

# Research Note: Effects of riboflavin on reproductive performance and antioxidant status of duck breeders

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**ABSTRACT** An experiment was conducted to investigate the effects of dietary riboflavin levels on reproductive performance, riboflavin status, and antioxidant status of laying duck breeders, to estimate the requirement of this vitamin for duck breeders. Different levels crystalline riboflavin (0, 2.5, 5, 10, and 15 mg/kg) were supplemented to a corn–soybean–corn gluten meal basal diet to produce 5 dietary treatments with different analyzed total riboflavin levels (1.48, 3.20, 6.30, 11.71, and 16.83 mg/kg). A total of 80 White Pekin duck breeders aged 40 wk were allotted to 5 dietary treatments of 16 birds each (8 replicates per treatment and 2 breeders per replicate), and all birds were raised individually for 9 wk. At the end of the experiment, reproductive performance, tissue riboflavin concentrations, and antioxidant status of White Pekin duck breeders were measured. The results showed that body weight, egg weight, egg production, and egg fertility were not affected by dietary riboflavin levels. However, among all of the laying duck breeders, the

birds fed the basal diet without riboflavin supplementation had the lowest egg hatchability, plasma riboflavin, egg yolk riboflavin, and egg albumen riboflavin ( $P < 0.001$ ). In addition, the duck breeders fed the basal diet without riboflavin supplementation showed the lowest antioxidant capacity indicated by greatest plasma malondialdehyde (MDA) content and lowest reduced glutathione content, total superoxide dismutase (T-SOD) activities, and total antioxidant capacity in both plasma ( $P < 0.001$ ) and egg yolk ( $P < 0.001$ ). These results revealed that dietary riboflavin supplementation improved the reproductive performance and antioxidant status of the duck breeders. According to the broken-line model, the riboflavin requirements (based on dietary total riboflavin) of laying duck breeders in terms of the egg hatchability, plasma riboflavin, egg yolk riboflavin, egg albumen riboflavin, plasma T-SOD activity, and plasma MDA content were 3.19, 7.42, 3.88, 7.44, 6.45, and 8.84 mg/kg, respectively.

**Key words:** duck breeder, riboflavin, reproductive performance, antioxidant status

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## INTRODUCTION

Riboflavin is an essential precursor of flavin mononucleotide and flavin adenine dinucleotide, which serve as coenzymes for numerous redox reactions in primary metabolic pathways such as antioxidant system and energy metabolism (Powers, 2003; Lienhart et al., 2013).

It has been shown that riboflavin deficiency reduces reproductive performance and riboflavin status in laying hens and ducks (Naber and Squires, 1993; Squires and Naber, 1993; Tang et al., 2019). In particular, dietary riboflavin deficiency in laying hens decreased hen weight, egg weight, egg production, and egg hatchability, as well as riboflavin concentrations in egg yolk and albumen to result in embryonic death (Naber and Squires, 1993; Squires and Naber, 1993). In addition, a strain of Leghorn chicken genetically unable to deposit riboflavin in their eggs, lays riboflavin-deficient eggs, and all of the embryos suddenly die at mid-incubation (Maw, 1954; Abrams et al., 1995; White III, 1996; Cogburn et al., 2018). Similarly, we found that dietary riboflavin deficiency gradually

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decreased egg hatchability to approximate zero and markedly reduced riboflavin concentrations in plasma and egg yolk in laying duck breeders (Tang et al., 2019). The high mortality of riboflavin-deficient embryos may be because of impaired energy generation processes, leading to energy depletion and severe hypoglycemia (White, 1996; Cogburn et al., 2018; Tang et al., 2019).

Riboflavin plays important roles in antioxidant system. Glutathione reductase is flavin adenine dinucleotide-dependent enzyme that catalyzes the reduction of glutathione disulfide to reduced glutathione (GSH). It has been well documented previously that riboflavin deficiency decreased glutathione reductase activity and GSH concentration, thereby leading to oxidative stress in animals (Bamji, 1969; Bamji and Sharada, 1972; Levin et al., 1990; Camporeale and Zempleni, 2003; Manthey et al., 2005; Chen et al., 2015). Also, in our previous study, dietary riboflavin deficiency decreased antioxidant capacity in starter ducks (Tang et al., 2014). However, it is still unclear whether dietary riboflavin affects antioxidant status of laying duck breeders, which requires further investigation.

Because no work has been reported on the riboflavin requirement of laying duck breeders, the NRC (1994) refers the reader to the requirement for starter ducks from 1 to 2 wk of age (4.0 mg/kg). However, it has been demonstrated that the riboflavin requirements decrease with increasing age in both chicks and ducks (Heuser et al., 1938; Tang et al., 2015). Hence, it is unknown whether the riboflavin requirement of starter ducks also applies to birds in the egg-laying period. Therefore, the objectives of the present study were to examine the effects of graded riboflavin levels on reproductive performance, riboflavin status, and antioxidant status of White Pekin duck breeders and to evaluate the requirement of this vitamin for these birds.

## MATERIALS AND METHODS

All experimental procedures of the present study were approved by the animal care and use committee of Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, and performed according to the guidelines for animal experiments established by the National Institute of Animal Health.

### Birds and Housing

A dose-response experiment with 5 dietary riboflavin levels (1.48, 3.20, 6.30, 11.71, and 16.83 mg/kg) was conducted to investigate the effects of riboflavin on reproductive performance, riboflavin status, and antioxidant status of laying duck breeders. A total of 80 female White Pekin duck breeders (*Anas platyrhynchos*) aged 40 wk were obtained from the Pekin duck breeding center (Chinese Academy of Agricultural Sciences) and were divided into 5 treatment groups with 8 replicates, received 5 experimental diets for 9 wk. All the laying duck breeders were individually placed in plastic cages (1.00 m length  $\times$  0.80 m deep  $\times$  0.60 m height).

Meanwhile, 16 male ducks of the same age with females were used to mate with female ducks during the experimental period. Every 5 consecutive cages composed one batch and, there were 16 batches in total. In each batch, the male duck was placed with the female duck breeder fed the experimental diets with 15 mg/kg riboflavin supplementation on the first day and was moved to the female fed the basal diet without riboflavin supplementation on the second day at 08:00 h and was moved to the females fed the diets with 10, 2.5, 5 mg/kg riboflavin supplementation on the third, fourth, and fifth day at 08:00 h, respectively. The same steps were repeated during the experimental period, where all of the female duck breeders were naturally inseminated on each of 5 D. In each group, 2 birds from 2 batches are considered to be one replicate. All the ducks received *ad libitum* access to water and feed. Eighteen hours of light were provided daily from 04:00 to 22:00 h.

### Experimental Diets

All the female ducks were raised with common diets from hatch to 40 wk of age, and all nutrients met the recommendations for ducks as established in the Nutrient Requirements of Meat-type Ducks of China (2012). During the experimental period (from 40–49 wk of age), a riboflavin-deficient basal diet containing 1.48 mg free riboflavin/kg of diet was formulated (Table 1). The vitamin mixture was free of riboflavin. Except the

**Table 1.** Composition of riboflavin-deficient basal diet (% as-fed basis).

Ingredient	Content
Corn	56.0
Corn gluten meal (44.3% CP)	10.0
Soybean meal (44% CP)	23.8
Limestone	7.00
Dicalcium phosphate	1.50
Vitamin and trace mineral premix <sup>1</sup>	1.00
DL-methionine	0.10
Sodium chloride	0.30
Lysine hydrochloride	0.30
Total	100
Calculated composition	
Metabolizable energy <sup>2</sup> , kcal/kg	2,743
CP (%)	19.29
Calcium (%)	3.07
Non-phytate P (%)	0.35
Lysine (%)	1.04
Methionine (%)	0.46
Methionine + cysteine (%)	0.77
Threonine (%)	0.76
Tryptophan (%)	0.20
Arginine (%)	1.01
Riboflavin <sup>3</sup> , mg/kg	1.48

<sup>1</sup>Supplied per kilogram of total diet: Cu (CuSO<sub>4</sub>•5H<sub>2</sub>O), 10 mg; Fe (FeSO<sub>4</sub>•7H<sub>2</sub>O), 60 mg; Zn (ZnO), 60 mg; Mn (MnSO<sub>4</sub>•H<sub>2</sub>O), 80 mg; Se (NaSeO<sub>3</sub>), 0.3 mg; I (KI), 0.2 mg; choline chloride, 1,000 mg; vitamin A (retinyl acetate), 10,000 IU; vitamin D<sub>3</sub> (Cholcalciferol), 3,000 IU; vitamin E (DL- $\alpha$ -tocopheryl acetate), 20 IU; vitamin K<sub>3</sub> (menadione sodium bisulfate), 2 mg; thiamin (thiamin mononitrate), 2 mg; pyridoxine hydrochloride, 4 mg; cobalamin, 0.02 mg; calcium-D-pantothenate, 20 mg; nicotinic acid, 50 mg; folic acid, 1 mg; biotin, 0.2 mg.

<sup>2</sup>The values are calculated according to the AME of ducks (Ministry of Agriculture of China, 2012).

<sup>3</sup>The value was analyzed by high performance liquid chromatography.

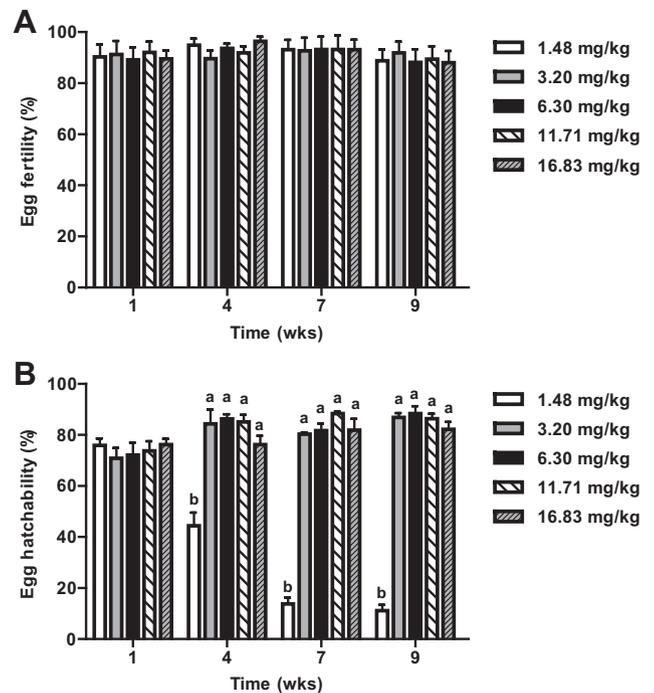
riboflavin content of the basal experimental diet, all nutrients met the recommendations for laying duck breeders by Ministry of Agriculture of China (2012). To produce 5 experimental diets, the basal diet was prepared as mash and then supplemented with 0, 2.5, 5, 10, and 15 mg crystalline riboflavin/kg diet, respectively. The riboflavin content of all experimental diets was analyzed by high performance liquid chromatography (HPLC). The analyzed values of 5 experimental diets were 1.48, 3.20, 6.30, 11.71, and 16.83 mg riboflavin/kg, respectively. All of the diets were cold-pelleted at room temperature. The crystalline riboflavin (purity, 99%) was obtained from Sigma Aldrich (St. Louis, MO).

## Productivity and Reproductive Performance

All laying duck breeders were weighed at the beginning and end of the experiment. During the experimental period, all eggs were collected and weighed daily on a replicate basis and stored at 16°C under relative humidity of 75%. Egg production and egg weight were calculated daily on a replicate basis and then expressed as averages for the 9-wk period from 40 to 49 wk of age. During the first, fourth, seventh, and ninth wks of experiment, 10 eggs from each replicate were selected and incubated in a commercial incubator (Yiai 12,096, Qingdao, China). The temperature was controlled at 37.8°C, 37.6°C, and 37.5°C during Days 1 to 14, 15 to 21, and 22 to 26 of incubation at 60% humidity. At day 5 of incubation, we candled the eggs to determine their fertility (expressed as the percentage of fertile eggs relative to incubated eggs). At day 26 of incubation, the eggs were transferred to a hatcher at 36.5°C and with humidity of 70%. At day 28 of incubation, the eggs that failed to hatch and the hatched ducklings were recorded. The hatchability of fertile eggs was calculated as percentage of hatched ducklings relative to the fertile eggs. Egg fertility and hatchability were calculated on a replicate basis.

## Sample Preparation and Data Collection

On the last day of the experiment (at 49 wk of age), blood samples from all the breeders were collected via a wing vein into heparin sodium-anticoagulant tubes and centrifuged at  $1,520 \times g$  for 15 min to obtain plasma. Plasma samples were stored at -20°C until



**Figure 1.** Effects of dietary riboflavin levels on egg fertility and hatchability of laying duck breeders. (A) Egg fertility ( $P > 0.05$ ,  $n = 8$  per group, error bars = SEM). (B) Egg hatchability ( $P < 0.001$ ,  $n = 8$  per group, error bars = SEM). Bars with different letters (a, b) differ significantly.

subsequent analysis. On the last day of the experiment, one egg from each replicate was broken to collect yolk and albumen samples, which were then stored at -20°C until further analysis.

At the end of the experiment, the male ducks were continuously raised for 5 D, received different experimental diets containing 16.83, 1.48, 11.71, 3.20, and 6.30 mg riboflavin/kg each day, respectively. Before changing experimental diets for male ducks, blood samples were collected every day at 08:00 h to obtain plasma. Plasma riboflavin contents of male ducks were determined, and no significant difference was found when the birds fed the different experimental diets for 1 D.

## Measurements

The riboflavin concentrations in feed, plasma, egg yolk, and egg albumen were determined by reversed-phase HPLC according to the methods described previously (Tang et al., 2013). Before HPLC analysis, feed

**Table 2.** Effects of dietary riboflavin levels on body weight and reproductive performance of laying duck breeders<sup>1</sup>.

Dietary riboflavin (mg/kg)	Initial body weight (kg)	Final body weight (kg)	Egg weight (g)	Egg production (%)
1.48	3.33	3.45	92.9	83.0
3.20	3.37	3.55	94.6	84.4
6.30	3.31	3.39	93.6	82.3
11.71	3.26	3.46	92.7	83.5
16.83	3.34	3.45	92.9	82.9
SEM	0.027	0.04	0.35	0.76
<i>P</i> -value	0.879	0.700	0.570	0.963

<sup>1</sup>Results are means with  $n = 8$  per group.

**Table 3.** Effects of dietary riboflavin levels on the riboflavin concentrations in plasma, egg yolk, and egg albumen of laying duck breeders<sup>1</sup>.

Dietary riboflavin (mg/kg)	Plasma riboflavin (μmol/L)	Egg yolk riboflavin (μg/g)	Egg albumen riboflavin (μg/g)
1.48	1.06 <sup>d</sup>	6.96 <sup>c</sup>	0.017 <sup>d</sup>
3.20	2.85 <sup>c</sup>	15.2 <sup>b</sup>	0.043 <sup>c</sup>
6.30	3.56 <sup>b</sup>	17.7 <sup>a</sup>	0.056 <sup>b</sup>
11.71	4.31 <sup>a</sup>	19.1 <sup>a</sup>	0.072 <sup>a</sup>
16.83	4.31 <sup>a</sup>	18.4 <sup>a</sup>	0.070 <sup>a</sup>
SEM	0.17	0.58	0.003
<i>P</i> -value	<0.001	<0.001	<0.001

<sup>a-d</sup>Means with different superscripts within the same column differ significantly ( $P < 0.05$ ).

<sup>1</sup>Results are means with  $n = 8$  per group.

and plasma samples were prepared according to the method described previously (Britton et al., 2003; Petteys and Frank, 2011), whereas egg yolk and egg albumen samples were prepared according to the method for animal tissue described previously (Batey and Eckhart, 1990). The peak of riboflavin was identified and quantified by analysis of authentic standard (Sigma Aldrich).

The malondialdehyde (MDA) and GSH contents and the total superoxide dismutase (T-SOD) and total antioxidant capacity (T-AOC) activities in plasma and egg yolk were measured using colorimetric methods with commercial kits (Nanjing Jiancheng Institute of Bioengineering, Nanjing, Jiangsu, China). These parameters were expressed as units per milliliter for plasma and units per milligram of protein for egg yolk. The egg yolk homogenate was assayed for contents of protein. One unit of T-SOD activity (U) was defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. One unit of T-AOC activity (U) was defined as the amount of enzyme per 1 mL plasma or per 1 mg egg yolk protein that would increase the absorbance by 0.01 at 37°C in 1 min.

### Statistical Analysis

Eight replicates were used as the experimental units. Data were analyzed by the one-way ANOVA procedure of SAS software (SAS Institute, 2011). The variability in the data was expressed as SEM. A probability level of  $P < 0.05$  was considered to be statistically significant. The broken-line regression analysis (Robbins et al., 2006) was used to estimate the riboflavin requirements

for ducks. The broken-line model was provided as follows:

$$y = l + u(r - x)$$

where,  $y$  = egg hatchability, riboflavin status (plasma riboflavin, egg yolk riboflavin, or egg albumen riboflavin), plasma T-SOD, or plasma MDA,  $x$  = dietary total riboflavin level (mg/kg),  $r$  = riboflavin requirement,  $l$  = the response at  $x = r$ ,  $u$  = the slope of the curve. In this model,  $y = l$  when  $x > r$ .

## RESULTS AND DISCUSSION

### Reproductive Performance

In the present study, dietary riboflavin levels did not affect the final body weight of the laying duck breeders, the overall period of egg weight and egg production ( $P > 0.05$ , Table 2), and egg fertility in the first, fourth, seventh, and ninth wks of the experiment ( $P > 0.05$ , Figure 1A), thereby confirming our previously findings (Tang et al., 2019). These results are also in line with previous findings in laying hens, showing that egg production did not change in laying hens fed a basal diet without riboflavin supplementation for 8 wk, although the egg production, hen weight, and egg weight were all decreased thereafter or at 27 wk (Squires and Naber, 1993). Previous studies showed that egg hatchability decreased to 5% in laying hens fed a riboflavin deficient diet for 3 wk (Naber and Squires, 1993; Squires and Naber, 1993), and egg hatchability was dropped to approximately zero at 6 wk in riboflavin-deficient laying duck breeders (Tang et al., 2019). In

**Table 4.** Effects of dietary riboflavin levels on plasma antioxidant status of laying duck breeders<sup>1</sup>.

Dietary riboflavin (mg/kg)	MDA (nmol/mL)	GSH (mg/L)	T-SOD (U/mL)	T-AOC (U/mL)
1.48	6.73 <sup>a</sup>	19.6 <sup>c</sup>	39.1 <sup>c</sup>	10.3 <sup>c</sup>
3.20	5.37 <sup>b</sup>	24.4 <sup>b,c</sup>	54.2 <sup>b</sup>	12.4 <sup>b</sup>
6.30	4.90 <sup>b,c</sup>	25.1 <sup>b</sup>	58.8 <sup>a,b</sup>	14.1 <sup>b</sup>
11.71	4.38 <sup>c</sup>	32.4 <sup>a</sup>	61.7 <sup>a</sup>	16.5 <sup>a</sup>
16.83	4.08 <sup>c</sup>	34.9 <sup>a</sup>	62.5 <sup>a</sup>	16.8 <sup>a</sup>
SEM	0.19	0.20	1.54	0.48
<i>P</i> -value	<0.001	<0.001	<0.001	<0.001

<sup>a-c</sup>Means with different superscripts within the same column differ significantly ( $P < 0.05$ ).

Abbreviations: MDA, malondialdehyde; GSH, reduced glutathione; T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity.

<sup>1</sup>Results are means with  $n = 8$  per group.

**Table 5.** Effects of dietary riboflavin levels on egg yolk antioxidant status of laying duck breeders<sup>1</sup>.

Dietary riboflavin (mg/kg)	MDA (nmol/mgprot)	GSH (mg/gprot)	T-SOD (U/mgprot)	T-AOC (U/mgprot)
1.48	2.61 <sup>a</sup>	4.01 <sup>c</sup>	5.42 <sup>c</sup>	0.27 <sup>c</sup>
3.20	2.11 <sup>b</sup>	4.87 <sup>b</sup>	7.32 <sup>b</sup>	0.30 <sup>b,c</sup>
6.30	1.59 <sup>c</sup>	5.10 <sup>b</sup>	9.65 <sup>a</sup>	0.44 <sup>b</sup>
11.71	1.44 <sup>c</sup>	6.22 <sup>a</sup>	11.1 <sup>a</sup>	0.58 <sup>a</sup>
16.83	1.30 <sup>c</sup>	6.63 <sup>a</sup>	11.1 <sup>a</sup>	0.58 <sup>a</sup>
SEM	0.10	0.17	0.40	0.025
<i>P</i> -value	<0.001	<0.001	<0.001	<0.001

<sup>a-c</sup>Means with different superscripts within the same column differ significantly ( $P < 0.05$ ).

Abbreviations: MDA, malondialdehyde; GSH, reduced glutathione; T-SOD, total superoxide dismutase; T-AOC, total antioxidant capacity.

<sup>1</sup>Results are means with  $n = 8$  per group.

the present study, among all of the laying duck breeders, the birds fed the basal diet without riboflavin supplementation had the lowest egg hatchability (11.1%) at 9 wk ( $P < 0.001$ , Figure 1B). This criterion increased as dietary riboflavin increased, reached a plateau when dietary riboflavin was above 3.20 mg/kg (Figure 1B). These results are consistent with previous observations in laying hens (Naber and Squires, 1993; Squires and Naber, 1993) and duck breeders (Tang et al., 2019). The low egg hatchability induced by riboflavin deficiency in laying duck breeders may be associated with impaired energy generation processes because a number of proteins involved in fatty acid beta oxidation, electron transport chain, and tricarboxylic acid cycle were down-regulated in the liver of riboflavin-deficient embryos (Tang et al., 2019).

The broken-line regression has been employed extensively to evaluate the riboflavin requirements of broilers (Ruiz and Harms, 1988; Chung and Baker, 1990) and ducks (Hegsted and Perry, 1948; Tang et al., 2013, 2014, 2015). Therefore, this regression was also used to estimate riboflavin requirements of laying duck breeders in the present study. According to this regression, the riboflavin requirement of laying duck breeders for egg hatchability was 3.19 mg riboflavin/kg diet ( $y = 84.80 - 43.10 \times [3.19 - x]$ ,  $x \leq 3.19$ ,  $P < 0.001$ ,  $R^2 = 0.934$ ), which is close to the value provided by NRC (1994) (4.0 mg/kg).

## Riboflavin Status

Tissue riboflavin is considered to be a useful biomarker for riboflavin status in animals. Previous studies have shown that riboflavin concentrations in the plasma, egg yolk, and egg albumen were markedly diminished in riboflavin deficient laying hens and duck breeders (Naber and Squires, 1993; Squires and Naber, 1993; Tang et al., 2019). In agreement with these previous studies, among all of the laying duck breeders, the birds fed the basal diet with no supplementation of riboflavin showed the lowest riboflavin concentrations in plasma, egg yolk, and egg albumen in the present study ( $P < 0.001$ , Table 3). All these parameters increased as dietary riboflavin increased (Table 3). Plasma riboflavin, egg yolk riboflavin, and egg albumen riboflavin reached a plateau when dietary riboflavin was

above 11.71, 6.30, and 11.71 mg/kg, respectively (Table 3). These results indicated that plasma riboflavin and egg albumen riboflavin levels exhibited stronger responses to dietary riboflavin than egg yolk riboflavin in laying duck breeders. Previous studies also indicated that riboflavin in egg albumen depleted more rapidly than that in egg yolk in hens fed riboflavin-deficient diets (Squires and Naber, 1993). This phenomenon can be explained by the shorter albumen secretion process compared with the long follicle maturation process, where albumen secretion takes only place when the yolk is present in the magnum of the oviduct (Salamon and Kent, 2013).

Notably, the dramatically decreased egg hatchability in laying duck breeders fed the basal diet without riboflavin supplementation was because of the markedly reduced riboflavin concentrations in the egg yolk and egg albumen, which is in line with the previous results in laying hens and duck breeders (Naber and Squires, 1993; Squires and Naber, 1993; Tang et al., 2019).

Similar to the previous studies in starter and growing ducks (Tang et al., 2013, 2014, 2015), tissue riboflavin concentrations increased with increasing riboflavin levels in the diets. Therefore, tissue riboflavin concentrations were used to estimate riboflavin requirements of laying duck breeders. According to the broken-line analysis, the riboflavin requirements of laying duck breeders for plasma riboflavin, egg yolk riboflavin, and egg albumen riboflavin were 7.42, 3.88, and 7.44 mg riboflavin/kg diet, respectively (plasma riboflavin:  $y = 4.32 - 0.50 \times [7.42 - x]$ ,  $x \leq 7.42$ ,  $P < 0.001$ ,  $R^2 = 0.743$ ; egg yolk riboflavin:  $y = 18.43 - 4.77 \times [3.88 - x]$ ,  $x \leq 3.88$ ,  $P < 0.001$ ,  $R^2 = 0.758$ ; egg albumen riboflavin:  $y = 0.071 - 0.0086 \times [7.44 - x]$ ,  $x \leq 7.44$ ,  $P < 0.001$ ,  $R^2 = 0.762$ ). The riboflavin requirements estimated for tissue saturation were much greater than that for egg hatchability (3.19 mg/kg), which is consistent with our previous findings in starter or growing ducks where we found that the riboflavin requirements for tissue riboflavin concentrations were greater than those for growth performance (Tang et al., 2013, 2014, 2015). To our best knowledge, this is the first study to estimate the riboflavin requirement of laying duck breeders. Because no work has been reported on the riboflavin requirement of laying duck breeders, the NRC (1994) refers the reader to the requirement for starter ducks aged from 1 to 2 wk (4.0 mg/kg). The

riboflavin requirements for plasma riboflavin and egg albumen riboflavin estimated by this study (7.42 and 7.44 mg/kg) were much greater than the NRC (1994) recommendation for laying duck breeders (4.0 mg/kg).

### Antioxidant Status

It has been reported that riboflavin deficiency can cause oxidative damage in rats (Levin et al., 1990), fish (Chen et al., 2015), and ducks (Tang et al., 2014). Similarly, among all of the laying duck breeders, we found that the birds fed the basal diet with no supplementation of riboflavin had the greatest MDA content as well as the lowest GSH content and T-SOD and T-AOC activities in both plasma and egg yolk in the present study ( $P < 0.01$ , Table 4;  $P < 0.01$ , Table 5). These results indicated that riboflavin deficiency could cause oxidative stress in laying duck breeders, which is consistent with previous studies (Levin et al., 1990; Tang et al., 2014; Chen et al., 2015). Riboflavin deficiency decreased glutathione reductase activity (Bamji, 1969; Bamji and Sharada, 1972; Levin et al., 1990) and GSH concentration (Camporeale and Zemleni, 2003; Manthey et al., 2005), thereby increasing the lipid peroxidation products in tissues (Levin et al., 1990). This explanation is supported by the markedly reduced GSH concentration found in riboflavin-deficient duck breeders in the present study. These effects could be another cause of embryonic death and the reduced egg hatchability induced by maternal riboflavin deficiency. According to the broken-line analysis, the riboflavin requirements of laying duck breeders for plasma T-SOD activity and MDA content were 6.45 and 8.84 mg riboflavin/kg diet, respectively (plasma T-SOD:  $y = 62.19 - 4.08 \times [6.45 - x]$ ,  $x \leq 6.45$ ,  $P < 0.001$ ,  $R^2 = 0.625$ ; plasma MDA:  $y = 4.08 + 0.20 \times [8.84 - x]$ ,  $x \leq 8.84$ ,  $P < 0.001$ ,  $R^2 = 0.389$ ), which were close to the values estimated for plasma riboflavin and egg albumen riboflavin in this study (7.42 and 7.44 mg/kg).

In conclusion, among all of the laying duck breeders, the birds fed the basal diet without riboflavin supplementation had the lowest the egg hatchability and riboflavin concentrations in plasma, egg yolk, and egg albumen. Meanwhile, these birds also had the lowest antioxidant capacity in both plasma and egg yolk, indicated by greatest plasma MDA content and lowest GSH content, T-SOD, and T-AOC activities. Fortunately, all these ill effects can be avoided by riboflavin supplementation. According to the broken-line model, the riboflavin requirements of laying duck breeders for egg hatchability, plasma riboflavin, egg yolk riboflavin, egg albumen riboflavin, plasma T-SOD activity, and plasma MDA content were 3.19, 7.42, 3.88, 7.44, 6.45, and 8.84 mg/kg, respectively.

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### REFERENCES

- Abrams, V. A. M., C. C. Han, and H.B. White, III. 1995. Riboflavin-deficient chicken embryos: hypoglycemia without dicarboxylic aciduria. *Comp. Biochem. Phys. B.* 111:233–241.
- Bamji, M. S. 1969. Glutathione reductase activity in red blood cells and riboflavin nutritional status in humans. *Clin. Chim. Acta* 26:263–269.
- Bamji, M. S., and D. Sharada. 1972. Hepatic glutathione reductase and riboflavin concentrations in experimental deficiency of thiamin and riboflavin in rats. *J. Nutr.* 102:443–448.
- Batey, D. W., and C. D. Eckhert. 1990. Identification of FAD, FMN, and riboflavin in the retina by microextraction and high-performance liquid chromatography. *Anal. Biochem.* 188:164–167.
- Britton, N. L., K. L. Riter, R. L. Smallidge, and J. Hillebrandt. 2003. Reversed-phase liquid chromatographic determination of riboflavin in feeds. *J. AOAC. Int.* 86:197–201.
- Camporeale, G., and J. Zemleni. 2003. Oxidative folding of interleukin-2 is impaired in flavin-deficient Jurkat cells, causing intracellular accumulation of interleukin-2 and increased expression of stress response genes. *J. Nutr.* 133:668–672.
- Chen, L., L. Feng, W. D. Jiang, J. Jiang, P. Wu, J. Zhao, S. Y. Kuang, L. Tang, W. N. Tang, Y. A. Zhang, X. Q. Zhou, and Y. Liu. 2015. Intestinal immune function, antioxidant status and tight junction proteins mRNA expression in young grass carp (*Ctenopharyngodon idella*) fed riboflavin deficient diet. *Fish. Shellfish. Immun.* 47:470–484.
- Chung, T. K., and D. H. Baker. 1990. Riboflavin requirement of chicks fed purified amino acid and conventional corn-soybean meal diets. *Poult. Sci.* 69:1357–1363.
- Cogburn, L. A., D. N. Smarsh, X. Wang, N. Trakooljul, W. Carré, and H. B. White. 2018. Transcriptional profiling of liver in riboflavin-deficient chicken embryos explains impaired lipid utilization, energy depletion, massive hemorrhaging, and delayed feathering. *BMC. Genomics.* 19:177.
- Hegsted, D. M., and R. L. Perry. 1948. Nutritional studies with the duck V. Riboflavin and pantothenic acid requirements. *J. Nutr.* 35:411–417.
- Heuser, G. F., H. S. Wilgus, and L. C. Norris. 1938. The quantitative vitamin-G requirement of chicks. *Poult. Sci.* 17:105–108.
- Levin, G., U. Cogan, Y. Levy, and S. Mokady. 1990. Riboflavin deficiency and the function and fluidity of rat erythrocyte membrane. *J. Nutr.* 120:857–861.
- Lienhart, W. D., V. Gudipati, and P. Macheroux. 2013. The human flavoproteome. *Arch. Biochem. Biophys.* 535:150–162.
- Manthey, K. C., Y. C. Chew, and J. Zemleni. 2005. Riboflavin deficiency impairs oxidative folding and secretion of apolipoprotein B-100 in HepG2 cells, triggering stress-response systems. *J. Nutr.* 135:978–982.
- Maw, A. J. G. 1954. Inherited riboflavin deficiency in chicken eggs. *Poult. Sci.* 33:216–217.
- Ministry of Agriculture of China 2012. *Nutrient Requirements of Meat-type Ducks of China*. China Agriculture Press, Beijing, China.
- Naber, E. C., and M. W. Squires. 1993. Research note: early detection of the absence of a vitamin premix in layer diets by egg albumen riboflavin analysis. *Poult. Sci.* 72:1989–1993.
- NRC 1994. *Nutrient Requirements of Poultry*, 9th rev. ed. Natl. Acad. Press, Washington, DC.
- Petteys, B. J., and E. L. Frank. 2011. Rapid determination of vitamin B<sub>2</sub> (riboflavin) in plasma by HPLC. *Clin. Chim. Acta* 412:38–43.
- Powers, H. J. 2003. Riboflavin (vitamin B-2) and health. *Am. J. Clin. Nutr.* 77:1352–1360.
- Robbins, K. R., A. M. Saxton, and L. L. Southern. 2006. Estimation of nutrient requirements using broken-line regression analysis. *J. Anim. Sci.* 84:E155–E165.
- Ruiz, N., and R. H. Harms. 1988. Riboflavin requirement of broiler chicks fed a corn-soybean diet. *Poult. Sci.* 67:794–799.

- Salamon, A., and J. P. Kent. 2013. Double and single yolked duck eggs: their contents and dimensions compared and the mechanical stimulation hypothesis for albumen secretion is supported. *Int. J. Poult. Sci.* 12:254–260.
- SAS Institute 2011. SAS User's Guide: Statistics Version 9.3. SAS Institute Inc., Cary, NC.
- Squires, M. W., and E. C. Naber. 1993. Vitamin profiles of eggs as indicators of nutritional status in the laying hen: riboflavin study. *Poult. Sci.* 72:483–494.
- Tang, J., J. Hu, Z. G. Wen, Y. Jiang, H. Al-Kateb, W. Huang, Y. M. Guo, M. Xie, and S. S. Hou. 2015. Effects of riboflavin supplementation on growth performance, carcass traits, and riboflavin status of growing male white Pekin ducks. *Anim. Feed Sci. Tech.* 209:274–279.
- Tang, J., J. Hu, M. Xue, Z. Guo, M. Xie, B. Zhang, Z. Zhou, W. Huang, and S. S. Hou. 2019. Maternal diet deficient in riboflavin induces embryonic death associated with alterations in the hepatic proteome of duck embryos. *Nutr. Metab.* 16:19.
- Tang, J., Z. G. Wen, Z. B. Guo, W. Huang, Y. M. Guo, M. Xie, and S. S. Hou. 2014. Dietary riboflavin supplementation improve the growth performance and antioxidant status of starter white Pekin ducks fed a corn–soybean meal diets. *Livest. Sci.* 170:131–136.
- Tang, J., M. Xie, J. Yang, Z. G. Wen, Y. W. Zhu, W. Huang, and S. S. Hou. 2013. Riboflavin requirements of white Pekin ducks from hatch to 21 d of age. *Br. Poult. Sci.* 54:407–411.
- White, III, H.B. 1996. Sudden death of chicken embryos with hereditary riboflavin deficiency. *J. Nutr.* 126:1303S–1307S.