Effects of riboflavin deficiency on the lipid metabolism of duck breeders and duck embryos

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ABSTRACT This study aimed to evaluate the effects of dietary riboflavin deficiency (**RD**) on the lipid metabolism of duck breeders and duck embryos. A total of 40 female 40-wk-old white Pekin duck breeders were randomly divided into 2 groups, received either RD diet (1.48 mg riboflavin/kg) or control diet (16.48 mg riboflavin/kg, **CON**) for 14 wk. Each group consisted of 20 duck breeders (10 replicates per group, 2 birds per replicate), and all experiment birds were single-caged. At the end of the experiment, reproductive performance, hepatic riboflavin, hepatic flavin mononucleotide (**FMN**), hepatic flavin adenine dinucleotide (**FAD**), hepatic morphology, hepatic lipid contents, and hepatic protein expression of duck breeders and duck embryos were measured. The results showed that the RD had no effect on egg production and egg fertility but reduced egg hatchability, duck embryo weight, hepatic riboflavin, FMN, and FAD status compared to results obtained in the CON group (all $P < 0.05$). Livers from RD ducks presented enlarged lipid droplets, excessive accumulation of total lipids, triglycerides, and free fatty acids (all $P < 0.05$). In addition to excessive lipids accumulation, medium-chain specific acyl-CoA dehydrogenase expression was downregulated ($P < 0.05$), and short-chain specific acyl-CoA dehydrogenase expression was upregulated in maternal and embryonic livers ($P < 0.05$). RD did not affect maternal hepatic acyl-CoA dehydrogenase family member 9 (**ACAD9**) expression, but duck embryonic hepatic ACAD9 expression was reduced in the RD group ($P < 0.05$). Collectively, dietary RD conditioned lower egg hatchability and inhibited the development of duck embryos. Increased accumulation of lipids, both maternal and embryo, was impaired due to the reduced flavin protein expression, which caused inhibition of hepatic lipids utilization. These findings suggest that abnormal duck embryonic growth and low hatchability caused by RD might be associated with disorders of lipid metabolism in maternal as well as embryos.

Key words: duck breeder, duck embryo, lipid metabolism, protein expression, riboflavin

INTRODUCTION

Maternal health during pregnancy or laying period is the basis to ensure the normal growth and development of the offspring. Previous studies have shown that riboflavin deficiency in female rats can decrease the birth weight of offspring (Roth-Maier et al., 2000). Riboflavin deficiency in laying hens could reduce egg hatchability and inhibit the development of chicken embryos (Squires and Naber, 1993; Cogburn et al., 2018), which

seriously hinders the chicken industry. In our previous study, riboflavin deficient female white Pekin laying duck breeders had reduced egg hatchability (Tang et al., 2019; Zhang et al., 2020b). Hence, strengthening the research on riboflavin in duck breeders, as well as maintaining maternal health and safety of embryo development, is essential for the duck industry.

Riboflavin (Vitamin B_2) is an essential precursor substance for varieties of flavin proteins with flavin mononucleotide (**FMN**) and flavin adenine dinucleotide (**FAD**) as coenzymes. Flavin proteins are catalysts for enzymes involved in fatty acids oxidation, such as medium-chain specific acyl-CoA dehydrogenase (**ACADM**) catalyzing C4~C16 fatty acids, acyl-CoA dehydrogenase family member 9 (**ACAD9**) catalyzing C16 and C18 fatty acids, and short-chain specific acyl-

CoA dehydrogenase (**ACADS**) catalyzing C4~C6 fatty acids (Zhang et al., 2002; Ghisla and Thorpe, 2004). Studies have demonstrated that patients with riboflavin transporter defect have symptoms of severe riboflavin deficiency (**RD**) accompanied by ACADM blemish (Gianazza et al., 2006; Zhuo et al., 2015). The mutation of hen riboflavin binding protein reduced lipids oxidation resulting in an energy deficit and the death of the chicken embryo (Abrams et al., 1995). Moreover, RD is associated with the excessive fat accumulation in the liver, and RD can cause deregulation and accumulation of hepatic triglycerides (**TG**) (Taniguchi and Nakamura, 1976; Olpin and Bates, 1982a, b; Duerden and Bates, 1985; Tang et al., 2017; Tang et al., 2019). Hepatic lipids accumulation might be connected to the reduced fatty acids β-oxidation (Cote et al., 2014). In addition to that, RD has been shown to increase hepatic TG contents and suppress the expression of various proteins in the reaction of catalyzing β-oxidation of fatty acids (ACADM, ACAD9, ACADS) (Tang et al., 2017). Our previous study demonstrated that the riboflavin deficient midembryonic stage (embryonic d 13, **E13**) duck embryo demonstrated a similar impact on hepatic TG and expression of the hepatic proteins (Tang et al., 2019).

Lipids play a role of reserve energy source through the β-oxidation of fatty acids. During incubation, poultry embryos mainly rely on lipids absorbed from egg yolk to be oxidized in the liver for energy (Noble and Cocchi, 1990). In the latter stages of incubation, 80% of yolk lipids are utilized (Noble and Cocchi, 1990; Noble and Mccartney, 1993). To date, there are no reports about RD on duck breeders and late-embryonic stage duck embryonic hepatic lipid metabolism. Therefore, this experimental study aimed to observe hepatic morphology, determine hepatic lipids content and protein expression to investigate the effect of dietary RD on hepatic lipid metabolism of duck breeders and late-embryonic stage duck embryos.

MATERIALS AND METHODS

Animals and Housing

This experimental research strictly complied with the National Institute of Animal Health regulation in experimental design, animal feeding, and sample acquisition. All animal procedures were approved by the Animal Care and Use Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences. A total of 40 female 40-wk-old white Pekin laying duck breeders (*Anas platyrhynchos*) were obtained from the Pekin duck breeding center (Chinese Academy of Agricultural Sciences). From hatching to 40 wk of age, the nutrients in the diet of these 40 female ducks met the nutritional requirements recommended by the Nutrient Requirements of Meat-type Ducks of China (NYT 2122-2012). All-female ducks were individually housed in plastic cages of the same size (length × deep × height = 1.00 m × 0.80 m × 0.60 m). The floor cages were covered

with rice husk to ensure the stability of humidity and to avoid the eggs from breaking. At 40 wk, these ducks were randomly divided into 2 groups with 10 replicates (2 birds per replicate) and received 2 experimental diets for 14 wk (40−54 wk).

Ten male ducks of the same age as laying duck breeders were used to mate naturally during the experimental period. The female ducks of the RD group and control (**CON**) group were alternately arranged, and every 4 female duck breeder mates with the same male duck. All the experimental animals received ad libitum access to water and food. All the birds were exposed to a total of 18 h of light from 4:00 am to 10:00 pm every day.

Experimental Design and Diets

This study was designed to investigate the effects of RD on the lipid metabolism of duck breeders and duck embryos. During the experimental period (from 40 to 54 wk of age), the basic diet was RD and included 1.48 mg/kg riboflavin in the diet was determined by high performance liquid chromatography (**HPLC**) (Table 1). The vitamin mixture was free of riboflavin. Except for riboflavin, the other nutrients in the diet met the requirements for laying duck breeders by the Ministry of Agriculture of China (2012, NY/T 2122-2012). Additional supplement with 15 mg/kg riboflavin in the basal diet as the CON group, the total riboflavin

Table 1. Composition of riboflavin-deficient basal diet (g/kg as-fed).

Ingredient	Content (g/kg)
Corn	560.0
Soybean (44% CP)	238.0
Corn gluten meal (44.3% CP)	100.0
Limestone	70.0
Dicalcium phosphate	15.0
Vitamin and trace mineral premix[1]	10.0
Sodium chloride	3.0
DL-methionine	1.0
Lysine hydrochloride	3.0
Calculated composition	
Metabolizable energy[2], Mcal/kg	2.743
CP	192.9
Calcium	30.7
Nonphytate phosphorus	3.5
Lysine	10.4
Methionine	4.6
Methionine + cysteine	7.7
Threonine	7.6
Tryptophan	2.0
Arginine	10.1
Riboflavin,[3] mg/kg	1.48

[1]Supplied per kilogram of total diet: Cu (CuSO$_4$•5H$_2$O), 10 mg; Fe (FeSO$_4$•7H$_2$O), 60 mg; Zn (ZnO), 60 mg; Mn (MnSO$_4$•H$_2$O), 80 mg; Se (NaSeO$_3$), 0.3 mg; I (KI), 0.2 mg; choline chloride, 1000 mg; vitamin A (retinyl acetate), 10000 IU; vitamin D$_3$ (Cholecalciferol), 3000 IU; vitamin E (DL-α-tocopheryl acetate), 20 IU; vitamin K$_3$ (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.
[2]The values are calculated according to the AME of ducks (Ministry of Agriculture of China, 2012, NY/T 2122-2012).
[3]The value was analyzed by high performance liquid chromatography.

concentrations for the CON group diets was 16.48 mg/kg. The HPLC analyzed riboflavin in RD and CON group diets were 1.48 and 16.83 mg/kg, respectively, close enough to the calculated values. All diets were cold-pelleted at room temperature. The crystalline riboflavin (purity, 99%) was manufactured by Sigma Aldrich (St. Louis, MO).

Sample Collection

On the last day of the experiment (at 54 wk of age), the breeders were weighed, and liver samples from all breeders were collected. The liver samples were collected in 3 parts. The first part was stored at 4% buffered-paraformaldehyde and 4°C for liver histological observation. The second part was stored at -20°C to determine hepatic lipids content and hepatic riboflavin content. The third part was placed in liquid nitrogen immediately and stored at -80°C for determining hepatic protein expression.

At the end of the experiment, all the 14th-wk-eggs were collected and counted to calculate the egg production. Before hatching, all the eggs were stored at 16°C and relative humidity of 75%. All the stored eggs were incubated in an incubator (12,096, Yiai, Qingdao, China). On the 5 d of incubation, egg fertility was evaluated by means of egg imaging, with clear blood vessels in the form of spider webs indicating the fertilized eggs. At embryonic d 22 (**E22**), 10 fertilized eggs were randomly selected from each treatment group to determine egg weight, embryo weight, embryonic liver weight, and calculate liver index (liver weight /embryo weight × 100%). Similar to duck breeders, the embryonic livers were divided into 3 parts and stored for subsequent analysis, as described above. The remaining fertilized eggs were proceeded to hatch for calculating the egg hatchability.

Measurements

HPLC determined hepatic riboflavin, FMN, and FAD concentrations from duck breeders and duck embryos according to the methods described previously (Batey and Eckhert, 1990; Tang et al., 2013). The hepatic riboflavin, FMN, and FAD contents were identified and calculated based on the peak area and authentic standard (PHR1054, Sigma Aldrich, St. Louis, MO).

Liver samples, about 1 cm^3 were cut from the bottom of the right lobe of duck breeders and duck embryos, fixed in 4% buffered-paraformaldehyde for histological observation using hematoxylin-eosin (**HE**) staining.

Hepatic total lipids (**TL**) were extracted and measured by chloroform-methanol (2:1) mixture, as described before (Folch et al., 1957; Wen et al., 2014). The extracts were dried and weighed. Afterward, the contents of TG (catalog number: A110-1-1), total cholesterol (**T-CHO**) (catalog number: A111-2-1), and free fatty acids (**FFA**) (catalog number: A042-1-1) were measured using commercial kits purchased from Nanjing Jiancheng Institute of Bioengineering (Nanjing, China).

A portion of the liver samples (5 out of 10 for each group) was used for protein expression determination. Protein extraction and protein concentration determination in livers were performed with reagent protocols using RIPA-protease K inhibitor (R0010, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) (30 mg sample of frozen tissue was taken, and 300 μL protein lysate was added) and BCA protein quantitative kits (23227, Thermo Fisher Scientific, Waltham, MA). Next, 30 μg protein sample was added to 4−20% Bis-Tris SurePAGE gel (M00656, Genscript, Co., Ltd., Nanjing, China). The gels ran in 1×Mops electrophoresis buffer and were transferred to PVDF membranes (66543, PALL Co., Pensacola, FL). Afterward, the membranes were blocked for 1 h at room temperature in blocking buffer with 5% skimmed milk powder (232100, BD Difco, Sparks, MD) in 1×TBST (1000 mL 1×TBST was prepared with 50 mL 20×TBS (T1080, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) + 950 mL ultrapure water + 2.34 mL Tween 20 (T8220, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The primary antibodies, including ACADM (1:5000 dilution, ab92461, Abcam, Cambridge, MA), ACAD9 (1:5000 dilution, ab113917, Abcam, Cambridge, MA), ACADS (1:5000 dilution, ab154823, Abcam, Cambridge, MA), and β-Tubulin (1:5000 dilution, HX1829, Huaxingbio, Beijing, China) were incubated at 4°C overnight. The secondary antibodies goat antirabbit (1:5000 dilution, SE131, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and goat antimouse (1:5000 dilution, SE134, Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) were incubated at room temperature for 1 h. The blots were visualized by ECL reagents (LY-0500, Beijing Lan Y Science & Technology Co., Ltd., Beijing, China). The signals were recorded with a Tanon 5200 Multi (Tanon Sciences & Technology Co., Ltd., Shanghai, China) and analyzed by the Tanon Gis 1D software (Tanon Sciences & Technology Co., Ltd., Shanghai, China). The β-Tubulin protein was employed to normalize the expression levels of other proteins, and the protein expression was calibrated with CON value.

Statistical Analyses

ALL data were analyzed using the 2-tailed Student t test procedure of SAS 9.4 software (SAS Institute, 2014). Data were expressed as the means and the standard error of the mean (**SEM**). In the 2 treatment groups, $P < 0.05$ indicated statistical significance.

RESULTS
Reproductive Performance and Embryonic Development

As shown in Table 2, the weight of duck breeders, egg production, egg fertility, and egg weight were not affected by riboflavin depletion for 14 wk. However, egg hatchability at 14 wk in RD was markedly lower than that in the CON group (2.42% vs. 89.1%, $P < 0.05$).

Table 2. Reproduction performance of duck breeders and growth performance of duck embryos in the RD group and the CON group[1].

Variable	RD	CON	SEM	P value
Duck breeder weight (kg)	3.41	3.50	0.076	0.568
Egg production (%)	86.1	83.1	3.04	0.633
Egg fertility (%)	87.0	87.6	2.57	0.910
Egg hatchability (%)	2.42[b]	89.1[a]	13.3	<0.001
Egg weight (g)	83.1	86.8	1.19	0.240
E22 embryo weight (g)	32.5[b]	37.2[a]	0.812	0.002
E22 embryonic liver weight (g)	0.623	0.674	0.013	0.218
E22 embryonic liver index (%)	1.94[a]	1.79[b]	0.035	0.026

Abbreviations: CON, control; RD, riboflavin deficiency; SEM, standard error of the mean.

[1]Results are means with n = 10 per group.

[a-b]Means with different superscripts within the same column differ significantly ($P < 0.05$).

Table 3. FAD, FMN, and riboflavin contents of liver in the RD group and the CON group of duck breeders and duck embryos[1].

Variable	RD	CON	SEM	P value
Maternal liver				
FAD (μg/g)	0.894[b]	1.54[a]	0.102	<0.001
FMN (μg/g)	3.64[b]	5.93[a]	0.367	<0.001
Riboflavin (μg/g)	3.09[b]	4.01[a]	0.156	0.002
E22 duck embryonic liver				
FAD (μg/g)	0.489[b]	0.977[a]	0.0625	<0.001
FMN (μg/g)	1.18[b]	1.55[a]	0.0463	<0.001
Riboflavin (μg/g)	2.49[b]	3.90[a]	0.169	<0.001

Abbreviations: CON, control; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; RD, riboflavin deficiency; SEM, standard error of the mean.

[1]Results are means with n = 10 per group.

[a,b]Means with different superscripts within the same column differ significantly ($P < 0.05$).

Compared with the CON group, duck embryo weight was lower (32.5 g vs. 37.2 g, $P < 0.05$), and the embryonic liver index was higher (1.94% vs. 1.79%, $P < 0.05$) in the RD group.

Hepatic Riboflavin Status

The hepatic riboflavin, FAD, and FMN contents of duck breeders and duck embryos in the RD group were significantly lower than those in the CON group (all $P < 0.05$). The detailed data were provided in Table 3.

Hepatic Histological Morphology, Hepatic Lipids

Hepatic HE staining sections were demonstrated in Figure 1. Compared with the CON group, the maternal hepatic HE histological analysis demonstrated that large drop hepatic steatosis diffusely involved all zones, with the highest concentration in the third zone of acini in the RD group. In comparison, small-drop local hepatic steatosis was also observed in the CON group, with lipid drops much smaller in size, randomly scattered throughout the hepatic lobule. In the embryonic liver, small- and medium-drop steatosis was observed only in the RD group, along with the initial traces of diffuse hydropic degeneration of hepatocytes and violations of the architecture of the hepatic lobules. Compared with the CON group, a decrease in the hepatic T-CHO was observed in duck breeders of the RD group. ($P < 0.05$, Table 4). Besides, hepatic TL, TG, and FFA levels of duck breeders and duck embryos in the RD group were higher than those of the CON group ($P < 0.05$, Table 4).
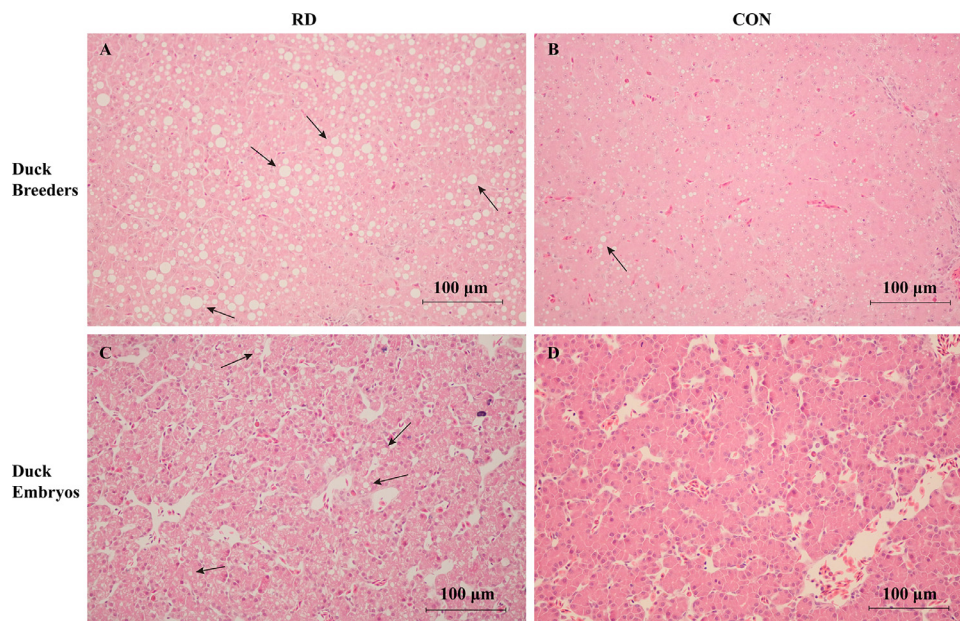
Figure 1. Hepatic hematoxylin-eosin staining analysis of duck breeders and E22 duck embryos.
The black arrows point white vacuole-like lipid droplets (magnification: 10×40). (A) Liver morphology of duck breeders in the riboflavin deficiency (RD) group. (B) Liver morphology of duck breeders in the control (CON) group. (C) Liver morphology of duck embryos in the RD group. (D) Liver morphology of duck embryos in the CON group.

Table 4. Total lipid, triglyceride, total cholesterol, and free fatty acids contents of liver in the RD group and the CON group of duck breeders and duck embryos[1].

Variable	RD	CON	SEM	P value
Duck breeder liver				
TL (%)	20.0[a]	8.85[b]	1.53	<0.001
TG (μmol/g)	86.9[a]	35.7[b]	7.51	<0.001
T-CHO (μmol/g)	9.55[b]	12.0[a]	0.405	0.001
FFA (μmol/g)	154[a]	134[b]	3.34	0.002
E22 duck embryonic liver				
TL (%)	12.3[a]	10.6[b]	0.331	0.005
TG (μmol/g)	494[a]	355[b]	28.4	0.010
T-CHO (μmol/g)	1,596	1439	499	0.088
FFA (μmol/g)	187[a]	146[b]	9.33	0.023

Abbreviations: CON, control; FFA, free fatty acids; RD, riboflavin deficiency; SEM, standard error of the mean; T-CHO, total-cholesterol; TG, triglyceride; TL, total lipids.

[1]Results are means with n = 10 per group.

[a, b]Means with different superscripts within the same column differ significantly ($P < 0.05$).

Hepatic ACADM, ACAD9, and ACADS Protein Expression

As illustrated in Figure 2 and Figure 3, the Western Blot determined the hepatic protein expression of duck breeders and duck embryos. Compared with the CON group, decreased hepatic ACADM and increased hepatic ACADS protein expression of the maternal and embryos in the RD group ($P < 0.05$). Hepatic ACAD9 expression has not been affected by RD in duck breeders ($P > 0.05$) but reduced in duck embryos of the RD group ($P < 0.05$).

DISCUSSION

The previous study has revealed that short-time RD (less than 8 wk) had no impact on egg production in hens (Squires and Naber, 1993). However, with the prolongation of fed with RD diet to 27 wk, the hen weight, egg production, and egg weight were all notably reduced (Squires and Naber, 1993). The final body weight, egg weight, egg production, and egg fertility of laying duck breeders fed with a basic diet without riboflavin for 9 wk did not change in our previous study (Zhang et al., 2020b). In line with previous results, the present study demonstrated that duck breeder weight, egg production, egg fertility, and egg weight were not affected by feeding a riboflavin deficient diet for 14 wk. Moreover, laying hens RD for 2−3 wk led to lower egg hatchability compared to the CON group (10% vs. 83%), and the reduced egg hatchability was approached to zero at 16 wk of RD (Naber and Squires, 1993; Squires and Naber, 1993). In
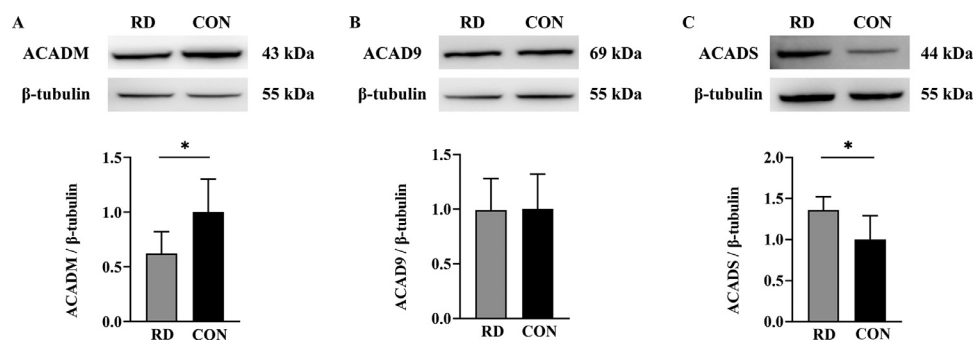
Figure 2. Western blot analysis of medium-chain specific acyl-CoA dehydrogenase (ACADM, A), acyl-CoA dehydrogenase family member 9 (ACAD9, B), and short-chain specific acyl-CoA dehydrogenase (ACADS, C) protein expression of duck breeders liver in the riboflavin deficiency (RD), and the control (CON) group[1].

The gray pillar represents the RD group, and the black pillar represents the CON group. ⁻ indicated significance difference between 2 groups, * indicated $P < 0.05$, ** indicated $P < 0.01$.

[1]Results are means with n = 5 per group.
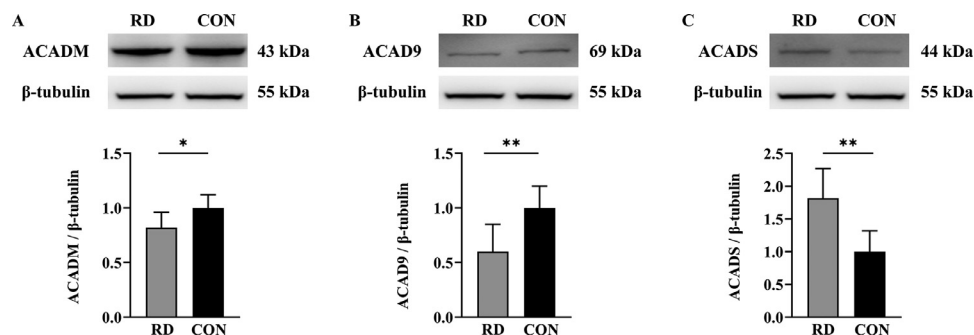
Figure 3. Western blot analysis of medium-chain specific acyl-CoA dehydrogenase (ACADM, A), acyl-CoA dehydrogenase family member 9 (ACAD9, B), and short-chain specific acyl-CoA dehydrogenase (ACADS, C) protein expression of E22 duck embryonic liver in the riboflavin deficiency (RD), and the control (CON) group[1].

The gray pillar represents the RD group, and the black pillar represents CON group. ⁻ indicated significance difference between 2 groups, * indicated $P < 0.05$, ** indicated $P < 0.01$.

[1]Results are means with n = 5 per group.

our previous study, the egg hatchability gradually decreased with the prolongation of RD (Tang et al., 2019), and egg hatchability reduced by 10% after 9 wk of riboflavin deficient diet (Zhang et al., 2020b). In the present study, egg hatchability was noted at 14 wk in the RD group to decrease to 2.42%. This result is consistent with previous reports and further confirms our previous findings.

In addition to that, some studies reported chicken embryonic underdevelopment, low contents riboflavin in vivo, and lower egg riboflavin content, caused by the innate absence of riboflavin binding protein in laying hens, explaining why these eggs could not normally hatch (Maw, 1954; Winter et al., 1967; Patterson and Bates, 1989; White et al., 1992). RD in pregnant rats and laying hens also impeded embryonic development and triggered hepatomegaly (Romanoff and Bauernfeind, 1942; Roth-Maier et al., 2000; Cogburn et al., 2018). Consistent with the previous studies, this study showed reduced duck embryo weight and increased liver index in the RD group. Maternal RD induced hepatomegaly led to poor development in offspring, which might be related to abnormal lipids metabolism (Abrams et al., 1995; Tang et al., 2019; Brocker et al., 2020).

Tissue riboflavin is a vital biomarker to evaluate animal riboflavin status. Previous studies have confirmed that dietary riboflavin absence could reduce the plasma riboflavin and hepatic riboflavin, FMN, and FAD of broilers and meat ducks (Ruiz and Harms, 1988; Tang et al., 2013; Tang et al., 2014, 2015). Additionally, maternal RD diminished the contents of egg yolk riboflavin and egg albumen riboflavin (Naber and Squires, 1993; Squires and Naber, 1993; Tang et al., 2019; Zhang et al., 2020a,b). Consistent with previous results, our study showed that RD markedly lessened the level of hepatic riboflavin, FMN, and FAD in duck breeders and duck embryos. These results revealed that dietary RD could result in the riboflavin deficient livers of duck breeders and duck embryos, which might affect the expression of flavin protein.

The liver is one of the main organs for fatty acid synthesis and utilization. RD could inhibit hepatic fatty acids β-oxidation and prompted accumulation of TG in mammals and birds (Taniguchi and Nakamura, 1976; Olpin and Bates, 1982a,b; Duerden and Bates, 1985; Tang et al., 2019). In the present study, HE staining suggested that the white lipids accumulation was more prominent in the liver of riboflavin deficient duck breeders and duck embryos. Content of hepatic TL, TG, and FFA in duck breeders and duck embryos was increased in the RD group as well. It has been confirmed in chicken embryonic livers that the duck hepatic T-CHO contents were higher in the embryonic stage (Noble and Qgunyemi, 1989; Liu et al., 2020). With embryo development, hepatic TG contents gradually increased, but hepatic T-CHO contents decreased (Liu et al., 2020). TG takes the major part of hepatic lipids in adult animals (Huang et al., 2018; Nii et al., 2020), hydrolyzed by lipase to glycerol and FFA as fuel for fatty acids β-oxidation. Our previous studies

demonstrated that riboflavin had a greater effect on TG than T-CHO (Tang et al., 2017, 2019). In previous reports, RD affected the expression of hepatic flavin proteins such as ACADM, ACAD9, ACADS, which catalyze fatty acid β-oxidation, in starter Pekin ducks and midembryonic stage (E13) duck embryos (Tang et al., 2017, 2019). The present study showed that the hepatic FAD, FMN, and their precursor riboflavin were decreased in the RD group and confirmed that the excessive accumulation of hepatic lipids might be related to the fatty acids β-oxidation-related flavin proteins expression of duck breeders and late-embryonic stage duck embryos.

In the fatty acid composition of animal liver tissue, C16 and C18 account for the highest proportion (Yamamoto et al., 1965). The 3 proteins ACADM, ACAD9, and ACADS, are all flavin proteins with FAD as the coenzyme catalyzing β-oxidation of C4-16, C18 and C20, as well as C4-6 fatty acids, respectively (Zhang et al., 2002; Ghisla and Thorpe, 2004). Previous studies have shown that RD impaired fatty acids oxidation by inhibiting the expression of fatty acyl-CoA dehydrogenase (Hoppel et al., 1979; Ross and Hoppel, 1987; Bates, 1990; Abrams et al., 1995; Tang et al., 2017; Tang et al., 2019). Human patients with profound muscle weakness were successfully treated with high-dose riboflavin to relieve the symptoms and alter the fatty acyl-CoA dehydrogenase deficiency (Gianazza et al., 2006; Zhuo et al., 2015). Proteomics results of HepG2 cells and duck liver indicated that the expressions of ACADM, ACAD9, and ACADS were declined in varying degrees of the RD group than those of the CON group (Tang et al., 2017, 2019; Xin et al., 2017). Similar to the previous studies, the present results confirmed that dietary RD could suppress the hepatic protein ACADM expression and hinder 4−16 carbon TG utilization in maternal ducks. In other words, RD triggered downregulation of ACADM and reduced utilization of medium-chain and long-chain fatty acids, which aggravated TG deposition. This might be the reason for the enlargement of lipids droplets in duck breeders. The increased hepatic ACADS expression of the maternal ducks in the RD group might be required to maintain energy in the body through consuming short-chain fatty acids. According to this, RD might have a similar effect in maternal animals and offspring, leading to decreased protein ACADM and compensatory increased the protein ACADS. However, the expression of ACAD9 in duck embryonic hepatic was also downregulated in the RD group, which aggravated TG deposition. As a result, abnormal lipid metabolism might further impede embryonic development.

The presented study was based on the preliminary stages of this experiment. It was found that under the conditions of the maternal riboflavin deficiency, midembryonic stage duck embryos nonalcoholic steatohepatitis might be connected to the reduced fatty acids β-oxidation (Tang et al., 2019). Therefore, the present experimental study was based on this finding, and we mainly discussed the effects of RD on hepatic lipids utilization

of duck breeders and late-embryonic stage duck embryos. However, further studies are needed to uncover other mechanisms that may lead to lipid accumulation.

In conclusion, dietary RD conditioned lower egg hatchability and inhibited the development of duck embryos. Increased accumulation of lipids, both maternal and embryo, was impaired due to the reduced flavin protein expression, which caused inhibition of hepatic lipids utilization. These findings suggest that abnormal embryonic growth and low hatchability caused by RD might be associated with disorders of lipid metabolism in duck breeders as well as duck embryos.

ACKNOWLEDGMENTS

DISCLOSURES

This manuscript has not been published or presented elsewhere in part or entirely and is not under consideration by another journal. This experimental research strictly complied with the National Institute of Animal Health regulation in experimental design, animal feeding, and sample acquisition. All animal procedures were approved by the Animal Care and Use Committee of the Institute of Animal Sciences, Chinese Academy of Agricultural Sciences. We have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

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