Maternal conjugated linoleic acid alters hepatic lipid metabolism via the AMPK signaling pathway in chick embryos

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ABSTRACT The effects of maternal conjugated linoleic acid (CLA) on embryonic development and hepatic lipid metabolism were investigated in chick embryos. A total of 180 Arbor Acres female broiler breeders (36 wk old) were randomly divided into the following 3 dietary treatment groups: a basic diet (control), a basic diet containing 0.5% CLA (CLA1), and a basic diet containing 1.0% CLA (CLA2). The females were fed for 8 wk, and the eggs from each group were collected and hatched during the last 2 wk. The results showed that the addition of dietary CLA increased the broken egg rate and reduced the fertilization rate and the egg hatchability ($P < 0.05$). CLA enrichment decreased the polyunsaturated and monounsaturated fatty acids and increased the saturated fatty acids in the yolk sac ($P < 0.05$). The yolk sac weight, body weight, and body length had a linear decrease with CLA supplementation ($P < 0.05$). In the developing chick embryo (at E14) and newly hatched chick (D0), the serum triglyceride concentration decreased with maternal CLA supplementation and was accompanied by a reduction in subcutaneous adipose tissue deposition. In addition, maternal CLA supplementation mediated the hepatic lipid metabolism by decreasing the mRNA expression of sterol regulatory element-binding proteins-1c (SREBP-1c), fatty acid synthase and acetyl-CoA carboxylase, and increasing the mRNA expression of adenosine 5′-monophosphate-activated protein kinase α (AMPKα), peroxisome proliferator-activated receptors α (PPARα), liver fatty acid-binding protein, adipose triglyceride lipase and carnitine palmitoyltransferase in embryonic chick livers ($P < 0.05$). A drop in SREBP-1c protein expression and an increase in the protein expression of p-AMPKα and PPARα were also observed in the liver of chick embryo ($P < 0.05$). In conclusion, maternal CLA supplementation regulated the fatty acid composition in the yolk sac, and mediated embryonic chick development and hepatic lipometabolism, and these effects may be related to the AMPK pathway. These findings suggest the potential ability of maternal CLA supplementation to reduce fat deposition in chick embryos.

INTRODUCTION

Conjugated linoleic acid (**CLA**), a group of linoleic acids with conjugated double bonds, is incorporated into various tissues, such as the liver, adipose tissue, skeletal muscles, and brain, and regulates lipid metabolism, inflammation, energy metabolism, and oxidative status (reviewed by Shen and McIntosh, 2016). Studies in different animal models have suggested that CLA, especially the trans-10, cis-12 isomer (t10,c12-CLA), reduces lipid accumulation in humans, rodents, and aves (Whigham et al., 2007; Shen et al., 2013; Wang et al., 2019). In 3T3-L1 adipocytes, CLA increased the rate of fatty acid oxidation and lipolysis, and decreased fatty acid biosynthesis in human adipocytes (Evans et al., 2002; Chung et al., 2005; Obsen et al., 2012). To reveal the regulatory mechanism of CLA in reducing lipid accumulation, various pathways were found to be involved in this process, including the adenosine 5′-monophosphate-activated protein kinase (**AMPK**), peroxisome proliferator-activated receptor (**PPAR**), and sterol regulatory element-binding protein (**SREBP**) pathways (Jiang et al., 2009; Lehnen et al., 2015; Lavandera et al., 2017), but much still remains to be studied.

Excessive maternal nutrient intake could induce metabolic disorders, including obesity, hepatic

steatosis, glucose intolerance, and inflammation in the offspring (Yokomizo et al., 2014; Oliveira et al., 2016; Li et al., 2017). The detrimental impact can be life-long, particularly when combined with additional stressors in adulthood (Hwang et al., 2007; Howie et al., 2009). The relationship between maternal diet and lipid metabolic alterations in the offspring has been noted and studied in humans and rodents (Gaillard et al., 2014; Keleher et al., 2018). In male rat offspring, the maternal CLA content showed a programming effect on lipid metabolic pathways, and led to a preventive effect on triacylglycerol (**TAG**) accretion in the adipose and liver tissues (Lavandera et al., 2017). In newly hatched chicks, maternal CLA intake reduced the liver TAG and carcass total fat contents (Cherian et al., 2005). However, the regulatory mechanism of maternal CLA on lipid metabolism in developing chick embryos remains unclear.

Therefore, the aim of this study was to investigate the effect of CLA supplementation in broiler breeder diets on embryonic development and hepatic lipid metabolism in chick embryo.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures performed in the present experiment were approved by the Institutional Animal Care and Use Committee of Shandong Academy of Agricultural Sciences. A total of 180 Arbor Acres female broiler breeders (36 wk old and 4.28 ± 0.12 kg BW) were allocated to 3 dietary treatment groups, with 5 replicates and 12 birds per replicate. A total of 4 birds were placed in 1 cage (45 cm × 45 cm × 45 cm), and 3 cages were grouped as replicates. All birds had free access to food and water, were on a 16-h light:8-h dark cycle and were assigned to a corn−soybean meal-based diet containing 0%, 0.5%, or 1.0% CLA mixture, which was fed to the birds for 8 wk. The CLA source containing 81% pure CLA (cis-9, trans-11 = 36.0%, trans-10, cis-12 = 41.7%, other isomers = 3.3%) was purchased from Aohai Biologic Limited Company (Qingdao, Shandong, China). To equalize the concentration of total fat in all diets, the CLA source was substituted for soybean oil on an equal weight basis and was included as 0, 0.62, and 1.23% of the diet to meet the assigned CLA additions (0%, 0.5%, and 1.0% pure CLA), as shown in Table 1. The composition of the control diet was based on the National Research Council (1994). The experimental diets were prepared weekly, and ethoxyquin (0.5%) was added to all diets to prevent fat oxidation.

Related Data Record and Sample Collection

During the last 2 wk, the egg number, egg weight, and broken egg rate in each replicate were recorded and calculated. A total of 30 eggs per treatment were collected for egg quality analysis. A total of 400 eggs of

Table 1. Ingredients and the analyzed and calculated chemical composition of the experimental diets.

Ingredients, %	Dietary treatments[1]		
	Control	CLA1	CLA2
Corn	50.0	50.0	50.0
Soybean meal	21.0	21.0	21.0
Wheat middlings	23.0	23.0	23.0
Soybean oil	**3.0**	**2.38**	**1.77**
CLA	**0**	**0.62**	**1.23**
Premix[2]	5.0	5.0	5.0
Limestone	8.0	8.0	8.0
Total	100	100	100
Calculated composition			
Available metabolic energy, kcal/kg	2,600	2,600	2,600
Crude protein, %	16.50	16.50	16.50
Calcium, %	3.5	3.5	3.5
Available phosphorus, %	0.4	0.4	0.4
Methionine	0.37	0.37	0.37

[1]Control diet contained 3% corn oil. CLA1 and CLA2 represent corn oil + 0.5% CLA or corn oil + 1% CLA, respectively.

[2]Supplied with the following nutrients per kg of diet: protein, 280 g; Met, 28 g; dicalcium phosphate, 160 g; vitamin A, 9,000 IU; vitamin D3, 2,000 IU; vitamin E, 11,000 IU; vitamin K3, 1 mg; vitamin B1, 1.20 mg; vitamin B2, 5.80 mg; vitamin B6, 2.6 mg; vitamin B12, 0.012 mg; niacin, 66 mg; biotin, 0.10 mg; pantothenic acid, 10 mg; folic acid, 0.7 mg; copper, 80 mg; iron, 80 mg; manganese, 100 mg; zinc, 75 mg; and ethoxyquin, 5 g.

a uniform size and weight were collected from each treatment, and hatched in an automatic incubator (FT-ZF 10, Chun Ming Tong Electronic Co., LTD, Beijing, China). The fertilization and hatchability of eggs were recorded. On embryonic day14 (E14) and on the hatched day (D0), 10 embryos from each group were randomly selected for measurements of BW, body length, and yolk sac weight. The serum was collected and stored at −20°C for triglyceride (**TG**) level detection. The liver, subcutaneous adipose tissue, and yolk sac were collected and snap-frozen in liquid nitrogen for further analysis. The concentration of TG in the serum was measured spectrophotometrically using commercial diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China). The subcutaneous adipose tissue was weighed and recorded.

Egg Quality Evaluation

The egg shape index was calculated by height/diameter. Eggshell strength on the vertical axis was measured by an Instron 3360 apparatus (Instron, Canton, MA, USA). The albumen height, yolk color, and Haugh units were measured using an Egg Multi Tester (EMT-7300, Tohoko Rhythm Co., LTD, Aizuwakamatsu, Japan). After breakout, the albumen and yolk were separated and weighed. Eggshells were then washed under slow flowing tap water to remove any adhering albumen (while keeping the shell membranes intact) and left to dry at room temperature for 1 h. Dry eggshells were weighed (0.0001 g) individually, and the eggshell thickness was measured at the sharp, blunt ends, and equator after removing the shell membranes using a micrometer, and the average eggshell thickness was calculated.

Table 2. Gene-specific primers of related genes.

Gene name	GenBank accession number	Primer sequences (5′→3′)	Product size (bp)
β-Actin	NM_205518.1	F: GAACCCCAAAGCCAACAG R: GGGCGTAGCCTTCATAGA	182
FAS	NM_205155.2	F: AATCTGCCGTCTGGAACTGAATGG R: CATCCTGTGACTGGTCGTGTTCTC	169
ACC	NM_205505.1	F: TCCAGCAGAACCGCATTGACAC R: GTATGAGCAGGCAGGACTTGGC	187
CPT1	NM_001012898.1	F: CGAGTCAGACACCACAGCAACAC R: CACCGTAACCATCATCAGCCACAG	99
AMPKα	NM_001039603.1	F: GCCTCGCCATACTCTTGATGAGC R: TTCTTCCGTCGAACACGCAAGTAG	195
ATGL	NM_001113291.1	F: AAGTCCTGCTGGTCCTCTCCTTG R: AGTGTTGTCCTCCATCTGGTCCTC	94
L-FABP	NM_204192.3	F: GAAGAGTGTGAGATGGAGCTGCTG R: GGTGATGGTGTCTCCGTTGAGTTC	132
PPARα	NM_001001464.1	F: TGCTGTGGAGATCGTCCTGGTC R: CTGTGACAAGTTGCCGGAGGTC	166
SREBP-1c	NM_204126.2	F: TGGTGGTGGACGCCGAGAAG R: GTCGTTGATGGATGAGCGGTAGC	134

ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyl transferase 1; FAS, fatty acid synthetase; PPARα, peroxisome proliferator-activated receptor α; and SREBP-1c, sterol regulatory element-binding protein-1c.

Determination of the Fatty Acid Profile in the Yolk Sac

The fatty acid profile was detected using yolk sac freeze-dried powder and carried out according to the method described by Sahan et al. (2014). The fatty acid methyl esters were analyzed by gas chromatography with a Hewlett-Packard HP6890 Gas Chromatography System installed with an Agilent HP-88 chromatographic column (100 m × 0.25 mm × 0.20 μm). The gas chromatography conditions were as follows: 260°C injector temperature; 270°C detector temperature; He carrier gas; 1:50 split ratio; temperature program set for 100°C for 5 min, followed by an increase of 5°C/min to 240°C, and then maintained for 30 min. Peaks were identified by a comparison of retention times with those of the corresponding standards from Sigma-Aldrich (St Louis, MO). The fatty acid content of the yolk sac was given as percentages of the total fatty acids.

The Expression of Genes Regulating the Liver Fatty Acid Metabolism

Total RNA was extracted from the liver tissue using TRIzol (Invitrogen, Carlsbad, CA), and the quantity and quality of the isolated RNA were determined using a biophotometer (Eppendorf, Hamburg, Germany) and agarose gel electrophoresis, respectively. Reverse transcription and quantification of all gene transcripts was performed as previously described (Fu et al., 2017). The PCR protocol was performed with an Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Foster, CA). The primers were designed for exon−intron junctions using Primer 5.0 software (Primer-E Ltd., Plymouth, UK). Real-time PCR

was performed at 95°C for 10 s of predenaturation, followed by 40 cycles consisting of denaturation at 95°C for 5 s and annealing and extension at 60°C for 40 s, with a standard curve plotted to calculate the efficiency of the real-time PCR primers. The mRNA levels of target genes were normalized to that of β-actin (ΔCT). The comparative CT method ($2^{-\Delta\Delta\mathrm{CT}}$) was used to quantitate the mRNA expression, in accordance with Livak and Schmittgen (2001). The primer sequences are listed in Table 2. The specificity of the amplification product was verified by a melting curve analysis. All samples were run in duplicate.

Western Blotting Analysis

The method of Western blotting has been described previously (Fu et al., 2018). Briefly, samples from the liver tissue were homogenized on ice in radioimmunoprecipitation assay buffer (50 mmol/L Tris-HCl at pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsufonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mmol/L sodium orthovanadate, and 1 mmol/L sodium fluoride) and centrifuged at 12,000 × g for 5 min at 4°C. A bicinchoninic assay kit (Beyotime, Jiangsu, China) was used to determine the protein concentrations. Then, after boiling at 100°C for 5 min, the protein extracts (40 μg) were electrophoresed in SDS polyacrylamide gels (7.5 to 10%) as described by Laemmli (1970). Separated proteins were transferred onto nitrocellulose membranes at 100 V for 1 h at 4°C in tris-glycine buffer containing 20% methanol. Membranes were blocked for 1 h and immunoblotted overnight at 4°C with the following primary antibodies: phospho-AMPKα^{Thr172}, AMPKα,

Table 3. Effect of maternal CLA supplementation on the quality of eggs.

Items	Dietary treatments[1]			P value
	Control	CLA1	CLA2	
Egg shape index	1.35 ± 0.08	1.34 ± 0.06	1.34 ± 0.06	0.6227
Albumen height, mm	4.50 ± 1.07	4.63 ± 0.84	4.23 ± 0.92	0.4150
Albumen weight, g	30.78 ± 4.14	29.07 ± 2.36	29.47 ± 3.29	0.3779
Yolk color	8.18 ± 1.46	8.44 ± 1.06	8.94 ± 0.86	0.5894
Haugh unit	68.06 ± 10.67	67.72 ± 7.24	66.31 ± 10.01	0.8310
Shell weight, g	5.10 ± 0.75	5.08 ± 0.72	4.99 ± 0.64	0.8906
Shell thickness, mm	0.40 ± 0.04	0.40 ± 0.03	0.41 ± 0.02	0.9400
Shell strength, kg/cm^2	39.79 ± 6.76	39.38 ± 8.56	36.66 ± 9.21	0.5726

[1]Control diet contained 3% corn oil. CLA1 and CLA2 represent corn oil + 0.5% CLA or corn oil + 1% CLA, respectively.

Table 4. Effect of maternal CLA supplementation on the fertility and hatchability of eggs.

Items	Dietary treatments[1]			P value
	Control	CLA1	CLA2	
Egg production rate, %	90.25 ± 3.27	88.35 ± 2.65	87.80 ± 4.58	0.7569
Broken egg rate, %	$7.11 \pm 1.37^{a,b}$	4.67 ± 0.32^{b}	8.42 ± 1.04^{a}	0.0158
Fertilization rate, %	95.37 ± 0.77^{a}	77.00 ± 0.78^{c}	86.88 ± 0.67^{b}	<0.0001
Hatchability rate, %[2]	86.45 ± 0.48^{a}	54.25 ± 1.18^{b}	44.96 ± 0.67^{c}	<0.0001

[1]Control diet contained 3% corn oil. CLA1 and CLA2 represent corn oil + 0.5% CLA or corn oil + 1% CLA, respectively.

[2]Values are the proportions of fertile eggs set for incubation that hatched during 21 d of incubation.

[a–c]bMeans $\pm$ SDs with different superscripts within a row differ significantly ($P < 0.05$).

PPARα, and SREBP-1c. β-actin was used as an internal control (Beyotime, Jiangsu, China). Western blots were developed and quantified using BioSpectrum 810 with VisionWorksLS 7.1 software (UVP LLC, Upland, California, USA).

Statistical Analyses

The results were expressed as the means $\pm$ SDs. All the data were subjected to one-way ANOVA with the Statistical Analysis Systems statistical software package (Version 8e, SAS Institute, Cary, NC). Significant differences between the treatment means were determined by Tukey's multiple range test. Significance was based on $P < 0.05$.

RESULTS

CLA Supplementation Increased the Broken Rate and Decreased the Fertile Egg Performance

There was no significant difference between the control and the CLA groups in egg shape index, albumen height, albumen weight, yolk color, Haugh unit, shell weight, shell thickness, or shell strength (Table 3, $P > 0.05$). The egg production rate was not affected by maternal CLA supplementation (Table 4, $P > 0.05$). With an increase in dietary CLA supplementation, the broken egg rate improved significantly ($P < 0.05$). The

fertilization rate of eggs was severely reduced in the CLA groups, with the lowest level in the CLA1 group ($P < 0.05$). Meanwhile, the hatchability rate of fertile eggs was substantially lower in the CLA1 (-29.42%) and CLA2 (-47.84%) groups than that in the control group ($P < 0.05$).

Maternal CLA Supplementation Altered the Fatty Acid Profile of the Yolk Sac

The fatty acid profile of the yolk sac, expressed as the percentage of total fatty acids in the yolk sac, was significantly altered by maternal CLA supplementation during the hatching period (Table 5). Maternal CLA supplementation resulted in a significant increase in the saturated fatty acids (**SFAs**) proportion ($P < 0.05$) but decreased the proportion of monounsaturated fatty acids (**MUFAs**) and polyunsaturated fatty acids (**PUFAs**) ($P < 0.05$) in the yolk sac at E14 and D0, with no differences for SFAs and MUFAs between the CLA1 and CLA2 groups ($P > 0.05$). The proportion of PUFAs in the yolk sac was linearly decreased at D0 ($P < 0.05$) but not at E14 ($P > 0.05$). It showed that at E14 and D0, the deposition of c9, t11-CLA and t10,c12-CLA, 2 major isomers of CLA, increased linearly in yolk sacs with maternal CLA supplementation ($P < 0.05$). The relative ratio of c9, t11-CLA was consistently higher than that of the t10,c12 isomer in the CLA1 and CLA2 groups ($P < 0.05$). Of the individual SFAs, the proportions of C14:0, C16:0, and C18:0 were

Table 5. Effect of maternal CLA supplementation on the fatty acid profiles of embryo yolk sacs at E14 and D0.

[1]Fatty acid/%	Incubation day	Dietary treatments[2]			P value
		Control	CLA1	CLA2	
14:0	E14	0.42 ± 0.01^b	0.77 ± 0.05^a	0.69 ± 0.01^a	0.0004
	D0	0.35 ± 0.03^b	0.74 ± 0.0392^a	0.79 ± 0.03^a	0.0001
16:0	E14	27.43 ± 0.69^b	35.75 ± 1.10^a	36.99 ± 0.31^a	0.0002
	D0	24.38 ± 0.43^b	36.12 ± 1.13^a	36.64 ± 1.10^a	0.0001
18:0	E14	9.06 ± 0.27^b	16.68 ± 0.64^a	17.94 ± 0.05^a	<0.0001
	D0	9.55 ± 0.34^c	17.15 ± 0.50^b	18.97 ± 0.51^a	<0.0001
18:1 n-9	E14	44.53 ± 0.40^a	25.92 ± 0.15^b	26.22 ± 0.69^b	<0.0001
	D0	45.66 ± 0.19^a	27.48 ± 1.24^b	27.91 ± 0.64^b	<0.0001
18:2 n-6	E14	11.81 ± 0.83^a	8.30 ± 0.49^b	8.71 ± 0.32^b	0.0106
	D0	13.78 ± 0.11^a	12.82 ± 0.36^a	8.31 ± 0.47^b	<0.0001
18:3 n-3	E14	0.29 ± 0.02^a	0.21 ± 0.01^b	0.16 ± 0.01^b	0.0010
	D0	0.41 ± 0.03^a	$0.33 \pm 0.02^{a,b}$	0.27 ± 0.01^b	0.0227
20:3 n-6	E14	0.15 ± 0.00	0.17 ± 0.00	0.15 ± 0.00	0.0651
	D0	0.17 ± 0.00^a	0.14 ± 0.00^b	0.12 ± 0.00^c	0.0026
20:4 n-6	E14	1.69 ± 0.05^a	1.27 ± 0.04^b	1.22 ± 0.05^b	0.0009
	D0	1.60 ± 0.06^a	1.06 ± 0.04^b	0.94 ± 0.01^b	<0.0001
22:6 n-3	E14	0.33 ± 0.02^a	0.25 ± 0.01^b	0.23 ± 0.00^b	0.0035
	D0	0.32 ± 0.04^a	0.17 ± 0.00^b	0.11 ± 0.00^b	0.0015
24:1	E14	0.06 ± 0.00^a	0.03 ± 0.00^b	0.03 ± 0.00^b	0.0001
	D0	0.06 ± 0.01^a	0.03 ± 0.00^b	0.03 ± 0.00^b	0.0048
Total SFA	E14	28.61 ± 0.70^b	37.48 ± 1.17^a	38.62 ± 0.32^a	<0.0001
	D0	35.11 ± 0.44^b	55.11 ± 1.34^a	57.50 ± 1.63^a	<0.0001
Total MUFA	E14	48.18 ± 0.57^a	28.45 ± 0.29^b	28.54 ± 0.62^b	<0.0001
	D0	48.47 ± 0.27^a	29.47 ± 1.26^b	29.60 ± 0.62^b	<0.0001
Total PUFA	E14	14.27 ± 0.91^a	10.20 ± 0.46^b	10.48 ± 0.29^b	0.0057
	D0	16.24 ± 0.27^a	14.53 ± 0.32^b	9.68 ± 0.52^c	<0.0001
C9,t11-CLA	E14	0.03 ± 0.00^c	0.72 ± 0.04^b	1.51 ± 0.04^a	<0.0001
	D0	0.05 ± 0.00^c	0.61 ± 0.01^b	1.36 ± 0.05^a	<0.0001
C10,t12-CLA	E14	0.01 ± 0.00^c	0.41 ± 0.01^b	0.62 ± 0.03^a	<0.0001
	D0	0.01 ± 0.00^b	0.33 ± 0.02^b	0.53 ± 0.02^a	<0.0001
Total CLA	E14	0.04 ± 0.00^c	1.13 ± 0.05^b	2.13 ± 0.03^a	<0.0001
	D0	0.06 ± 0.00^c	0.94 ± 0.03^b	1.72 ± 0.05^a	<0.0001

[1]Fatty acids (%) = fatty acids contents/total fatty acids methyl esters.
[2]Control diet contained 3% corn oil. CLA1 and CLA2 represent corn oil + 0.5% CLA or corn oil + 1% CLA, respectively.
a–cMeans $\pm$ SDs with different superscripts within a row differ significantly ($P < 0.05$, n = 10).
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

increased significantly in the CLA groups ($P < 0.05$). For the MUFAs, the proportions of C18:1n-9 and C24:1 decreased significantly in the CLA groups ($P < 0.05$). In addition, the proportions of 18:2n-6 and other PUFAs were linearly reduced from the control to the CLA groups ($P < 0.05$).

Maternal CLA Supplementation Mediated the Body Size of the Chick Embryos

As shown in Table 6, we detected the effect of maternal CLA on embryonic development. Maternal CLA supplementation did not affect the initial egg weight or yolk weight at E14 or D0 ($P > 0.05$). At E14, the egg weight, wet embryo weight, and embryo length were linearly reduced as CLA increased ($P < 0.05$), and the yolk sac weight was increased in the CLA groups ($P < 0.05$). At D0, no significant difference was observed in chick weight between the control and CLA2 groups

($P > 0.05$), but maternal 1% CLA supplementation (CLA2 group) decreased the residual yolk sac weight, yolk-free chick weight, and chick length ($P < 0.05$). No significant difference was found for all parameters between the control and CLA1 groups on D0 ($P > 0.05$).

### Maternal CLA Supplementation Alters Hepatic Lipid Metabolism in Chick Embryos

As shown in Figure 1, CLA supplementation in the diet of broiler breeders decreased the serum TG concentration and the subcutaneous fat deposition of chick embryos at E14 and D0, in a dose-dependent manner ($P < 0.05$). Moreover, in the liver of chick embryos, maternal CLA supplementation increased the mRNA expression of liver fatty acid-binding protein (**L-FABP**), especially in the CLA1 group, and the expression was significantly higher in the CLA1 group than in the CLA2 group (Figure 2A, $P < 0.05$). The mRNA

Table 6. Effect of maternal CLA supplementation on the body size of E14 embryos and the body weight of D0.

| Incubation day | Items | Dietary treatments[1] | | | P value |
		Control	CLA1	CLA2	
Before incubation	Initial egg weight, g	52.83 ± 1.29	51.17 ± 0.82	51.12 ± 1.90	0.5464
	Initial yolk weight, g	16.99 ± 0.34	17.19 ± 0.49	16.76 ± 0.72	0.8446
E14	Egg weight, g	53.13 ± 1.37^a	$49.65 \pm 1.12^{a,b}$	47.92 ± 2.19^b	0.1081
	Wet embryo weight, g	10.44 ± 0.57^a	$8.93 \pm 0.56^{a,b}$	7.87 ± 0.60^b	0.0209
	Embryo length, cm	9.05 ± 0.20^a	$8.68 \pm 0.22^{a,b}$	7.88 ± 0.40^b	0.0367
	Yolk sac weight, g	11.90 ± 0.38^b	14.09 ± 0.75^a	15.52 ± 0.33^a	0.0005
D0	Chick weight, g	33.05 ± 1.19	35.31 ± 0.82	34.51 ± 1.70	0.3963
	Residual yolk sac weight, g	5.41 ± 0.25^b	6.15 ± 0.39^b	9.94 ± 1.32^a	0.0006
	Yolk-free chick weight, g	27.64 ± 0.92^a	29.16 ± 0.82^a	24.58 ± 0.65^b	0.0088
	Chick length, cm	16.76 ± 0.42^a	15.85 ± 0.37^a	14.45 ± 0.55^b	0.0071

[1]Control diet contained 3% corn oil. CLA1 and CLA2 represent corn oil + 0.5% CLA or corn oil + 1% CLA, respectively.

[a,b]Means $\pm$ SDs with different superscripts within a row differ significantly ($P < 0.05$, n = 10).
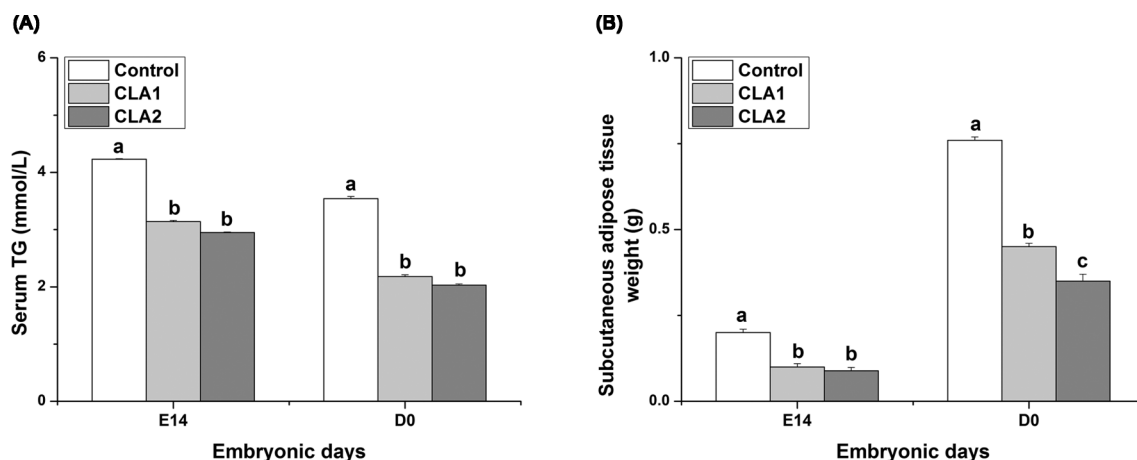
Figure 1. The effect of the maternal CLA content on serum TG levels (A) and subcutaneous adipose tissue deposition (B) in chick embryos at E14 and D0.

levels of adipose triglyceride lipase (**ATGL**) and carnitine palmitoyltransferase (**CPT1**) were upregulated with the increase in maternal CLA supplementation (Figure 2A, B, $P < 0.05$), but those of fatty acid synthase (**FAS**) and acetyl-CoA carboxylase (**ACC**) were inhibited (Figure 2B, $P < 0.05$). Furthermore, maternal CLA supplementation induced the mRNA and protein expression levels of AMPKα and PPARα in a dose-dependent manner (Figures 2C and 3, $P < 0.05$), and reduced the mRNA and protein expression of SREBP-1c ($P < 0.05$).

DISCUSSION

As broilers' excess fat deposition decreases feed efficiency and reduces the nutritional value of carcass parts and the commercial value, it has a negative impact on poultry production (Jennen et al., 2004; Zhou et al., 2007; Baéza and Le Bihan-Duval, 2013). Dietary CLA was suggested to reduce body fat accumulation and modulate lipid metabolism, which could have a programming effect on offspring lipid metabolism (Cherian et al., 2005; Gray et al., 2015; Reynolds et al., 2015;

Lavandera et al., 2017). Taking into account maternal CLA content in regulating offspring lipid metabolism, the aim of this study was to investigate the effect and mechanism of maternal CLA supplementation on embryonic development and lipid metabolism in developing chick embryos.

In the current study, we found that dietary CLA supplementation did not affect the quality of eggs, and these results were consistent with those of previous studies (Cherian et al., 2007; Qi et al., 2011). The egg production rate was not affected by CLA supplementation. Nevertheless, it has been reported that when the dietary CLA addition exceeds 2%, the egg production rate might decrease, mainly due to the reduced feed intake of laying hens (Shang et al., 2005; Kim et al., 2007).

Maternal CLA intake significantly increased the broken egg rate and severely decreased the egg fertilization rate and hatchability rate of fertile eggs, as reported by Aydin et al. (2001) and Cherian et al. (2005), and this consumption contributed to the inhibition of Δ-9 desaturase activity and the change in fatty acid composition in yolk, especially for the content and
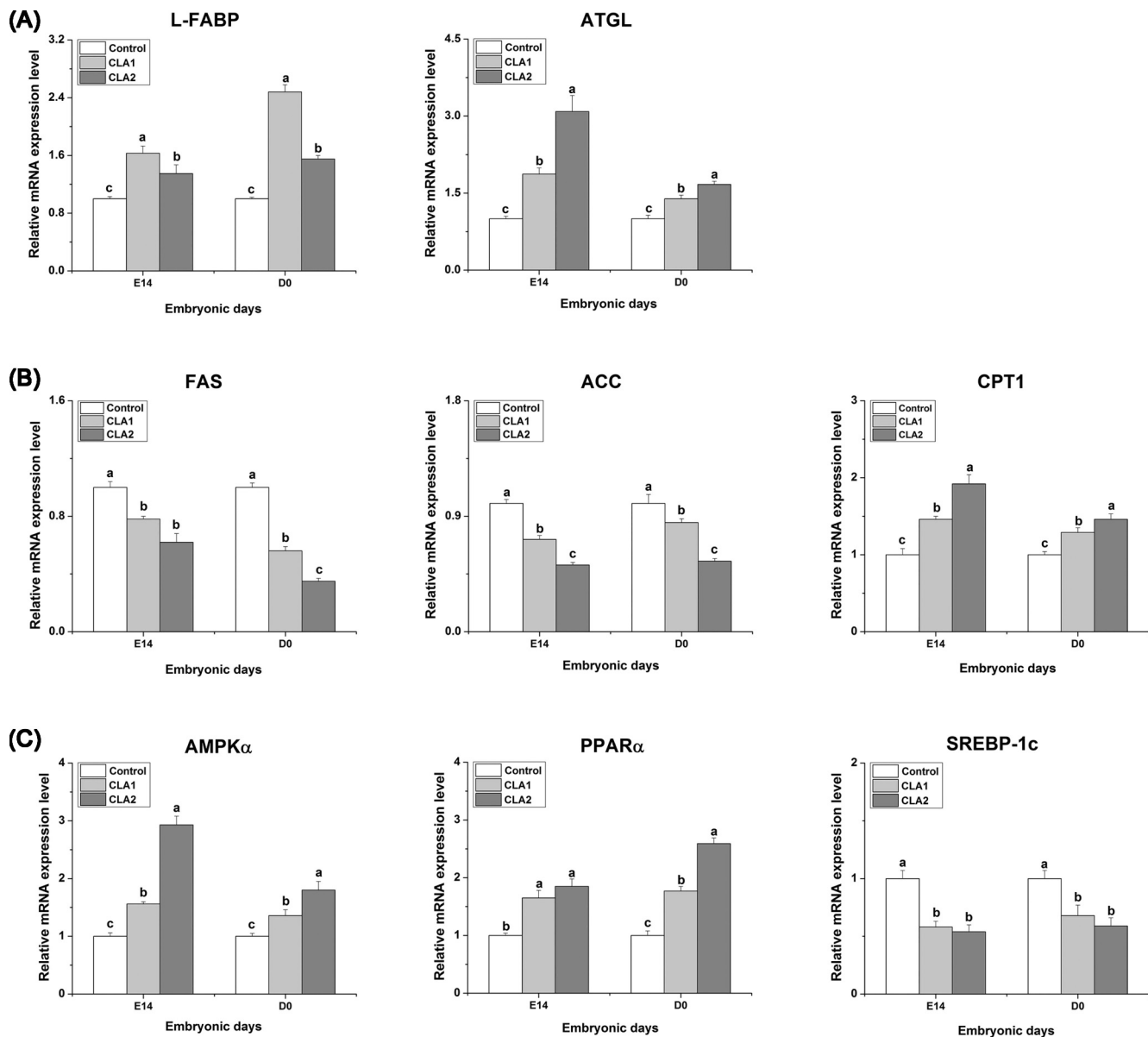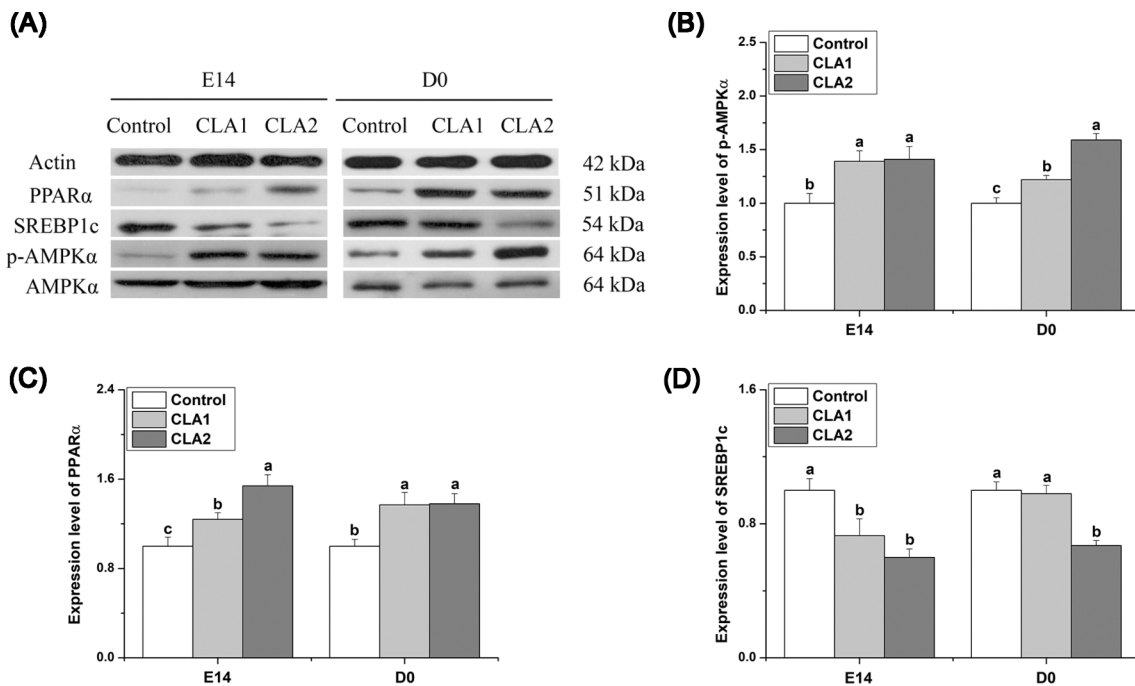
Figure 2. The effect of the maternal CLA content on the mRNA expression of genes related to lipid metabolism in the embryonic liver. The effect of maternal CLA treatment on the mRNA expression of genes related to triglyceride metabolism (L-FABP and ATGL) (A), fatty acid metabolism rate-limiting enzymes (FAS, ACC, and CPT1) (B), and related upstream regulators (AMPKα, PPARα, and SREBP-1c) (C). [a,b]Means with different letters are significantly different, $P < 0.05$. ACC, acetyl-CoA carboxylase; AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; CPT1, carnitine palmitoyl transferase 1; FAS, fatty acid synthetase; PPARα, peroxisome proliferator-activated receptor α; SREBP-1c, sterol regulatory element-binding protein-1c.

ratio of C18:0 and C18:1(n-9) (Noble and Cocchi, 1990; Tullet, 1990; Li and Watkins, 1998). The fertilization rate was significantly lower in the CLA1 group than in the control and CLA2 groups. The hatchability rate of fertile eggs in the CLA2 group was the lowest among all the treatments. The yolk is vital for embryonic development and is the only source of lipids for embryonic tissue growth (Speake et al., 1998). As FA delivery, partitioning and tissue uptake during incubation affects embryonic health and hatchability (Latour et al., 2000; Aydin et al., 2001; Cherian et al., 2005), the alteration of the yolk's fatty acid composition can have drastic effects on embryonic survival (Aydin et al., 2001). Then, the fatty acid composition in the yolk sac was

detected. Consistent with our previous study in laying hens (Liu et al., 2017), the maternal CLA content mediated the fatty acid composition in the yolk sac of developing chick embryos, with an increase in SFAs and a decrease in PUFAs and MUFAs. Stearoyl-coenzyme A desaturase (**SCD-1**) is a limiting enzyme for the conversion of SFAs to MUFAs, and it was showed that the CLA-mediated inhibition of SCD-1 activity induced an increase in SFAs and a decrease in MUFAs (Shang et al., 2005).

The fatty acid composition of egg yolk might be manipulated by diet, and the yolk is metabolized and transported to the embryo during the incubation of the chicken egg (Speake et al., 1998). Therefore, the

Figure 3. The effect of the maternal CLA on the expression of proteins related to lipid metabolism in the embryonic liver at E14 and D0. (A) Western blot image, (B) phospho-AMPKα^{Thr172}, (C) PPARα, and (D) SREBP-1c. [a,b]Means with different letters are significantly different, $P < 0.05$. AMPK, AMP-activated protein kinase; PPARα, peroxisome proliferator-activated receptor α; SREBP-1c, sterol regulatory element-binding protein-1c.

fertile egg is a useful research tool for studying the role of maternal nutrition on offspring performance. In this study, a linear increase in the CLA content in the yolk sac was found as a result of increasing the amount of CLA added to the broiler breeder diet. Interestingly, although the concentrations of both isomers in the diets were similar, the relative content of c9,t11-CLA in the yolk sac was much higher than that of t10,c12-CLA in the yolk sac, and this phenomenon might have resulted from the increased catabolism or absorption rate of the t10, c12 isomer compared to that of c9,t11 CLA (Park et al., 1999). In addition, the incorporation of CLA in egg yolk also altered the fatty acid profile and led to embryonic mortality by disrupting the lipid utilization by embryos (Leone et al., 2010). Thus, the change in embryonic performance and lipid metabolism could be attributed to maternal CLA supplementation, which was administered to alter fat deposition during embryo incubation.

Studies performed by Latour et al. (2000) noted that the maternal CLA content did not influence the BWs of E15 chick embryos, but decreased the relative yolk weights during incubation. Cherian et al. (2005) and Aydin and Cook (2009) found that maternal CLA lowered the body fat and increased the residual yolk sac weight in newly hatched chicks. In the current study, we found that maternal CLA supplementation increased the residual yolk sac weight and influenced embryonic development by reducing body weight and length. However, the transport of fatty acids from the yolk sac to the yolk sac membrane was not impaired by ma-

ternal CLA (Leone et al., 2010), suggesting that the phagocytosis of lipids into the membrane might not be influenced in the embryo by maternal CLA feeding. Although maternal CLA supplementation exhibited a severe influence on offspring development, classic studies have also demonstrated that the increased incorporation of linoleic acid and linolenic acid in yolk, by fish oil or soybean oil supplementation in the breeder diet, might improve reproductive performance by mediating the fatty acid composition in yolk (Alvarez et al., 2004; Muma et al., 2006).

Given that CLA is known to regulate lipid metabolism in rodents and avians, it is necessary to evaluate the extent to which maternal CLA influences hepatic lipid metabolism in developing chick embryos. In agreement with previous studies in different animal models (Martinez et al., 2013; Malinska et al., 2015; Wang et al., 2019), the present results showed that maternal CLA supplementation reduced the serum TG levels and subcutaneous adipose tissue weights in chick embryos, suggesting that the maternal CLA content altered lipid metabolism and fat deposition in developmental embryos. CLA was shown to elevate the metabolic rate of fat by inhibiting fatty acid synthesis and/or accelerating fatty acid oxidation and lipolysis (Evans et al., 2002; Chung et al., 2005; Obsen et al., 2012).

The liver is the major organ for lipid metabolism in avians. Cherian et al. (2005) indicated that maternal CLA reduced the hepatic lipid content in newly hatched chicks. The present study found that the maternal CLA

content mediated the inactivation of FAS and ACC and the activation of L-FABP, ATGL, and CPT1, and these effects resulted in a decrease in lipogenesis and an increase in β-oxidation, in agreement with the results of previous studies (Purushotham et al., 2007; Lavandera et al., 2017). In addition, maternal CLA supplementation increased the expression of AMPKα and PPARα, and decreased the expression of SREBP-1c. Several studies have reported that CLA reduced the development of hepatic steatosis and induced lipid oxidation by activating PPARα, a transcription factor involved in lipid metabolism in the liver (Purushotham et al., 2007; Yu et al., 2012; Lavandera et al., 2017). SREBP-1c, a key lipogenic transcription factor in cellular lipid metabolism and homeostasis, was reported to regulate the activity of lipogenic genes, such as FAS. AMPK, activated by increases in the AMP/ATP ratio and various cellular stresses (Daval et al., 2006; Hardie, 2008), has been demonstrated to be closely related to the regulation of lipid and carbohydrate metabolism (Viollet et al., 2006), especially in the liver (Li et al., 2018). Moreover, activated AMPK in hypothalamic nuclei is involved in the control of feed intake and energy balance (Song et al., 2012). It was reported that the hepatic AMPK activity in chicken embryos from larger eggs was higher than that from normal eggs (Hu et al., 2013), indicating a physiological change in response to a higher nutrient composition and faster embryonic growth. The activity of FAS and ACC, as target genes, is inhibited by phosphorylated AMPK (Browning and Horton, 2004; Smith et al., 2016). In addition, AMPK activation suppressed SREBP-1c cleavage and nuclear translation, and inhibited ACC activity and malonyl CoA production, resulting in fat synthesis suppression and fatty acid oxidation stimulation (Yang et al., 2009; Li et al., 2011). Moreover, AMPK is reported to activate β-oxidation through a PPARα-dependent pathway (Osler and Zierath, 2008). Taking the present study into account, we proposed that maternal CLA alterations to hepatic lipid metabolism in chick embryos might be mediated by the AMPK-PPARα/SREBP-1c pathways. The cross-regulation relationship among these downstream proteins in the maternal CLA-mediated lipometabolism of offspring remains unclear.

In conclusion, the current study demonstrated that maternal CLA supplementation significantly altered the fatty acid composition in the yolk sac, embryonic development, and hepatic lipometabolism. These results suggest that maternal CLA might decrease the hepatic lipid synthesis via AMPK signaling. The present study provides further evidence that the CLA content in the maternal diet mediates lipid metabolism in the offspring. This knowledge could be important to develop dietary strategies to reduce excess lipid accumulation in broilers, provided that the adverse effects of CLA on the hatchability of fertile eggs and embryo development may be recovered by regulating the fatty acid composition in the yolk sac.

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