

The response of meat ducks from 15 to 35 d of age to gossypol from cottonseed meal

Q. F. Zeng,^{*,1} P. Bai,^{*} J. P. Wang,^{*} X. M. Ding,^{*} Y. H. Luo,^{*} S. P. Bai,^{*} Y. Xuan,^{*} Z. W. Su,^{*}
S. Q. Lin,[†] L. J. Zhao,[†] and K. Y. Zhang^{*}

^{*}*Institute of Animal Nutrition, Key Laboratory for Animal Disease-Resistance Nutrition of China Ministry of Education, Sichuan Agricultural University, Chengdu, Sichuan, China, 611130; and* [†]*ChengDu Feed Measure Center, Chengdu, Sichuan, China, 610041*

ABSTRACT The objective of this study was to investigate the responses of meat ducks of 15 to 35 d of age to free gossypol (FG) from cottonseed meal (CSM) and to establish the maximum limits of dietary FG concentration based on growth performance, blood parameters, and tissue residues of gossypol. Nine hundred 15-d-old ducks were randomly allocated to 5 treatments with 10 cages/treatment and 18 ducks/cage on the basis of BW. Five isonitrogenous and isocaloric experimental diets were formulated on a digestible amino acid basis to produce diets in which 0% (without FG), 25% (36 mg FG/kg), 50% (75 mg FG/kg), 75% (111 mg FG/kg), and 100% (153 mg FG/kg) of protein from soybean meal were replaced by that from CSM. Increasing dietary FG content, BW, and ADG decreased (linearly, $P < 0.05$, except for ADG of days 29 to 35),

and F/G linearly increased ($P < 0.05$). At 35 d, blood hemoglobin, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration linearly decreased ($P < 0.05$), while serum total protein, albumin, and globulin content linearly decreased ($P < 0.05$), and the residue of gossypol in liver, kidney, heart, breast, and leg muscle linearly increased ($P < 0.001$) with increases in dietary FG concentration. Ducks fed 36 mg FG/kg (5.83% CSM of diet) diet had a normal histological structure of liver, and muscle (breast and leg) had no residue of gossypol. The maximum limit of dietary FG concentration was estimated to range from a low of 36 mg/kg to maximize serum globulin concentration to a high of 124 mg/kg to minimize feed intake for 22 to 28d on the basis of a quadratic broken-line model.

Key words: duck, gossypol, hepatic histopathology, hematology, serum biochemical profile, residue of gossypol

2015 Poultry Science 94:1277–1286
<http://dx.doi.org/10.3382/ps/pev070>

INTRODUCTION

Cottonseed meal (CSM) is both a cheap coproduct and a good source of protein, similar to soybean meal (SBM), and could be used to replace SBM. Although lysine content and its digestibility are low in CSM, this can be partially offset by diets formulated on a digestible amino acid basis (Gamboa et al., 2001a). The risk of gossypol toxicity is the primary concern of the poultry industry, limiting the incorporation of CSM in poultry feeds. Several studies conducted on broilers have proved that gossypol can reduce performance, increase mortality and concentrations of free gossypol

(FG) in tissues of chickens (Henry et al., 2001; Gamboa et al., 2001a; Lordelo et al., 2005; Adeyemo and Longe, 2007; Adeyemo, 2010). Henry et al. (2001) found that broilers fed 1,600 mg purified gossypol/kg of diet resulted in significantly enlarged gallbladder, perivascular lymphoid aggregate formation, biliary hyperplasia, and hepatic cholestasis. This indicated that liver toxicity was the critical effect of gossypol on the health of the chicken. Blevins et al. (2010) observed that gamma glutamyltransferase was also elevated in chickens fed 1,000 mg gossypol/kg of diet at day 14, and its activities increased further by day 21. Zeng et al. (2014) found that the dietary total gossypol (TG) and FG concentration from CSM should be lower than 929 and 77 mg/kg of diet based on growth performance and health of 0 to 21d of age of meat ducks, respectively, and observed that meat ducks were more sensitive to dietary FG concentration than broilers. However, no research has been done on the effects of CSM or gossypol from CSM on the growth performance and health of meat ducks from 15 to 35d of age.

The other toxic effect of FG is that can combine with ferric ion in blood, subsequently affecting erythrocyte

© The Author 2015. Published by Oxford University Press on behalf of Poultry Science Association. This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com.

Received September 15, 2014.

Accepted January 12, 2015.

¹Corresponding author: zqf@sicau.edu.cn

oxygen-carrying or oxygen-releasing capacity (Brocas et al., 1997). Yildirim et al. (2003) revealed that the red blood cell (**RBC**) count of fish fed the two highest levels of dietary gossypol (1,200 and 1,500 mg) was significantly lower than that of fish fed a control diet; hemoglobin significantly decreased in fish fed 900 mg or more gossypol diets; hematocrit was significantly affected at each incremental level of dietary gossypol beyond 600 mg/kg or higher. In contrast, Velasquez-Pereira et al. (2002) reported that blood hemoglobin and hematocrit in superovulated beef heifers was not affected by dietary gossypol. To date, no reports have focused on the effect of gossypol or CSM on the hematology of ducks.

Gossypol has a strong tendency to accumulate in animal tissues and is not easily cleared from the animal body (Sharma et al., 1966). This has generated considerable concern about the safety of CSM. Sharma et al. (1966) reported that the levels of gossypol in pig organs were directly related to the level of gossypol in the diet and to the length of time the diet was fed. Larger amounts of gossypol are found in the liver, kidney, heart, and spleen, with lesser amounts found in brain and muscle (Roehm et al., 1967). To our knowledge, there have been no reports of gossypol residue in tissues of meat duck fed CSM. Therefore, the objectives of this study were to determine the effects of graded levels of gossypol from CSM in the diets of 15- to 35-d-old meat ducks on growth performance, hematology, serum biochemical profile, liver histopathology, and gossypol tissue residue and to establish the maximum limits of dietary FG concentration based on growth performance, hematology, and serum biochemical profile.

MATERIALS AND METHODS

Birds, Experimental Design, and Management

One-d-old ducks were obtained from a local duck breeding farm. During the first 2 weeks, ducks were acclimated to the experimental conditions and fed a commercial diet (corn–soybean basal diet, ME = 2,900 Kcal/kg; CP = 19.5%). At 15 d, 900 male ducks (initial weight 669 ± 10 g) were weighed and sorted into 50 cages, with 10 cages/treatment and 18 ducks/cage, according to a completely randomized design. Five isonitrogenous and isocaloric experimental diets were formulated on a digestible amino acid (**AA**) basis. The experimental diets were formulated to produce diets in which 0% (diet 1, control diet, a corn–soybean basal diet, without TG and FG), 25% (diet 2, with 594 mg TG/kg and 36 mg FG/kg), 50% (diet 3, with 1,096 mg TG/kg and 75 mg FG/kg), 75% (diet 4, with 1,725 mg TG/kg and 111 mg FG/kg), and 100% (diet 5, with 2,229 mg TG/kg and 153 mg FG/kg) of protein from SBM were replaced with that from CSM. The TG and FG concentrations of all diets were the analyzed values. The composition and calculated nutrient and energy levels

of all diets are presented in Table 1. Diets 2, 3, and 4 were generated by blending diets 1 and 5 at a ratio of 3:1, 1:1, and 1:3, respectively. Diet 5 was supplemented with L-lysine, HCl, DL-methionine, L-threonine, and L-tryptophan, and it contained the same level of these AA as the control diet on a digestible basis. All diets were formulated to meet or exceed nutrient requirements of meat duck according to NRC (1994) (Table 1). Diets were fed in pellet form. Feed and water were provided ad libitum throughout the experimental period. All ducks were housed in an environmentally controlled facility at 24°C. The experimental protocol used in this study was approved by the Animal Care and Use Committee of Sichuan Agricultural University.

Sampling and Measurement

On days 21, 28, and 35, after 12 h feed withdrawal, ducks were weighed, and feed consumption was recorded by cage. Average daily gain, ADFI, and F/G were calculated. Mortality was recorded daily. One duck with weight closest to the cage average was selected and bled. Approximately 2 mL blood was collected via the vena brachialis and was placed into tubes containing EDTA-Na₂ (an anticoagulant) for subsequent determination of hematological assays, which included the hematocrit (**HCT**, %), hemoglobin (**HGB**, g/L), mean corpuscular volume (**MCV**, fl), mean corpuscular hemoglobin (**MCH**, pg), mean corpuscular hemoglobin concentration (**MCHC**, g/L), and the **RBC** count ($\times 10^2$ /L). These blood parameters were analyzed using an automated hematology analyzer (Alfa Basic 16p, Boule Medical AB, Sweden) within 2 h after blood collection. Approximately 5 mL blood sample were collected from the jugular vein and immediately placed on ice, transported to the laboratory within 3 h of collection, and centrifuged at $2,000 \times g$ at 4°C for 15 min. Serum was collected and stored at –20°C until analysis for biochemical parameters. Serum alanine aminotransferase (**ALT**) and aspartate aminotransferase (**AST**) activity, total protein (**TP**), globin (**GLB**), and albumin (**ALB**) contents were analyzed using biochemistry Analyzer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH).

Once blood was collected, birds were euthanized by cervical dislocation. Samples of liver (right lobes, 10 ducks/treatment) were collected for gossypol content determination and the left lobes (5 ducks/treatment) of the liver were collected to examine the liver for histopathological lesions according to Zeng et al. (2014). The fixed tissues were trimmed and embedded in paraffin. Thin sections (5 μ m) were sliced, mounted on a slide, and stained with hematoxylin and eosin for histopathological examination by a pathologist. Based on the severity of liver steatosis, the severity of lesions was scored subjectively as follows: 0 = normal histological structure (400 \times); 1 = approximately 25% liver cytoplasmic vacuolation and slight funicular hyperplasia of bile duct epithelium were recognizable

Table 1. Dietary ingredient and calculated energy and nutrient contents of per-treatment diets (as-fed basis).

Items	Diet1*	Diet 2	Diet 3	Diet 4	Diet 5
Ingredient (%)					
Corn	71.5	69.8	68.2	66.6	65.0
Soybean oil	1.53	2.32	3.10	3.90	4.69
Soybean meal (CP) (43%)	23.3	17.5	11.6	5.83	—
Cottonseed meal (CP)(43%)	—	5.83	11.6	17.5	23.3
L-lysine.HCl	0.13	0.23	0.32	0.42	0.51
DL-methionine	0.17	0.18	0.19	0.19	0.20
Limestone	0.75	0.79	0.83	0.86	0.90
Dicalcium phosphate	1.03	0.98	0.93	0.87	0.82
L-theronine	0.00	0.04	0.09	0.13	0.17
L-tryptophan	0.07	0.08	0.08	0.09	0.09
Rice bran and hull	0.57	1.26	1.94	2.63	3.31
Sodium chloride	0.35	0.35	0.35	0.35	0.35
Choline chloride	0.15	0.15	0.15	0.15	0.15
Vitamin and mineral premix ¹	0.53	0.53	0.53	0.53	0.53
Total	100	100	100	100	100
Energy and nutrient composition ²					
ME (MJ/kg)	12.54	12.54	12.54	12.54	12.54
Crude protein (%)	16.00	16.00	16.00	16.00	16.00
Ca (%)	0.60	0.60	0.60	0.60	0.60
Available P (%)	0.30	0.30	0.30	0.30	0.30
Digestible lysine (%)	0.81	0.81	0.81	0.81	0.81
Digestible methionine (%)	0.39	0.39	0.39	0.39	0.39
Digestible theronine (%)	0.54	0.54	0.54	0.54	0.54
Digestible tryptophan (%)	0.22	0.22	0.22	0.22	0.22
Free gossypol (analyzed)(mg/kg)	0.00	36	75	111	153
Total gossypol (analyzed)(mg/kg)	0.00	594	1,096	1,725	2,229

¹Vitamin and mineral premix supplied per kilogram diet: vitamin A, 9,000 IU; vitamin D₃, 1,500 IU; vitamin E, 7.5 IU; vitamin B₁, 0.6 mg; vitamin B₂, 4.8 mg; pantothenic acid, 1.5 mg; vitamin B₁₂, 0.009 mg; folic acid, 0.15 mg; niacin 20 mg; Cu (CuSO₄•5H₂O), 8 mg; Fe (FeSO₄•7H₂O), 80 mg; Zn (ZnSO₄•7H₂O), 90 mg; Mn (MnSO₄•H₂O), 70 mg; Se (NaSeO₃), 0.3 mg; I (KI), 0.4mg.

²Calculated values unless indicated otherwise.

*Diet 1 =control group, a basal diet; Diet 2 = 25% protein of SBM in basal diet replaced by that of CSM; Diet 3 = 50% protein of SBM in basal diet replaced by that of CSM; Diet 4 = 75% protein of SBM in basal diet replaced by that of CSM; Diet 5 = 100% protein of SBM in basal diet replaced by that of CSM.

(400×); 2 = approximately 50% liver cytoplasmic vacuolation and serious funicular hyperplasia of bile duct epithelium were observed (400×); 3 = 80 to 100% liver cytoplasmic vacuolation and much more massive hyperplasia of bile duct epithelium were found (400×). The pathologist was blinded to treatment when evaluating slides. On day 35, other organs or tissues, such as kidney, heart, breast muscle, and leg muscle of ducks (10 ducks/treatment), were collected. All samples were packed in ice as they were collected and stored at -20°C until being analyzed for gossypol.

Gossypol Determination by HPLC

All test diets were assessed for TG content by HPLC as described by Hron et al. (1990) and assessed for FG content by HPLC according to Zeng et al. (2014). The TG in tissue was analyzed as follows: freeze-dried and finely ground tissue samples were mixed with extraction reagent composed of 2% 3-amino-1-propanol and 10% glacial acetic acid in N, N-dimethylformamide and vigorously vortexed (30 min). The vortexed samples were treated by ultrasonic at 40°C for 30 min and then heated at 90°C for 30 min, cooled on ice, and then centrifuged (4°C) at 1,500 × g for 5 min. After centrifu-

gation, an aliquot of the supernatant was diluted with the mobile phase to obtain a desirable concentration, centrifuged again at 1,500 × g for 5 min, and filtered through a syringe filter (0.45 μm) before injection into the HPLC system as described by Lee and Dabrowski (2002). The conditions of HPLC were as follows: column of chromatogram sepax sapphire C₁₈ 5 μm 120A (250 × 4.6 mm) mobile phase acetonitrile/water (1.2% H₃PO₄) = 80/15 (V/V), UV254-nm detector, column temperature 25°C, flow rate 1 mL/min as described by Hron et al. (1990).

Statistical Analysis

The effects of gossypol were assessed by ANOVA followed by a linear contrast to test the dose effect of gossypol on growth performance, blood parameters, and tissue residues of gossypol among treatments were assessed using the GLM procedure of SAS software (SAS Institute Inc., 2006). Mortality data were arcsin transformed prior to the analysis. In the present study, broken-line regression analysis (Robbins et al., 2006) was used to estimate the maximum level of dietary FG concentration using the nonlinear regression (NLIN) procedure of SAS

Table 2. Effects of gossypol from CSM on growth performance of 15- to 35-d-old ducks.¹

Item	Dietary FG concentration (mg/kg)					SEM	P-value	
	0	36	75	111	153		ANOVA	Linear
BW, g								
Day 15	668	672	665	669	672	3.27	0.56	0.80
Day 21	1,235 ^a	1,251 ^a	1,254 ^a	1,234 ^a	1,196 ^b	8.48	<0.001	0.008
Day 28	1,832 ^a	1,835 ^a	1,839 ^a	1,825 ^a	1,726 ^b	10.5	<0.0001	<0.001
Day 35	2,508 ^{3a}	2,515 ^a	2,521 ^a	2,502 ^a	2,371 ^b	16.3	<0.0001	<0.001
ADG(g)								
Days 15–21	80.9 ^a	82.7 ^a	84.1 ^a	81.0 ^a	74.8 ^b	1.22	<0.001	0.007
Days 22–28	84.5 ^a	83.5 ^a	83.6 ^a	83.5 ^a	74.8 ^b	1.48	0.001	<0.001
Days 29–35	96.3	97.2	95.8	96.8	88.3	2.61	0.06	0.11
Days 15–35	87.2 ^a	87.3 ^a	88.4 ^a	87.3 ^a	80.9 ^b	0.87	<0.0001	<0.001
ADFI(g)								
Days 15–21	148 ^{a,b}	152 ^{b,c}	153 ^c	154 ^c	145 ^a	1.29	<0.001	0.27
Days 22–28	161 ^a	166 ^b	168 ^b	170 ^b	159 ^a	1.65	<0.001	0.85
Days 29–35	201	207	207	209	199	3.53	0.23	0.91
Days 15–35	168 ^a	175 ^b	175 ^b	177 ^b	166 ^a	1.76	<0.001	0.68
FG (g/g)								
Days 15–21	1.82 ^a	1.84 ^{a,b}	1.85 ^{a,b}	1.91 ^{b,c}	1.95 ^c	0.02	0.001	<0.001
Days 22–28	1.91 ^a	1.99 ^{a,b}	2.02 ^{a,b}	2.02 ^b	2.11 ^c	0.03	0.002	0.003
Days 29–35	2.06 ^a	2.11 ^{a,b}	2.13 ^{a,b}	2.16 ^{b,c}	2.21 ^c	0.02	0.002	<0.001
Days 15–35	1.93 ^a	1.98 ^b	1.98 ^b	2.03 ^c	2.08 ^d	0.01	<0.001	<0.001
Mortality (%)								
Days 15–21	0	0	0	0.56	0	0.25	0.48	0.42
Days 22–28	0	0	0.59	0	0.59	0.37	0.32	0.56
Days 29–35	0	0	0	0	1.25	0.56	0.42	0.16
Days 15–35	0	0	0.56	0.56	1.67	0.64	0.36	0.06

¹Means represent 10 replicates/treatment.

^{a-d}Means in the same row with different superscripts are significantly different ($P < 0.05$).

(SAS Institute Inc., 2006). The 1-slope broken-line model and 2-slope broken-line model were as follows: $Y = L - U*(X - R)$ and $Y = L - U1*(R - X) - U2*(X - R)$, respectively, where Y = response index, X = dietary FG concentration (mg/kg), R = breakpoint (maximum limit), L = response at $X = R$, U = slope of curve. Data are expressed as means and SEM. Probability values greater than 0.05 were considered significant.

RESULTS

Growth Performance

The growth performance of ducks fed graded levels of dietary FG is presented in Table 2. Dietary FG concentration reduced ($P < 0.01$) BW and ADG in a linear manner at 21, 28, and 35 d of age, except for ADG from 29 to 35 d of age. Ducks fed a diet of 153 mg FG/kg had the lowest BW and ADG ($P < 0.05$) compared with ducks fed the other 4 diets. ADFI presented an increase and then a decrease ($P < 0.01$) with the increase in dietary FG content during the periods from 15 to 21 d, 22 to 28 d, and 15 to 35 d. The feed-to-gain ratio in each period and during the whole experiment linearly increased ($P < 0.01$) with increases in dietary FG concentration.

Blood Parameters

The blood parameters of ducks fed graded levels of dietary gossypol are presented in Table 3 and Table 4. As

dietary FG concentration increased, blood HGB, MCH, and MCHC level linearly decreased ($P < 0.05$) at 35d of age. Compared with ducks in the control group, blood HGB concentration of ducks at 35d of age in both the 111 and 153 mg FG/kg diet groups showed a 5% decrease ($P < 0.05$). A linear increase ($P < 0.05$) was observed in serum ALT activity, and a linear decrease ($P < 0.05$) was observed for AST/ALT with an increasing level of dietary CSM at 28 d of age. At 35 d, serum TP, ALB, and GLB linearly decreased ($P < 0.05$); therefore, the ALB-to-GLB ratio linearly increased ($P < 0.01$) with increasing levels of dietary FG.

Hepatic Histopathology

The score of hepatic histopathology is presented in Table 5, and the corresponding pictures from which hepatic scores were based are shown in Figure 1. The liver cells of birds fed control diets and diets of 36 mg FG/kg showed small amounts of lipid droplets in the cytoplasm of hepatocytes, and all the liver samples had a normal histological structure at 21, 28, and 35 d; thus, the total score was 0. The total scores for ducks fed a diet of 153 mg FG/kg on days 21, 28, and 35 were the highest; ducks fed a 111 mg FG/kg diet were intermediate, followed by the scores of ducks fed diets of 75 mg FG/kg. The length of gossypol intake had no clear effects on total scores of hepatic histopathology among ducks fed diets of 75 mg FG/kg, 111 mg FG/kg, and 153 mg FG/kg.

Table 3. Effects of gossypol from CSM on hematology of 15- to 35-d-old ducks.¹

Item	Dietary FG level (mg/kg)				SEM	P-value		
	0	36	75	111		ANOVA	Linear	
Red blood cell (RBC) count $\times 10^2$ /L								
Day 21	2.11	2.10	2.18	2.10	2.06	0.06	0.73	0.62
Day 28	2.01	1.86	1.87	1.92	1.92	0.06	0.49	0.56
Day 35	2.01	2.07	2.11	2.05	1.98	0.04	0.18	0.55
Hemoglobin (HGB) (g/L)								
Day 21	95.6	96.0	99.0	95.8	89.4	2.67	0.19	0.15
Day 28	156	144	143	143	147	4.24	0.19	0.18
Day 35	159 ^a	160 ^a	156 ^{ab}	151 ^b	151 ^b	2.68	<0.05	<0.01
Hematocrit (HCT) (%)								
Day 21	33.6	34.2	35.0	34.2	32.2	0.01	0.28	0.32
Day 28	30.7	28.6	28.8	29.0	29.4	0.94	0.55	0.48
Day 35	31.5	32.5	32.2	31.5	30.8	0.62	0.31	0.22
Mean corpuscular volume (MCV) (fL)								
Day 21	159	163	162	163	156	14.6	0.20	0.57
Day 28	153	155	154	151	154	17.0	0.85	0.73
Day 35	156 ^a	158 ^a	153 ^b	152 ^b	156 ^a	11.5	<0.01	0.12
Mean corpuscular hemoglobin (MCH) (pg)								
Day 21	45.2	45.7	45.4	45.7	43.3	0.73	0.14	0.10
Day 28	77.8	77.4	76.0	75.1	76.8	1.10	0.57	0.27
Day 35	77.8 ^a	77.5 ^a	73.9 ^b	73.6 ^b	75.2 ^{ab}	1.00	<0.01	<0.01
Mean corpuscular hemoglobin concentration (MCHC) (g/L)								
Day 21	285	280	281	281	277	2.73	0.41	0.11
Day 28	508	502	495	497	500	6.64	0.73	0.36
Day 35	498	492	485	480	484	5.42	0.16	<0.05

¹Means represent 10 replicates/treatment of 1 duck/replicate.

^{a,b}Means in the same row with different superscripts are significantly different ($P < 0.05$).

Tissue Residues of Gossypol

The gossypol residue on tissues of ducks fed different levels of FG is presented in Table 6. Concentrations of gossypol in liver, kidney, heart, and muscle of ducks at 35 d of age linearly increased ($P < 0.001$) with increasing dietary gossypol levels. Liver had the highest accumulation of gossypol. However, the gossypol residues in the breast muscle of ducks fed diet containing 153 mg/kg FG and 2,229 mg/kg TG was 17.84 mg/kg on day 35, which was higher than that in the kidney, heart, and leg muscles. When the dietary FG and TG contents were respectively 75 mg/kg and 1,096 mg/kg or even lower, the gossypol in the breast muscle was undetectable. Similarly, when ducks were fed a diet containing 36 mg/kg FG and 593 mg/kg TG, respectively, the gossypol in leg muscle was undetectable. As the length of feeding diets containing gossypol increased, the accumulation of gossypol in liver linearly increased ($P < 0.001$).

Maximum Limits of Dietary FG Concentration in Duck from 15 to 35 d of Age

The regression equation and maximum limits using 1-slope and 2-slope broken-line analyses are presented in Table 7, and the representative pictures of the broken-

line regression model are shown in Figure 2. Using a 1-slope broken-line analysis, the maximum limits for dietary FG concentration in diet for ducks from 15 to 35d of age increased with the duck's age. For example, the maximum limits for dietary FG concentration based on BW at 21, 28, and 35d were 97, 107, and 108 mg/kg, respectively. A 2-slope broken-line analysis revealed that the maximum limit of dietary FG concentration based on ADFI was higher than that based on BW or ADG. On the other hand, the maximum limit of dietary FG concentration based on growth performance was higher than that based on HGB, TP, ALB, GLB, or ALB:GLB. The maximum limit of the dietary FG concentration was estimated to range from a low of 36 mg/kg to maximize serum globulin concentration to a high of 124 mg/kg to minimize feed intake during the period from 22 to 28 d using a quadratic broken-line model.

DISCUSSION

Berardi and Goldblatt (1980) suggested that poor growth, anorexia, and high mortality are symptoms indicative of the toxic effect of gossypol. In the present study, visible signs of gossypol toxicity, including dyspnea, anorexia, weakness, and sudden death, were not observed in any of the ducks during the experimental

Table 4. Effects of gossypol from CSM on serum biochemical profiles in 15- to 35-d-old ducks.¹

Item	Dietary FG level (mg/kg)					SEM	P-value	
	0	36	75	111	153		ANOVA	Linear
Alanine aminotransferase (ALT) (U/L)								
Day 21	57.5	50.8	53.3	51.0	53.3	7.64	0.85	0.72
Day 28	41.8	45.8	46.3	45.5	54.7	3.80	0.22	<0.05
Day 35	40.8	45.6	39.4	44.5	47.5	3.81	0.44	0.31
Aspartate aminotransferase (AST) (U/L)								
Day 21	75.2	69.0	69.8	79.5	64.6	19.6	0.74	0.45
Day 28	77.2	62.0	77.2	68.7	70.7	9.13	0.71	0.44
Day 35	70.6	74.1	56.4	60.3	63.7	6.33	0.09	0.41
Aspartateaminotransferase:Alanineaminotransferase (AST/ALT)								
Day 21	1.34	1.32	1.28	1.58	1.34	0.17	0.29	0.65
Day 28	1.86	2.16	1.64	1.54	1.38	0.26	0.21	<0.05
Day 35	1.54	1.57	1.36	1.33	1.53	0.66	0.38	0.72
Total protein (TP) (g/L)								
Day 21	29.3	29.4	32.1	29.4	29.3	2.41	0.28	0.99
Day 28	30.7	30.7	28.4	31.2	31.4	0.89	0.25	0.47
Day35	32.6 ^{a,b}	34.7 ^a	32.2 ^a	30.0 ^b	30.2 ^b	1.06	<0.01	<0.05
Albumin (ALB) (g/L)								
Day 21	13.8	15.4	16.3	15.4	15.1	2.30	0.08	0.70
Day 28	13.7	13.6	12.6	13.4	14.0	0.46	0.34	0.62
Day 35	14.4 ^{a,b}	15.1 ^a	14.2 ^{a,b}	14.1 ^b	13.5 ^b	0.45	<0.05	<0.05
Globin (GLB) (g/L)								
Day 21	15.5	14.0	15.4	14.4	13.9	0.62	0.13	0.18
Day 28	17.0	17.2	15.8	17.8	17.4	0.56	0.32	0.46
Day 35	18.9 ^a	19.6 ^a	18.0 ^{ab}	16.9 ^b	16.8 ^b	0.68	<0.01	<0.05
Albumin:Globin (ALB/GLB)								
Day 21	0.90 ^a	1.12 ^b	1.04 ^b	1.09 ^b	1.07 ^b	0.15	<0.05	0.45
Day 28	0.80	0.82	0.80	0.78	0.80	0.03	0.95	0.71
Day 35	0.71 ^a	0.71 ^a	0.73 ^a	0.79 ^b	0.80 ^b	0.02	<0.01	<0.01

¹Means represent 10 replicates/treatment of 1 duck/replicate.

^{a,b}Means in the same row with different superscripts are significantly different ($P < 0.05$).

period. The maximum limit of dietary FG concentration is 97 to 121 mg/kg based on the BW or ADG of ducks from 15 to 35 d of age. This limit is lower than that reported for broilers, which is estimated to be around 200mg/kg (Adeyemo and Longe, 2007; Mush-taq et al., 2008). These results indicate that meat ducks are more sensitive to dietary FG than broilers. Moreover, the maximum limit of dietary FG level based on ADG was 98 mg/kg on days 14 to 21, 109 mg/kg on days 22 to 28, and 121 mg/kg on days 29 to 35. This result indicates that the tolerance of duck to dietary FG concentration increased with increases in the duck's age. This is in line with those reports that mentioned that young birds are more sensitive to dietary FG concentration (Lordelo et al., 2005; Nagalashmi et al., 2007). However, in the present study, the ADFI had two breakpoints. The first breakpoint may have been caused by the formation of protein (lysine)-gossypol complexes (Lyman et al., 1959), resulting in protein or AA deficiencies in diets containing CSM (Kornegay et al., 1961). Moreover, CSM shows lower energy utilization efficiency in comparison to SBM for meat ducks. The second breakpoint was the toxic effect of FG. This confirms what was reported earlier, i.e., that

reductions in BW gain and feed intake are common signs of gossypol toxicosis (Henry et al., 2001; Blevins et al., 2010).

In general, the cytotoxic activity of gossypol is associated with decreased hemoglobin concentration and hematocrit; as a result, anemia develops (Berardi and Goldblatt, 1980). In the present study, we found that HGB, MCH, and MCHC in ducks 35d of age decreased linearly with increases in dietary FG concentration, and the maximum limit of dietary FG content on HGB was 43 mg/kg. The foregoing results were in line with those of Adeyemo and Longe (2007), who found that RBC count and HCT significantly decreased when broilers were fed a diet containing 74 mg FG/kg. Gao et al. (2011) reported that 14-d-old layer chickens fed diets of 70 to 140 mg/kg FG had a lower RBC count, HCT, and HGB in comparison to chicken fed the control diet without FG. Fang (2004) reported that laying hens fed a diet with 200 mg FG/kg showed lower serum Fe²⁺ concentration compared with the control group. The same effects were observed in fish (Mbahinzireki et al., 2001; Yue and Zhou, 2008). These reports suggested that dietary gossypol reduced iron bioavailability and its absorption for hemoglobin formation by binding

Table 5. Effects of gossypol from CSM on histopathology of livers in 15- to 35-d-old ducks.¹

Item	Score 0 ²	Score 1 ²	Score 2 ²	Score 3 ²	Total score ³
Day 21					
Diet 1* (0 mg FG/kg)	5	0	0	0	0
Diet 2 (36 mg FG/kg)	5	0	0	0	0
Diet 3 (75 mg FG/kg)	4	0	1	0	0.5
Diet 4 (111 mg FG/kg)	2	2	1	0	0.8
Diet 5 (153 mg FG/kg)	3	0	0	2	1.2
Day 28					
Diet 1 (0 mg FG/kg)	5	0	0	0	0
Diet 2 (36 mg FG/kg)	5	0	0	0	0
Diet 3 (75 mg FG/kg)	4	1	0	0	0.2
Diet 4 (111 mg FG/kg)	3	1	1	0	0.6
Diet 5 (153 mg FG/kg)	2	2	1	0	0.8
Day 35					
Diet 1 (0 mg FG/kg)	5	0	0	0	0
Diet 2 (36 mg FG/kg)	5	0	0	0	0
Diet 3 (75 mg FG/kg)	4	0	1	0	0.4
Diet 4 (111 mg FG/kg)	3	1	1	0	0.6
Diet 5 (153 mg FG/kg)	2	1	2	0	1

¹Means represent 5ducks/treatment with 3 slices of 1 duck.

²Score 0 = normal histological structure (A400); Score 1 =approximately 25% liver cytoplasmic vacuolation and slight funicular hyperplasia of bile duct epithelium were recognizable (B400); Score 2 =approximately 50% liver cytoplasmic vacuolation and serious funicular hyperplasia of bile duct epithelium were observed (C400); Score 3 = almost 100% liver cytoplasmic vacuolation and much more massive hyperplasia of bile duct epithelium were found (D400).

³Total score = (score 0×number of ducks + score 1× number of ducks + score 2×number of ducks +score3×number of ducks)/total number of ducks, for example, at 21 d of age, total score of Diet 5=(0 × 3)+(1 × 0)+(2 × 0)+(3 × 2)/5 = 1.2.

*Diet 1 =control group, a basal diet; Diet 2 = 25% protein of SBM in basal diet replaced by that of CSM; Diet 3 = 50% protein of SBM in basal diet replaced by that of CSM; Diet 4 = 75% protein of SBM in basal diet replaced by that of CSM; Diet 5 = 100% protein of SBM in basal diet replaced by that of CSM.

Table 6. Residues of gossypol in tissues of 15- to 35-d-old ducks.¹

Item	Dietary FG level (mg/kg)					SEM	P-value	
	0	36	75	111	153		ANOVA	Linear
Day 35								
Kidney	ND ^{1,a}	6.60 ^b	9.06 ^c	12.97 ^d	17.73 ^e	0.58	<0.001	<0.001
Heart	ND ^a	4.77 ^b	6.99 ^c	8.71 ^d	11.11 ^e	1.11	<0.001	<0.001
Leg muscle	ND ^a	ND ^a	4.31 ^b	8.58 ^c	11.53 ^d	1.01	<0.001	<0.001
Breast muscle	ND ^a	ND ^a	ND ^a	9.92 ^b	17.84 ^c	0.86	<0.001	<0.001
Liver								
Day 21	ND ^a	25.5 ^{b,A}	37.2 ^{c,A}	46.7 ^{d,A}	58.8 ^{e,A}	0.30	<0.001	<0.001
Day 28	ND ^a	25.3 ^{b,A}	45.5 ^{c,B}	59.3 ^{d,B}	93.7 ^{e,B}	1.13	<0.001	<0.001
Day 35	ND ^a	35.9 ^{b,B}	53.8 ^{c,C}	80.7 ^{d,C}	101.8 ^{e,C}	1.23	<0.001	<0.001

¹Means represent 10 replicates/treatment of 1 duck/replicate.

^{a-e}Means in the same row with different superscripts are significantly different ($P < 0.05$).

^{A,B,C}Means in the same column with different superscripts are significantly different ($P < 0.05$).

dietary iron (Clawson et al., 1975; Herman and Smith, 1973).

In the present study, it was found that the maximum limits of dietary FG content on TP, ALB, GLB, and ALB:GLB were 37, 78, 36, and 52 mg/kg, respectively. This is consistent with Adeyemo and Longe (2007), Anwar et al. (2008), and Sharma et al. (1966), who reported that the serum total protein was reduced by approximately 20% when animals were fed diets containing CSM. Herman (1970) observed a severe reduction in plasma protein concentration in fish receiving as

little as 53 mg FG/kg. Serum protein was significantly reduced at 900 mg/kg or higher gossypol from gossypol-acetic acid in fish diets (Yildirim et al., 2003). The decrease in serum protein concentration may be an indication of protein synthesis and metabolism disturbance. One reason for this may be the reduced protein digestibility and retention resulting from the formation of protein-gossypol complexes (Lyman et al., 1959). Another reason may be that the liver is the main target organ of gossypol. Direct evidence of liver damage by gossypol can be further identified by histopathological

Table 7. Summary of maximum limit of dietary FG content for Pekin ducks from 15 to 35 d of age.

Items	Maximum limit of dietary FG content ¹	
	Estimated value (mg/kg)	R ²
21 d BW (kg)	97	0.9995
28 d BW (kg)	107	0.9999
35 d BW (kg)	108	0.9996
15 to 21 d ADG (g)	98	0.9978
22 to 28 d ADG (g)	109	0.9971
29 to 35 d ADG (g)	121	0.9932
15 to 35 d ADG (g)	108	0.9993
15 to 21 d ADFI ² (g)	55 or 115	0.9993
22 to 28 d ADFI (g)	54 or 124	0.9991
15 to 35 d ADFI (g)	42 or 116	0.9990
35 d HGB ³ (g/L)	43	0.9978
35 d TP ³ (g/L)	37	0.9981
35 d ALB ³ (g/L)	77	0.9954
35 d GLB ³ (g/L)	36	0.9998
35 d ALB/GLB	52	0.9997

¹The maximum limit of dietary FG (lowest FG response) is based on analyzed dietary FG (0, 36, 75, 111, and 153 mg/kg) to achieve either the maximal or minimal response.

²Two-slope broken line is $Y = L - U1*(R-X) - U2*(X-R)$, respectively, where Y = response index, X = dietary FG concentration (mg/kg), R = breakpoint (maximum limit), L = response at X = R, U = slope of curve.

³HGB = hemoglobin; TP = serum total protein; ALB = serum albumin; GLB = serum globin.

examination. Cytoplasmic vacuolation of hepatocytes, a type of liver steatosis, and hyperplasia of bile duct epithelium are typical syndromes of gossypol toxicity (Henry et al., 2001). The present study showed that the

hepatic histopathological score according to cytoplasmic vacuolation and hyperplasia of bile duct epithelium increased with increasing dietary FG, where ducks fed 153 mg FG/kg had the highest histopathological score. Blevins et al. (2010) reported that lipidosis scores were higher in chickens fed 1,000 mg purified gossypol/kg of diet than those fed the control diet, which is indicative of lipid storage and subsequent liver malfunction (Plaa and Charbonneau, 2008). Ilyas et al. (2007) observed liver fatty changes consisting of liver parenchyma with an infiltrating aggregate of cells, newly formed bile ducts in the parenchyma in the hepatocytes of chickens fed diet containing 27% CSM, and yet damage to the liver was more severe in chickens fed a diet containing 41% CSM.

This report is the first to show the gossypol residue of tissue in meat ducks. It found that when ducks from 15 to 35 d of age were fed diets with CSM (FG: 36 to 153 mg/kg, TG: 594 to 2,229 mg/kg) for 21 d, the concentration of gossypol also linearly increased in the liver, kidney, heart, and muscle with increasing dietary levels of FG. Gossypol was undetectable in breast muscle of ducks fed diets containing 36 mg FG/kg and 75 mg FG/kg, respectively, and in leg muscle of ducks fed a diet containing 36 mg FG/kg. These results accord with those of Gamboa et al. (2001a and 2001b), which found the tissue residue of gossypol in broilers fed diets with CSM (FG: 92 to 504 mg/kg, TG: 2,626 to 4,085 mg/kg) for 21 d, increased linearly in

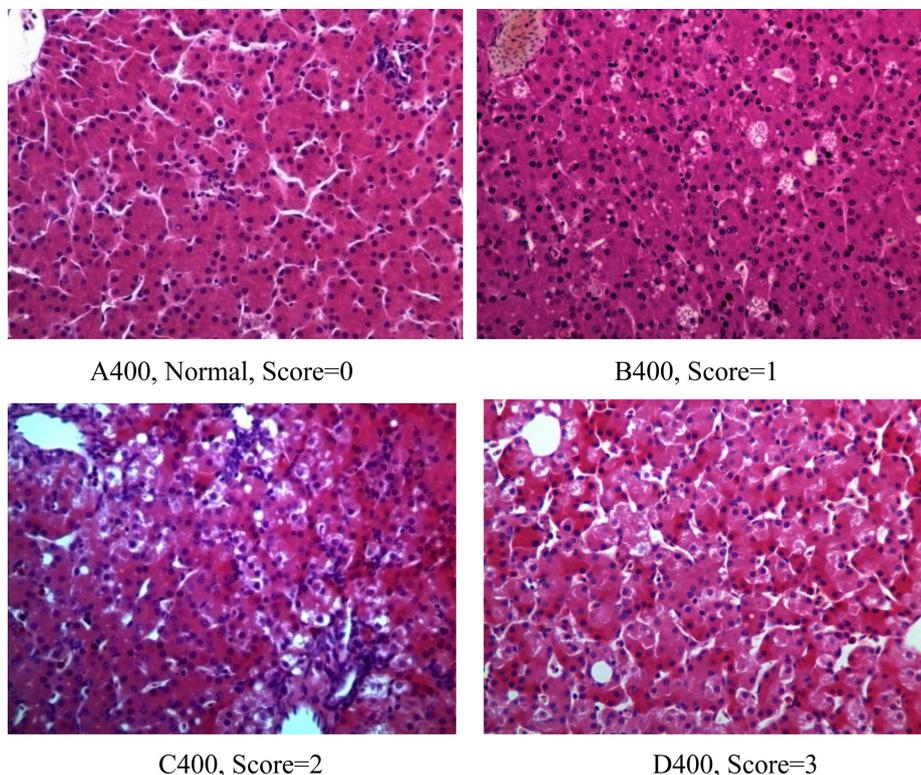


Figure 1. Morphology of duck livers from control and gossypol-containing groups from day 35. A400: control normal histological structure (400×)(score =0); B400: approximately 25% liver cytoplasmic vacuolation and slight funicular hyperplasia of bile duct epithelium were recognizable (400×)(score =1); C400: approximately 50% liver cytoplasmic vacuolation and serious funicular hyperplasia of bile duct epithelium were observed (400×)(score =2); D400: 80 to 100% liver cytoplasmic vacuolation and much more massive hyperplasia of bile duct epithelium were found (400×)(score =3).

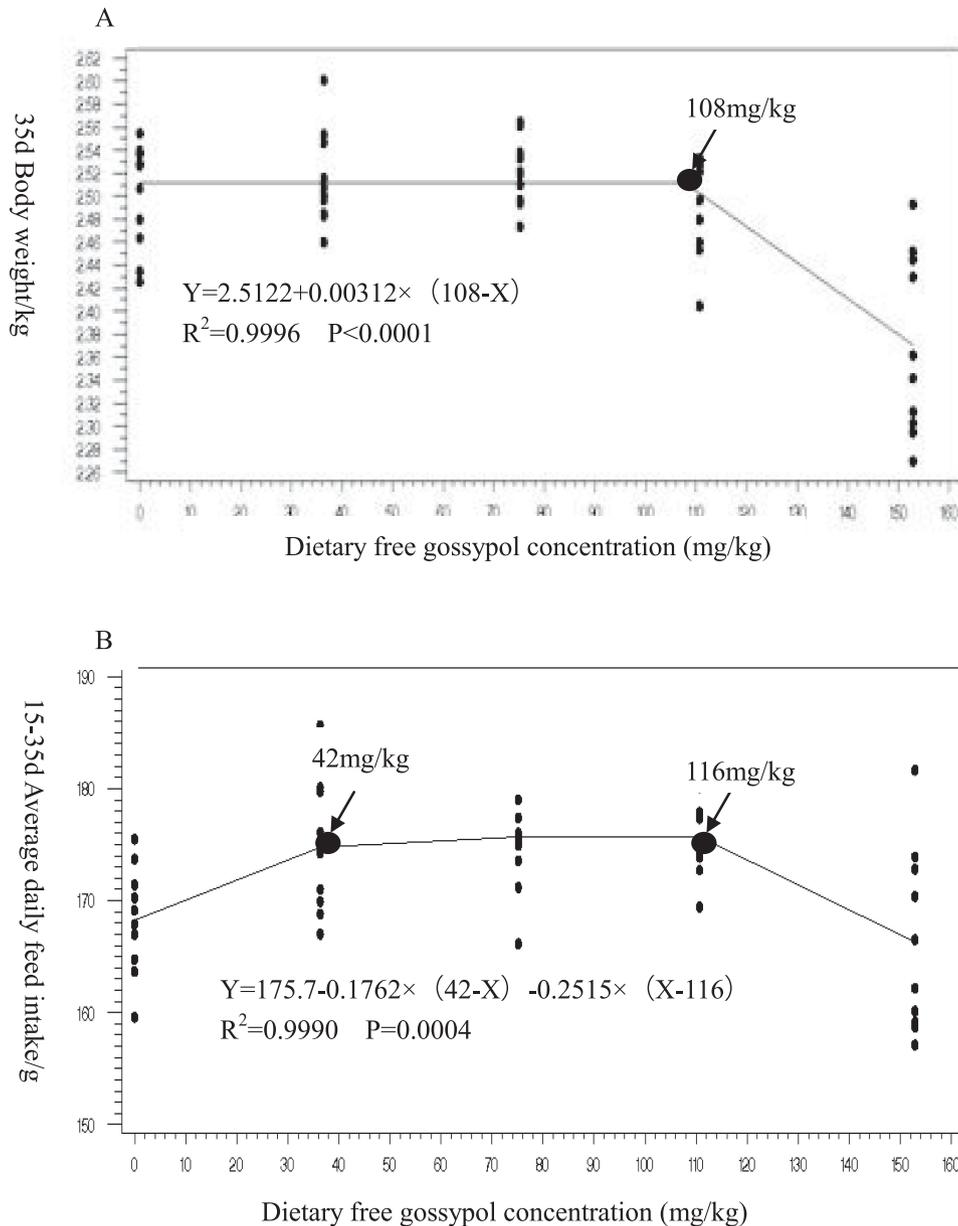


Figure 2. Quadratic broken-line analysis of BW (day 35)(A) and average daily feed intake (days 15 to 35)(B) with dietary FG concentration. (A) A breakpoint in 35d BW occurred at 108 mg/kg of dietary FG concentration based on 1-slope broken-line model; equation: $Y=2.5122 + 0.00312 \times (108-X)$; breakpoint (BP) = 108 mg/kg (if dietary FG concentration is less than BP, then $X = 0$; if dietary FG concentration is less than BP, then $X = \text{dietary free gossypol concentration} - \text{BP}$). (B) Two breakpoints of ADFI (days 15 to 35) occurred at 42 and 116 mg/kg dietary FG concentration based on 2-slope broken-line model; equation: $Y = 175.7 - 0.1762 \times (42-X) - 0.2515 \times (X-116)$; BP = 42 or 116 mg/kg (if dietary FG concentration is less than BP, then $X = 0$; if dietary FG concentration is greater than BP, then $X = \text{dietary FG concentration} - \text{BP}$).

plasma, liver, kidney, and muscle with increasing dietary levels of FG. Liver had the highest concentration of gossypol (71.4 to 313.6 mg/kg DM) followed by kidney (9.2 to 36.3 mg/kg DM), and muscle (2.1 to 9.8 mg/kg DM). The maximum residue limits of FG in food determined by the Food and Agriculture Organization (FAO) and the US Food and Drug Administration (FDA) were 600 mg/kg and 450 mg/kg, respectively. In the present study, it was found that tissue gossypol residue in duck organs was considerably lower than the maximum residue limits set by the FAO and FDA.

There are still no reports that directly identify a definitive amount of gossypol in food that presents a risk to human health, and so this matter requires further study.

In conclusion, the maximum limits of dietary FG concentration varied from 36 to 124 mg/kg, depending on the parameter evaluated. Meat ducks from 15 to 35 d of age fed diets containing 153 mg FG/kg (23.3% CSM diet) presented poor performance, serious hepatic lesions, and high levels of gossypol residue in liver. The CSM (5.83% CSM, 594 mg/kg TG, 36 mg/kg FG)-fed

ducks performed well compared to the standard of SBM-fed ducks.

ACKNOWLEDGMENTS

The authors thank Dr. Todd J. Applegate and Dr. S. A. Adedokun (Animal Science Department, Purdue University, West Lafayette, IN) for revising this manuscript. This research was supported by grants from the National Scientific and Technical Supporting program (2014BAD13B02), from the meat duck industry chain in Sichuan Province (2014NZ0030), and from the Sichuan Agricultural University 211 Foundation of China.

REFERENCES

- Adeyemo, G. O., and O. G. Longe. 2007. Effects of graded levels of cottonseed cake on performance, haematological and carcass characteristics of broilers fed from day old to 8 weeks of age. *Afri. J. Biotechnol.* 6:1064–1071.
- Adeyemo, G. O. 2010. Effects of exposure duration to cottonseed cake-based diets on broiler performance. *Int. J. Poult. Sci.* 9:162–166.
- Anwar, M. S., M. Z. Khan, I. Javed, A. Khan, and M. K. Saleem. 2008. Pathological effects of cottonseed meal with and without ferrous sulphate in male Japanese quails (*Coturnix Japonica*). *Pakistan. Vet. J.* 28:51–56.
- Berardi, L. C., and L. A. Goldblatt. 1980. Gossypol. Pages 184–237 in *Toxic Constituents of Plant Feedstuff*. I. E. Lienar, ed., Academic Press, New York.
- Blevins, S., P. B. Siegel, D. J. Blodgett, M. Ehrlich, G. K. Saunders, and R. M. Lewis. 2010. Effects of silymarin on gossypol toxicosis in divergent lines of chickens. *Poult. Sci.* 89:1878–1886.
- Brocas, C., R. M. Rivera, F. F. Paula-Lopes, L. R. McDowell, M. C. Calhoun, C. R. Staples, N. S. Wilkinson, A. J. Boning, P. J. Chenoweth, and P. J. Hansen. 1997. Deleterious actions of gossypol on bovine spermatozoa, oocytes and embryo. *Biol. Reprod.* 57:901–907.
- Clawson, A. J., J. H. Maner, G. Gomez, O. Mejia, Z. Flores, and J. Buitrago. 1975. Unextracted cottonseed in diets for monogastric animals. I. The effect of ferrous sulfate and calcium hydroxide in reducing gossypol toxicity. *J. Anim. Sci.* 40:640–647.
- Fang, Q. Y. 2004. Effects of graded levels of cottonseed meal in diets on performance, quality of egg, serum biochemical profiles in laying hen. M.S. thesis. Sichuan Agriculture University, Ya'an, China.
- Gao, Y. S., M. J. Tang, and J. X. Liu. 2011. Effects of high cottonseed meal content in diets on performance and quality of egg in laying hen. *Chinese Poult. Sci.* 34:16–19.
- Gamboa, D. A., M. C. Calhoun, S. W. Kuhlmann, A. U. Haq, and C. A. Bailey. 2001a. Tissue distribution of gossypol enantiomers in broilers fed various cottonseed meals. *Poult. Sci.* 80:920–925.
- Gamboa, D. A., M. C. Calhoun, S. W. Kuhlmann, A. U. Haq, and C. A. Bailey. 2001b. Use of expander cottonseed meal in broiler diets formulated on a digestible amino acid basis. *Poult. Sci.* 80:789–794.
- Henry, M. H., G. M. Pesti, and T. P. Brown. 2001. Pathology and histopathology of gossypol toxicity in broiler chicks. *Avian Dis.* 45:598–604.
- Herman, R. L. 1970. Effects of gossypol on rainbow trout *Salmogairdneri Richardson*. *J. Fish Biol.* 2:293–303.
- Herman, D. L., and F. H. Smith. 1973. Effects of bound gossypol on the absorption of iron by rats. *J. Nutr.* 103:882–889.
- Hron, R. J. Sr., M. S. Kuk, and G. Abraham. 1990. Determination of free and total gossypol by high performance liquid chromatography. *J. Am. Oil Chem. Soc.* 3:182–187.
- Ilyas, M., M. K. Saleemi, F. Mahmood, and M. Z. Khan. 2007. Pathological effects of feeding cottonseed meal with and without lysine in male Japanese quails. *Pakistan Vet. J.* 27:55–62.
- Kornegay, E. T., A. J. Clawson, F. H. Smith, and E. R. Barrick. 1961. Influence of protein source on toxicity of gossypol in swine rations. *J. Anim. Sci.* 20:597–602.
- Lee, K. J., and K. Dabrowski. 2002. High performance liquid chromatographic determination of gossypol and gossypolone enantiomers in fish tissues using simultaneous electrochemical and ultraviolet detectors. *J. Chromatogr. B.* 779:313–319.
- Lordelo, M. M., A. J. Davis, M. C. Calhoun, M. K. Dowd, and N. M. Dale. 2005. Relative toxicity of gossypol enantiomers in broilers. *Poult. Sci.* 84:1376–82.
- Lyman, C. M., B. P. Baliga, and M. W. Slay. 1959. Reactions of proteins with gossypol. *Arch. Biochem. Biophys.* 84:486–497.
- Mbahinzireki, G. B., K. Dabrowski, K. J. Lee, D. El-Saidy, and E. R. Wisner. 2001. Growth, feed utilization and body composition of tilapia fed cottonseed meal-based diets in a recirculating system. *Aquacult. Nutr.* 7:189–200.
- Mushtaq, T., M. Sarwar, G. Ahmad, M. A. Mirza, T. Ahmad, M. Athar, M. M. H. Mushtaq, and U. Noreen. 2008. Influence of pre-press solvent-extracted cottonseed meal supplemented with exogenous enzyme and digestible lysine on performance, digestibility, carcass and immunity responses of broiler chickens. *J. Anim. Physiol. An.* 93:253–262.
- Nagalakshmi, D., V. R. Savaram, K. P. Arun, and R. B. Vadali. 2007. Cottonseed meal in poultry diets: A review. *J. Poult. Sci.* 44:119–134.
- National Research Council. 1994. *Nutrient Requirements of Poultry*, 9th rev. ed. Natl. Acad. Press, Washington, DC.
- Plaa, G. L., and M. Charbonneau. 2008. Detection and evaluation of chemically induced liver injury. Pages 1456–1495 in *Principles and Methods of Toxicology*. A. W. Hayes, ed., CRC Press, Boca Raton, FL.
- Robbins, K. J., A. M. Saxton, and L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. *J. Anim. Sci.* 84:E155–E165.
- Roehm, J. N., D. J. Lee, and R. O. Sinnhuber. 1967. Accumulation and elimination of dietary gossypol in the organs of rainbow trout. *J. Nutr.* 92:425–428.
- SAS Institute Inc. 2006. *SAS/STAT User's Guide*. Release 9.1. SAS Institute, Inc., Cary, NC.
- Sharma, M. P., F. H. Smith, and A. J. Clawson. 1966. Effects of levels of protein and gossypol, and length of feeding period on the accumulation of gossypol in tissues of swine. *J. Nutr.* 88:434–438.
- Yildirim, M., C. Lim, P. J. Wan, and P. H. Klesius. 2003. Growth performance and immune response of channel catfish (*Ictalurus punctatus*) fed diets containing graded levels of gossypol-acetic acid. *Aquacult.* 219:751–768.
- Yue, Y. R., and Q. C. Zhou. 2008. Effect of replacing soybean meal with cottonseed meal on growth, feed utilization, and hematological indexes for juvenile hybrid tilapia. *Aquacult.* 284:185–189.
- Velasquez-Pereira, J., C. F. Arechiga, L. R. McDowell, P. J. Hansen, P. J. Chenoweth, M. C. Calhoun, C. A. Risco, T. R. Batra, S. N. Williams, and N. S. Wilkinson. 2002. Effects of gossypol from cottonseed meal and dietary vitamin E on the reproductive characteristics of superovulated beef heifers. *J. Anim. Sci.* 80:2485–2492.
- Zeng, Q. F., G. L. Yang, G. N. Liu, J. P. Wang, S. P. Bai, X. M. Ding, Y. H. Luo, and K. Y. Zhang. 2014. Effects of dietary gossypol concentration on growth performance, blood profiles, and hepatic histopathology in meat ducks. *Poult. Sci.* 93:1–10.