripts are different ($P < 0.001$)

Figure 4.1 Average body weight across lines for chickens fed gossypol (**G**), gossypol-silymarin (**G-S**), silymarin (**S**), and control (**C**) diets by wk. Gossypol and G-S fed chickens weighed less than S and C fed chickens at wk 3 (P <0.001) with this difference increasing thereafter.

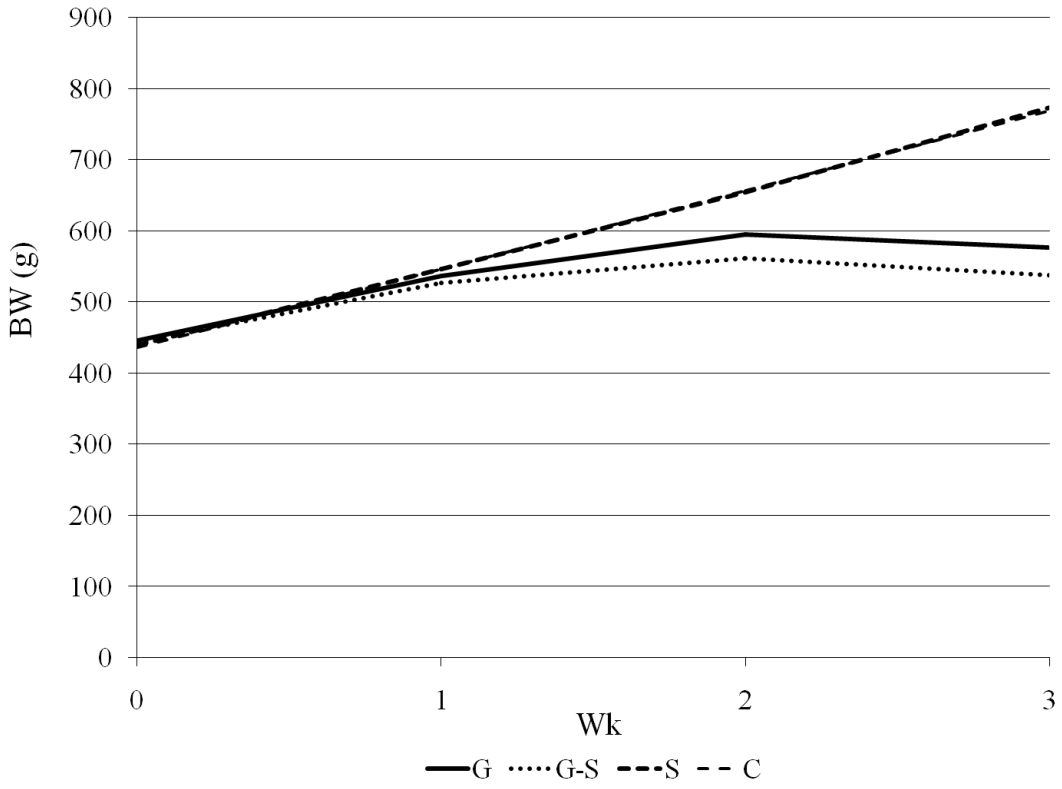


Figure 4.2 Average weekly feed intake (per bird) across lines for chickens fed gossypol (**G**), gossypol-silymarin (**G-S**), silymarin (**S**), and control (**C**) diets by wk. At wk 3 G and G-S fed chickens consumed less feed than C and S ($P = 0.001$).

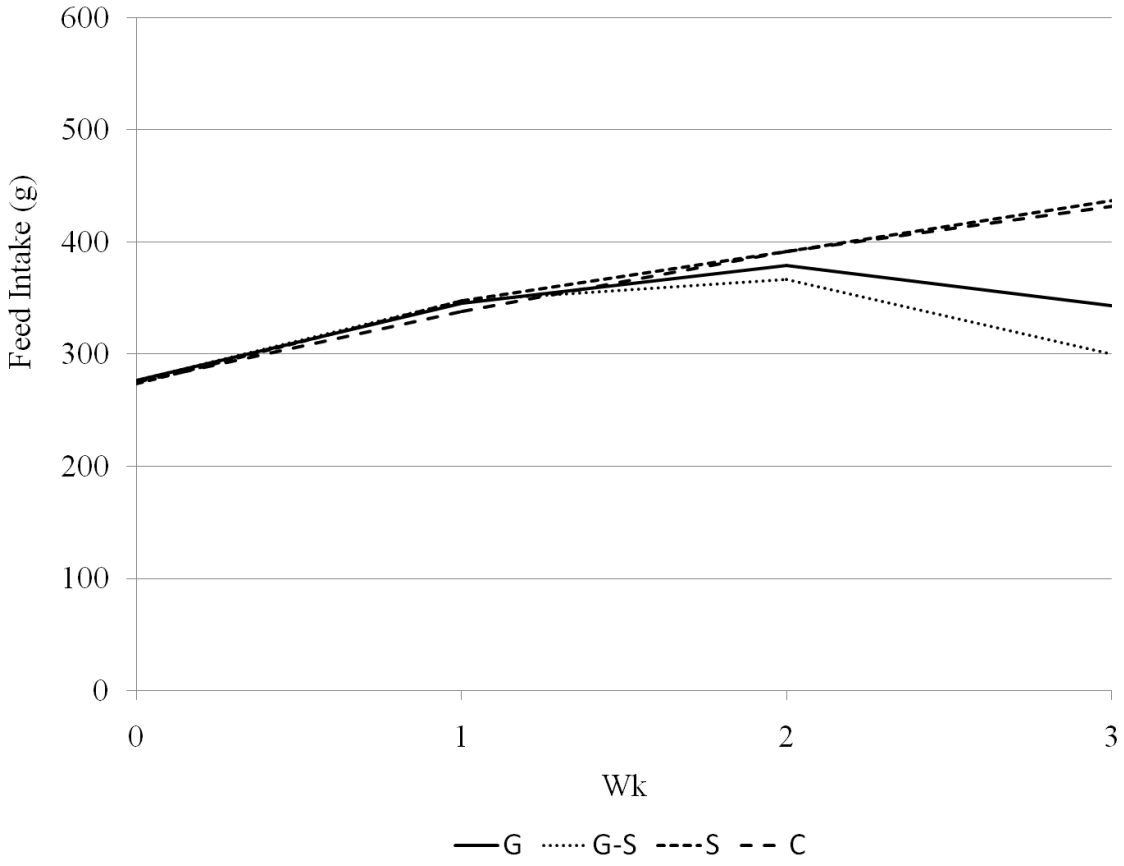


Figure 4.3 Gamma glutamyltransferase activities across lines for a chicken fed gossypol (**G**), gossypol-silymarin (**G-S**), silymarin (**S**), and control (**C**) diets by wk. Chickens fed G and G-S had higher GGT values at wk 2 which became substantially larger by wk 3 ($P < 0.001$).

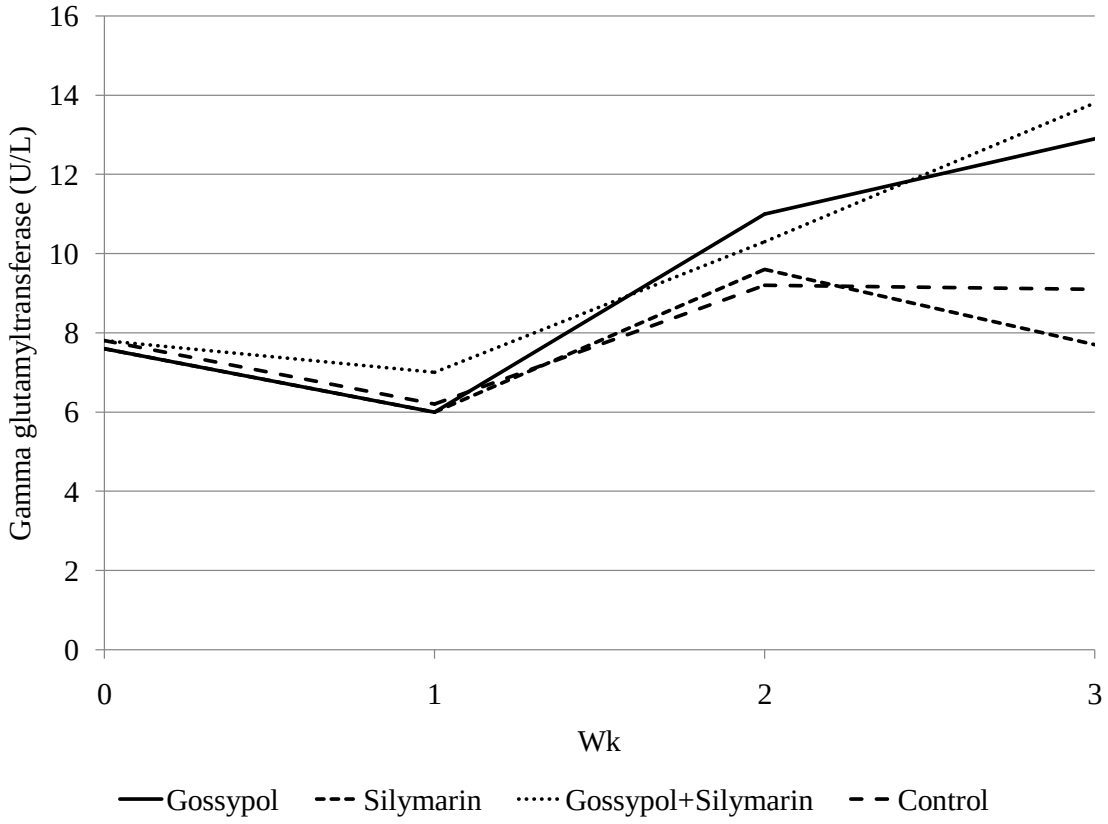
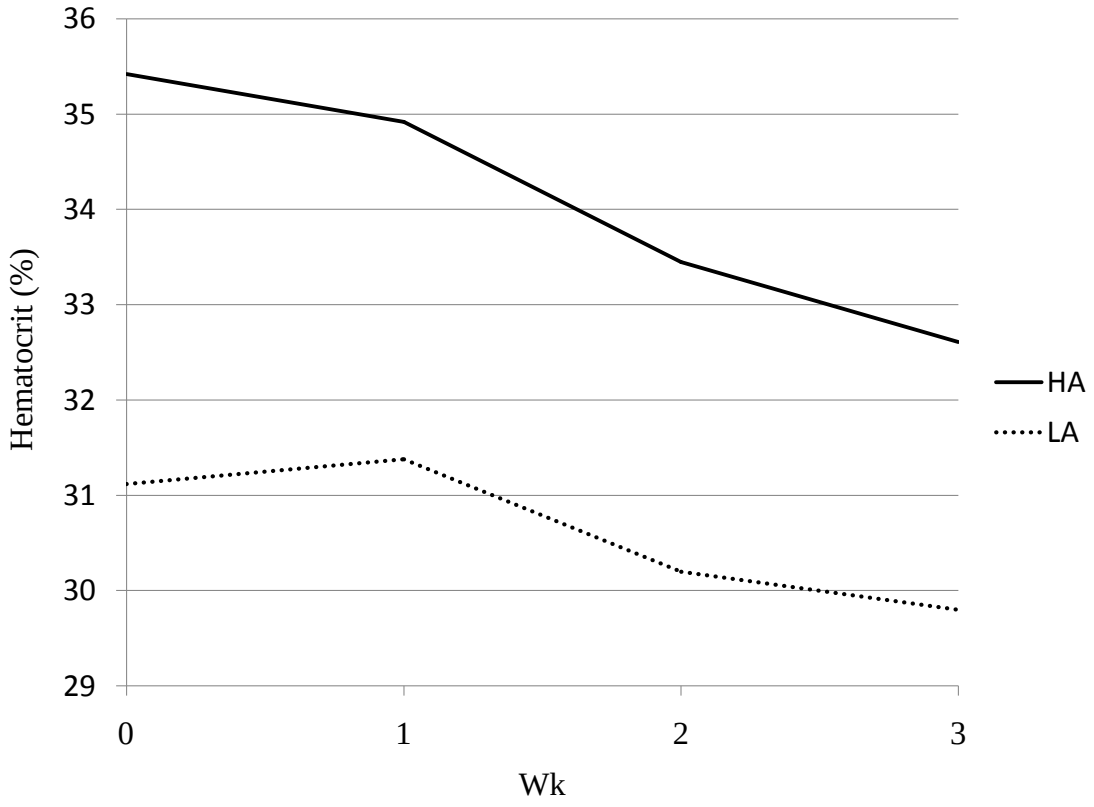


Figure 4.4 Hematocrit across treatments for chickens of high antibody (**HA**) and low antibody (**LA**) response lines . High antibody select chickens had higher hematocrits that LA chickens throughout the experimental period ($P < 0.001$).



Chapter 5: Conclusions

Chapter 2

An understanding of basal activities of drug metabolizing enzymes is essential to any toxicology study. Thus the study in chapter 2 was conducted to establish these activities. However, due to unavoidable circumstances, chickens in this study came from 3 separate hatches. Therefore, the possibility of a hatch effect could not be ruled out. Although all birds came from the same parents, at times differences in enzyme activity seemed to coincide with a difference in hatch. The possibility of a hatch effect corroborates the idea that not only age, genetic background, and sex impact enzyme activity but factors such as environment and age of sire and dam. Therefore, literature values can be used as a reference but should not be considered absolute. Although reference values are important, they can never replace data obtained from a control population in a toxicological experiment.

In this experiment, glutathione-S-transferase (**GST**) and quinone reductase (**QR**) activity changed very little over time. It is possible that the time points chosen (i.e., 4, 8, 12, 16, 20 wk of age) reflect ages where physiological maturity had very little effect on enzyme activity. More dynamic time periods may be in younger birds, particularly in the early days post hatch. Young birds are developing quickly and, therefore, enzyme activity may concurrently need to develop early on to help the chick cope with its environment. Also, it is possible that older birds would be more interesting. Birds under more physiological stress, such as a female during lay, may also experience a change in enzyme activity.

Chapters 3 and 4

Silymarin did not have an effect on either gossypol treated or untreated chickens. This prompts the question: "at what concentration of silymarin were these birds exposed?" Silymarin was added to the diet at a concentration of 1000 ppm with no response from the bird. It is likely that the bioavailability of silymarin was less than 100%. In humans, i.v. injections of milk thistle compounds have had more success than oral doses (Ferenci et al., 2008). Although not practical for use in production systems, i.v. injection of silymarin provides an alternative to ensure that silymarin reaches the blood stream unaltered by digestion. It may be helpful to bleed birds every hour for a few hours and then assay the plasma for antioxidant content. In theory an elevated plasma antioxidant content would indicate that the birds are being exposed to silymarin.

Dietary concentrations of gossypol and silymarin for future studies need to be carefully considered. The goal was to use a concentration of gossypol that caused a toxicosis. This goal was met. However, it is possible that this concentration coincided with the plateau area of a toxic dose-response curve for gossypol. Lower concentrations of gossypol may elicit a toxic response yet may be more responsive to silymarin treatment. The ideal would be to find the concentration that on the threshold of the dose-response plateau.

Gossypol is a very potent toxin for male reproduction. No indices of fertility or reproductive competence were assessed in either study. Males fed 1000 ppm gossypol had visibly smaller testes and underdeveloped secondary sex characteristics (i.e. combs). Measures such as serum testosterone, testes weight, and sperm motility and morphology would be useful data to collect to corroborate visual observations. Furthermore, adrenal glands appeared to be enlarged. Serum cortisol would provide insight into why these glands may be enlarged and provide further insight into the amount of stress placed upon the bird.

While not as significant as the liver, the kidney does have the ability to detoxify toxins. The kidney is difficult to dissect in birds, particularly in an ovulating female because it lies in the back of the body cavity. Also, the kidney has a sponge-like consistency, which makes it difficult to keep intact. Despite these difficulties, it would be interesting to study enzyme activities in the kidney. Toward the end of each experiment, birds with 1000 ppm gossypol in their diet (both with and without 1000 ppm silymarin added) appeared to produce substantially more urates than feces. This might have reflected a reduction in water intake, although this was not measured. Something was happening in the kidney not quantified elsewhere in this study.

Overall

There were similarities in enzyme activities levels among control birds across the 3 experiments. Chickens in the second experiment (chapter 3) were approximately 10 wk old when the experiment began, and were about 14 wk when livers were collected. The enzyme data from the control birds can be compared to the 12 and 16 wk data from the first experiment (chapter 2). Quinone reductase activity in birds from the second experiment was approximately half that of 12 and 16 wk birds from the first experiment. In males, cytochrome P450 3A4 (**CYP450 3A4**) activity was much lower in 12 and 16 wk birds (1.19 and 0.73 μmoles 7-benzyloxyquinoline (**7-BQ**) oxidized/min per mg protein) compared to experiment 2 birds (2.59

μmoles 7-BQ oxidized/min per mg protein). However, females from experiment 2 had CYP450 3A4 activity that was remarkably similar to that of 12 wk birds (2.75 and 2.66 μmoles 7-BQ oxidized/min per mg protein, respectively). Female GST activity was basically the same between experiment 2 and 12 wk birds from experiment 1 (0.092 and 0.091 μmoles GSH conjugated/min per mg protein). In males, neither CYP450 3A4 nor GST activities were similar among birds from experiments 1 and 2.

Birds from the third experiment (chapter 4) were approximately 5 wk old when the experiment began and were thus10 wk of age when livers were collected. Control birds from this experiment therefore could be compared to 8 wk old birds from experiment 1. Quinone reductase activity was very similar between experiments 1 and 3 (2.46 and 2.75 nmoles NADH oxidized/min per mg protein, respectively). This is unsurprising because the birds were all untreated and approximately the same age. Glutathione-S-transferase was approximately 8 times greater in experiment 1 birds, and CYP450 3A4 activity was approximately 12 times greater. As mentioned previously, in experiment 1 there was concern about a hatch effect. These birds came not only from different hatches but also from different parents. This could account for some of the difference. A more likely reason for these differences, however, is that the birds from experiment 1 were managed differently than those from experiment 3. Birds from the first experiment had a different lighting schedule (14 h light; 10 h dark) and were also housed on wood shavings in floor pens compared to 24 h light exposure and caged housing.

Finally, a comparison between the 1000 ppm gossypol and silymarin treated birds of experiments 2 and 3 can be made. Average QR activity was exactly the same between the two experiments. This is unsurprising. Quinone reductase is responsible for reducing environmental quinones into quinols. Due to its chemical structure, gossypol is an excellent substrate for QR. Quinone reductase activity however was quite different between silymarin treated birds (1.67 and 2.82 nmoles NADH oxidized/min per mg protein). This could be attributed to the difference in age of the birds. However, no age differences in QR activity were found in the basal enzyme study. Glutathione-S-transferase activity was very similar among gossypol treated birds, but not silymarin treated birds. Cytochrome P450 3A4 activity was much larger in experiment 2 birds than experiment 3. These differences may go back to the fact that the birds came from different hatches.

Final Conclusions

Silymarin has been used extensively to treat liver disorders in humans. It remains a potential hepatoprotective agent against gossypol toxicosis. However, there is much more to be done in order to harness the protective power of silymarin. Dietary concentrations of silymarin and gossypol and the type of biomarkers measured collected are just a few of the factors that should be considered in future studies.

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