

## ORIGINAL ARTICLE

## Effect of expanded cottonseed meal on laying performance, egg quality, concentrations of free gossypol in tissue, serum and egg of laying hens

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## ABSTRACT

Three hundred and sixty Hy-Line Brown hens, 40 week of age, were allocated to five treatments, each of which included four replicates of 18 hens. After an expanded process of cottonseed meal (CSM), free gossypol content in CSM was decreased from 1.24 to 0.40 g/kg. The dietary treatments were corn-soybean meal based diets including 6% CSM and 6%, 8% and 10% expanded cottonseed meal (ECSM). Hens fed 8% ECSM had higher ( $P < 0.05$ ) laying rate and average egg weight than those fed 6% CSM. The albumen height and Haugh unit in the control group, 6% and 8% ECSM groups were superior ( $P < 0.05$ ) to other treatments. Hens fed 6% CSM resulted in severe ( $P < 0.05$ ) egg yolk discoloration. Free gossypol (FG) concentrations in yolk and albumen and tissues of the 6% CSM group were greater ( $P < 0.05$ ) than those in any ECSM treatments. Hens fed 6% CSM and 10% ECSM had the highest ( $P < 0.05$ ) FG concentrations in the liver compared with those in the kidney and muscle, and higher ( $P < 0.05$ ) FG residues in yolk than those in albumen. In conclusion, FG in CSM can be reduced by 68% through an expanded process and ECSM can be available in laying hens at up to 10% of the total diet and an appropriate replacement of soybean meal with ECSM may improve performance in laying hens.

**Key words:** egg quality, expanded cottonseed meal, gossypol, laying hens, laying performance.

## INTRODUCTION

China is the largest producer of cotton around the world, and cottonseed meal (CSM), a byproduct of the process of extracting the oil from cotton seeds, has long been considered as an alternative protein source due to the increased cost of soybean meal. Although CSM is substantially lower in energy and protein than soybean meal, it still can be used successfully in layer diets, because laying hens have lower energy and protein requirements than broilers (Davis *et al.* 2002). However, CSM utilized in poultry feed as a protein supplement is limited to low lysine levels and has gossypol toxicity (Robinson & Li 2008). Problems related to lysine level and nutrient density can be rectified by addition of synthetic lysine (Mahmood *et al.* 2011; Saki *et al.* 2012). However, solutions to the problems related to gossypol in CSM have been elusive.

Gossypol is a highly reactive compound that rapidly binds to a range of substances, including minerals and amino acids (Braga *et al.* 2012). Previous studies found

that effects of free gossypol (FG) on birds were different. Several investigations reported that FG could inhibit growth performance and increase mortality in broilers (Henry *et al.* 2001a; Khadiga *et al.* 2009), while other findings demonstrated that broiler performance was not significantly affected when the content of dietary FG was less than 250 mg/kg (Hermes *et al.* 1983). The presence of FG in CSM or cake given to layers resulted in egg yolk discoloration (Fitzsimmons *et al.* 1989; Nelson 2004). Similarly, due to the sensitivity to FG, researchers suggested that CSM was not satisfactory for incorporating into layer diets (Nelson 2004). Accordingly, the inclusion of CSM in layer diets largely depends on the content of FG in CSM.

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In order to utilize CSM effectively, great efforts have been devoted to reduce and detoxify the FG, for example, ferrous sulfate treatment (Panigrahi & Plumb 1996; Tabatabai *et al.* 2002), adding lysine (Martin 1990; Saki *et al.* 2012) into diet and using the fermentation process for detoxification of gossypol in CSM (Zhang *et al.* 2007; Tang *et al.* 2012). Meanwhile, the expander-expeller process has been demonstrated as an available method to reduce the content of FG in CSM (Noftsker *et al.* 2000). However, there is little information about the effects of expanded cottonseed meal (ECSM) on laying hens. Therefore, the objective of this study was to evaluate the effects of ECSM on laying performance, egg quality, concentrations of FG in tissue, serum and egg of laying hens.

## MATERIALS AND METHODS

### Experimental design, birds and management

A total of 360 Hy-Line Brown commercial laying hens at 40 weeks of age were randomly allocated to 20 units (each with six cages in three levels, three birds/cage) that were then randomly assigned to five dietary treatments (five units/treatment). Each cage had 2250 cm<sup>2</sup> (45 × 50 cm) floor space and was equipped with two nipple drinkers and one feeder. The dietary treatments were corn-soybean meal-based diets supplemented with 0, 6%, 8% and 10% ECSM and 6% CSM (Zhejiang Xinxin Feed Co. Ltd, Jiaxing, Zhejiang, China) (Table 1). Cages were randomly located in a ventilated room with the temperature between 21 to 27°C and 16 h/day of illumination (10 to 20 lx). Diets were offered twice daily for

*ad libitum* intake and laying hens had free access to water throughout the entire experimental period. The experiment lasted 9 weeks, including a 1 week acclimation period and an 8 week experimental period. Feed residues were collected and weighted weekly to enable estimation of average daily feed intake (ADFI). Eggs from each replicate were counted and weighted daily to calculate laying rate, average egg weight and feed conversion. Mortalities and health status were visually observed and recorded daily throughout the entire experimental period. All experimental procedures were approved by the Animal Care and Use Committee of Animal Science College of Zhejiang University (Hangzhou, Zhejiang, China).

### Sampling

At the age of 49 weeks, 16 birds per treatment were sacrificed by bleeding of the jugular vein after 12 h fasting (water was offered *ad libitum*), and samples from liver, kidney and the left Pectoralis major muscle were collected. Blood samples collected during bleeding from jugular vein were centrifuged for 10 min (958 × *g*) to separate out serum. All samples were kept at -80°C prior to analysis. In addition, 36 eggs (nine eggs from each replicate) for each treatment were randomly selected for egg quality and FG content determination.

### Experimental parameters measured

Approximately 200 g of each diet, CSM and ECSM were chosen and stored at -20°C for analysis of crude protein (CP), calcium and phosphorus according to the method of AOAC (2002). The dry matter (DM) content of CSM and ECSM was analyzed by drying the samples at 105°C for 5 h, ashing by incineration at 550°C for 24 h in a muffle furnace (RWF, Verder Retsch Shanghai Trading Co., Ltd, Shanghai, China) and fat content was measured by extracting the oil

**Table 1** Ingredients and nutrient composition of experimental dietst

Item (% unless noted)	Control	6% CSM	6% ECSM	8% ECSM	10% ECSM
Corn	65.75	66.30	66.30	66.50	66.75
Soybean meal (44.2% CP)	20.89	12.84	12.84	10.02	7.19
Fish meal (60.2% CP)	2.70	4.20	4.20	4.80	5.40
Cottonseed meal	—	6.00	—	—	—
Expanded cottonseed meal	—	—	6.00	8.00	10.00
Limestone	8.66	8.66	8.66	8.68	8.66
Sodium chloride	0.30	0.26	0.26	0.25	0.23
Calcium hydrogen phosphate	0.72	0.50	0.50	0.42	0.37
DL-Methionine	0.14	0.13	0.13	0.12	0.12
Zeolite powder	0.34	0.61	0.61	0.71	0.78
Premix‡	0.50	0.50	0.50	0.50	0.50
Nutrient levels§					
ME (kcal/kg)	2,690	2,680	2,680	2,678	2,676
CP, analyzed	16.01	16.03	16.03	16.05	16.04
Calcium, analyzed	3.62	3.52	3.55	3.49	3.58
Total phosphorous, analyzed	0.67	0.71	0.68	0.73	0.76
Lys	0.83	0.81	0.81	0.81	0.80
Met	0.41	0.41	0.41	0.40	0.41
Met + Cys	0.65	0.65	0.65	0.65	0.65

†The control group was fed the basal diet. The other treatment diets were supplemented with 60 g CSM and 60, 80 and 100 g ECSM per kg of diet. CP, crude protein; ME, metabolism energy; Lys, lysine; Met, methionine; Cys, cysteine; CSM, cottonseed meal; ECSM, expanded cottonseed meal. ‡Supplied per kilogram of diet: vitamin A, 7000 IU; vitamin D<sub>3</sub>, 2500 IU; vitamin E, 30 IU; vitamin K<sub>3</sub>, 1 mg; vitamin B<sub>1</sub>, 1.5 mg; vitamin B<sub>2</sub>, 4 mg; vitamin B<sub>6</sub>, 2 mg; vitamin B<sub>12</sub>, 0.02 mg; niacin, 30 mg; folic acid, 0.55 mg; pantothenic acid, 10 mg; biotin, 0.16 mg; choline chloride, 400 mg; Cu, 10 mg; Fe, 70 mg; Mn, 100 mg; Zn, 70 mg; I, 0.4 mg; Se, 0.5 mg. §Values were calculated from data provided by Feed Database in China (2009).

with diethyl ether according to Wang *et al.* (2004). The crude fiber content was determined by the method of American Oil Chemists Society (AOCS 2009). FG concentrations were determined by high-power liquid chromatography (HPLC: Hitachi L-8900 Amino Acid Analyzer, Tokyo, Japan) according to the method of AOCS (2009). Eggs were weighed and cracked, and albumen height, Haugh unit, yolk color, eggshell thickness and eggshell strength were measured with a digital egg tester (DET-6000; Nabel Co. Ltd, Kyoto, Japan). Eggshell thickness (without the eggshell membrane) was measured at the middle part of the egg. Albumen and yolk were separated and frozen at  $-80^{\circ}\text{C}$ . The concentrations of FG in albumen, yolk, liver, kidney, muscle and serum were determined by the method of AOCS (2009).

## Statistical analyses

Results were analyzed by one-way analysis of variance (ANOVA) and results with concentrations of FG were analyzed by general linear models using SPSS for Windows, version 16.0 (SPSS Inc., Chicago, IL, USA). Differences among all treatments were separated by the Tukey test for multiple comparisons, and probability values of less than 0.05 were considered as significant. Results are expressed as mean  $\pm$  SE.

## RESULTS AND DISCUSSION

The main findings of the current study (Table 2) showed that the expanded process of CSM dramatically decreased 68% of FG content in CSM in comparison with CSM (from 1.24 g/kg to 0.40 g/kg), whereas other nutrient compositions had little

**Table 2** Nutrient composition and contents of free gossypol of cottonseed meal and expanded cottonseed meal†

Item (% unless noted)	CSM	ECSM
DM	91.20	91.02
EE	0.63	0.57
CP	43.99	43.87
Ash	6.63	6.50
CF	5.97	5.74
Calcium	0.27	0.26
Total phosphorus	1.04	1.08
FG, g/kg	1.24	0.40

†Analytical values, means of triplicate analyses. CF, crude fiber; CP, crude protein; CSM, cottonseed meal; DM, dry matter; ECSM, expanded cottonseed meal; EE, ether extract; FG, free gossypol.

changes. The decrease in FG may be attributed to the expanded process, because gossypol may bind to protein, possibly to the epsilon-amino group of lysine when CSM was under heat treatment (Craig & Broderick 1981). In agreement with our findings, Henry *et al.* (2001b) showed that extrusion of CSM resulted in reduction in FG detected.

The inclusion of CSM in feeds for birds has been studied for decades; however, little research has been conducted to evaluate the effects of ECSM in laying hens. Our study found that all laying hens appeared healthy and no mortality occurred throughout the entire experimental period (data not shown). As shown in Table 3, no significant differences in ADFI were found between CSM and ECSM treatments. ADFI increased as the levels of ECSM increased, and hens fed 10% ECSM had higher ( $P < 0.05$ ) ADFI than those in the control group. Birds fed 8% ECSM had higher ( $P < 0.05$ ) laying rate and average egg weight than those fed 6% CSM. However, feed conversion ratios (FCR) in the 6% CSM group were higher ( $P < 0.05$ ) than those in the control group. Our study was in agreement with previous research that high gossypol in CSM was associated with reduced performance in chickens (Watkins *et al.* 1993; Azman & Yilmaz 2005). Henry *et al.* (2001b) found that broiler diets formulated with CSM require higher lysine levels to obtain performance due to unavailability of the lysine bound to gossypol during the heating of CSM. In this experiment, although lysine levels exceeded the NRC (1994) recommendations, our study showed that adding 6% CSM (FG content: 75.12 mg/kg) into diet had negative effects on hens' performances. In contrast, feed formulated with ECSM resulted in laying rate, average egg weight and FCR equivalent to the diets with whole soybean meal as the major protein source. It is suggested that the quality improvement of CSM was one of the factors affecting the laying performance of laying hens. Although no significant differences on laying performance were found among ECSM treatments, hens fed 8% ECSM had higher laying rates and average egg weight and lower FCR than the other two ECSM treatments.

Eggshell strength and eggshell thickness are two important indicators for reflection of eggshell quality.

**Table 3** Average feed intake, laying rate, average egg weight and feed conversion of laying henst

Item	Treatment				
	Control	6% CSM	6% ECSM	8% ECSM	10% ECSM
ADFI (g)	119.72 $\pm$ 1.78 <sup>b</sup>	123.57 $\pm$ 1.26 <sup>ab</sup>	122.00 $\pm$ 2.20 <sup>ab</sup>	123.81 $\pm$ 0.52 <sup>ab</sup>	124.95 $\pm$ 1.06 <sup>a</sup>
Laying rate (%)	95.71 $\pm$ 1.08 <sup>ab</sup>	92.96 $\pm$ 1.08 <sup>b</sup>	94.03 $\pm$ 0.60 <sup>ab</sup>	96.47 $\pm$ 0.36 <sup>a</sup>	95.52 $\pm$ 1.23 <sup>ab</sup>
Average egg weight (g)	57.92 $\pm$ 0.89 <sup>a</sup>	54.91 $\pm$ 0.97 <sup>b</sup>	56.53 $\pm$ 0.46 <sup>ab</sup>	57.74 $\pm$ 0.36 <sup>a</sup>	57.17 $\pm$ 0.88 <sup>ab</sup>
FCR (g:g)	2.07 $\pm$ 0.06 <sup>b</sup>	2.25 $\pm$ 0.06 <sup>a</sup>	2.16 $\pm$ 0.04 <sup>ab</sup>	2.14 $\pm$ 0.02 <sup>ab</sup>	2.19 $\pm$ 0.05 <sup>ab</sup>

<sup>a,b</sup>Means within a row with different superscript differ significantly ( $P < 0.05$ ). †Data are means for four replicates of 18 laying hens/replicate. ADFI, average daily feed intake; CSM, cottonseed meal; ECSM, expanded cottonseed meal; FCR, feed conversion ratio.

**Table 4** Egg quality of laying henst

Item	Treatment				
	Control	6% CSM	6% ECSM	8% ECSM	10% ECSM
Albumen height (mm)	6.66 ± 0.54 <sup>a</sup>	5.40 ± 0.85 <sup>b</sup>	6.81 ± 0.43 <sup>a</sup>	6.15 ± 0.51 <sup>ab</sup>	5.42 ± 0.86 <sup>b</sup>
Haugh unit	83.05 ± 1.75 <sup>a</sup>	73.42 ± 1.75 <sup>b</sup>	82.97 ± 2.41 <sup>a</sup>	77.96 ± 3.67 <sup>ab</sup>	74.09 ± 2.36 <sup>b</sup>
Yolk color	5.90 ± 0.43 <sup>b</sup>	7.19 ± 0.19 <sup>a</sup>	6.75 ± 0.41 <sup>ab</sup>	6.60 ± 0.30 <sup>ab</sup>	6.89 ± 0.35 <sup>ab</sup>
Eggshell strength (kgf/m <sup>2</sup> )	3.98 ± 0.28	3.50 ± 0.88	3.48 ± 0.69	4.38 ± 0.44	4.24 ± 0.30
Eggshell thickness (mm)	0.372 ± 0.012	0.388 ± 0.031	0.393 ± 0.027	0.385 ± 0.043	0.406 ± 0.034

<sup>a,b</sup>Means within a row with different superscript differ significantly ( $P < 0.05$ ). †Data are means for four replicates of nine eggs/replicate. CSM, cottonseed meal; ECSM, expanded cottonseed meal; kgf, kilogram force.

Eggshell strength ultimately affects the soundness of the shell, and weaker shelled eggs are more prone to have cracks and breakages followed by subsequent microbial contamination. There were no changes ( $P > 0.05$ ) among any treatments regarding eggshell strength and eggshell thickness (Table 4). Haugh unit and albumen height are important concerning internal egg characteristics. This study showed that birds fed 6% CSM and 10% ECSM had lower ( $P < 0.05$ ) albumen height and Haugh unit than birds fed control, 6% and 8% ECSM diets. However, previous studies reported that 15% to 60% of CSM replacement of soybean meal had no significant effect on albumen height and Haugh unit of layers (Davis *et al.* 2002; Odunsi *et al.* 2007). The differences in the values might be connected with Silversides and Scott's (2001) presumption that Haugh unit was influenced by age of hens and storage time.

Many of the original studies reporting egg discoloration when CSM was included in the feed were conducted using ground whole cottonseeds or with CSM produced by screwpress processing techniques (Davis *et al.* 2002). The primary determinant of yolk color is the xanthophyll content of the diet consumed. However, yolk color can be manipulated according to the ingredients in diet, for example, the inclusion of higher levels or incorrect ratios of pigments can lead to orange-red yolks and the inclusion of weed seeds may result in green yolks, and the inclusion of white corn or barley may yield light-colored yolks (Natalie 2009). As Table 4 indicated, hens fed 6% CSM diets produced egg yolk with severe ( $P < 0.05$ ) discoloration. Nelson (2004) found that yolk color score increased with supplemental levels of CSM. Similar findings demonstrated that birds fed 30% CSM had higher yolk color score than those fed similar levels of soybean meal (Davis *et al.* 2002). In contrast, previous studies had also noted that some laying hens seemed to be more susceptible to the effects of gossypol ingestion than others (Panigrahi *et al.* 1989; Panigrahi & Morris 1991). If this variation in susceptibility to gossypol is based on genetic differences, it could mean that a strain of laying hens could be selected for tolerance to gossypol (Davis *et al.* 2002). Egg discoloration in the current research was limited

to that associated with gossypol, which was assumed to cause egg yolk discoloration based on a chemical combination of gossypol with ferric iron ( $\text{Fe}^{+3}$ ) released from yolk proteins (Kemmerer *et al.* 1966). Lordelo *et al.* (2007) reported that severe egg yolk discoloration was only caused by feeding hens (+)-gossypol, but the mechanism is unknown.

As shown in Table 5, the 6% CSM group had the highest concentrations of FG, and liver and kidney were the main target tissues for FG residues. There was significant interaction between dietary levels of ECSM and different samples in concentrations of FG ( $P < 0.05$ ). No significant differences ( $P > 0.05$ ) were found in serum FG concentration between the CSM and ECSM groups. The highest concentration of FG in serum was in agreement with gossypol accumulating in the liver, kidney and other tissues (Gamboa *et al.* 2001; Lordelo *et al.* 2005). Hens fed 6% CSM had the highest ( $P < 0.05$ ) concentrations of FG in the liver, kidney and muscle than any ECSM treatments, and FG concentrations in the liver, kidney and muscle increased ( $P < 0.05$ ) with the increase of supplemental levels of ECSM. Meanwhile, the liver had the highest concentrations of FG followed by kidney and muscle, and hens fed 6% CSM and 10% ECSM had the highest ( $P < 0.05$ ) FG concentrations in the liver compared with those in the kidney and muscle. A similar pattern of FG accumulation was reported previously by Kim *et al.* (1996) in lambs and by Knabe *et al.* (1995) in pigs and by Gamboa *et al.* (2001) and Lordelo *et al.* (2005) in broilers. Dietary inclusion of 6% CSM also resulted in the highest ( $P < 0.05$ ) FG residues in yolk and albumen. FG concentrations in yolk and albumen of the 10% ECSM group were higher ( $P < 0.05$ ) than those in the other two ECSM groups. Moreover, hens fed 6% CSM and 10% ECSM had higher ( $P < 0.05$ ) FG residues in yolk than those in albumen. These results strongly indicate that higher FG concentration in yolk was probably associated with higher FG concentration in the liver, because the proteins of the yolks are continuously synthesized in the liver, while the proteins of the albumen are synthesized in the oviduct (Etches 1996). However, more research is needed to determine the mechanisms responsible for the deposition of FG in egg yolk.



**Table 5** Concentrations of FG in yolk, albumen, liver, kidney, muscle and serum of laying henst

Item	Treatment			
	6% CSM	6% ECSM	8% ECSM	10% ECSM
Yolk (mg/kg)	0.200 ± 0.012 <sup>ay</sup>	0.068 ± 0.012 <sup>cx</sup>	0.093 ± 0.010 <sup>cx</sup>	0.155 ± 0.018 <sup>by</sup>
Albumen (mg/kg)	0.103 ± 0.005 <sup>az</sup>	0.033 ± 0.007 <sup>cx</sup>	0.055 ± 0.010 <sup>cx</sup>	0.075 ± 0.006 <sup>bcz</sup>
Liver (mg/kg)	0.442 ± 0.020 <sup>aw</sup>	0.182 ± 0.019 <sup>dw</sup>	0.258 ± 0.014 <sup>cw</sup>	0.350 ± 0.012 <sup>bw</sup>
Kidney (mg/kg)	0.385 ± 0.011 <sup>ax</sup>	0.160 ± 0.006 <sup>dw</sup>	0.195 ± 0.006 <sup>cw</sup>	0.297 ± 0.013 <sup>bx</sup>
Muscle (mg/kg)	0.250 ± 0.028 <sup>ay</sup>	0.039 ± 0.002 <sup>dx</sup>	0.089 ± 0.002 <sup>cx</sup>	0.142 ± 0.010 <sup>by</sup>
Serum (µg/mL)	1.931 ± 0.009 <sup>a</sup>	0.523 ± 0.030 <sup>a</sup>	0.715 ± 0.029 <sup>a</sup>	0.892 ± 0.006 <sup>a</sup>
FG contents (mg/kg)				
Diets		Samples		
6% CSM	0.276 ± 0.033 <sup>x</sup>	Yolk	0.129 ± 0.017 <sup>y</sup>	
6% ECSM	0.096 ± 0.017 <sup>z</sup>	Albumen	0.067 ± 0.008 <sup>y</sup>	
8% ECSM	0.138 ± 0.021 <sup>yz</sup>	Liver	0.308 ± 0.030 <sup>x</sup>	
10% ECSM	0.204 ± 0.028 <sup>xy</sup>	Kidney	0.259 ± 0.027 <sup>x</sup>	
		Muscle	0.130 ± 0.024 <sup>y</sup>	
Analysis of variance	df	P-value		
Diets	3		0.000	
Samples	4		0.000	
Diets × samples	12		0.000	

<sup>a-d</sup>Means within a row with different superscripts differ significantly ( $P < 0.05$ ). <sup>w-z</sup>Means within a column with different superscript differ significantly ( $P < 0.05$ ). †Data are means for four replicates of four hens/replicate and nine eggs/replicate. CSM, cottonseed meal; ECSM, expanded cottonseed meal. FG, free gossypol.

In summary, FG in CSM can be reduced by 68% through an expanded process and ECSM can be available in laying hens at up to 10% of the total diet and an appropriate replacement of soybean meal with ECSM may improve performance in laying hens.

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