Expression patterns of AMPK and genes associated with lipid metabolism in newly hatched chicks during the metabolic perturbation of fasting and refeeding

Zhiyong Xu, Yan Yu, Huichao Zhao, Yanhong Zhang, Li Wang, and Jinyou Ma[1]

College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Xinxiang 453003, China

ABSTRACT Fasting−refeeding perturbation has been extensively used to reveal specific genes and metabolic pathways that control energy metabolism in chickens. In this study, 200 chickens were randomly assigned to 2 groups after hatching: the control group (C, fed ad libitum) and the fasting−refeeding group (T, water ad libitum). The chicks in Group T were fasted for 72 h, and then fed for another 48 h. Liver, hypothalamus, and adipose samples were collected at 0 (F0), 24 (F24), 48 (F48), and 72 h (F72) after fasting and 4 (FR4), 12 (FR12), 24 (FR24), and 48 h (FR48) after refeeding, respectively. Results showed that Group T had a significantly higher number of liver vacuoles ($P < 0.05$ or $P < 0.01$) and a significantly lower gray value of Sudan IIIstained sections ($P < 0.05$ or $P < 0.01$) than Group C at F48−FR48. In addition, compared with the Group C, fasting and refeeding reduced the expression of stearoyl CoA desaturase (**SCD**) mRNA ($P < 0.05$ or $P < 0.01$) in the liver and adipose tissues, the expression of glucocorticoid receptor (**GR**) mRNA ($P < 0.05$ or $P < 0.01$) in the liver, adipose, and hypothalamus tissues, and the expression of fatty acid synthase (**FAS**) mRNA ($P < 0.05$ or $P < 0.01$) in the liver at F24−FR24. Moreover, relative to those in Group C, fasting and refeeding increased the mRNA expression levels of adenosine monophosphate-activated protein kinase (AMPK) α, AMPKβ, and AMPKγ in the hypothalamus ($P < 0.05$ or $P < 0.01$) at F24−FR24. In conclusion, fasting and refeeding increased the fat content of the liver, and the expression of lipolytic genes in the hypothalamus (e.g., AMPKα, AMPKβ, and AMPKγ) but decreased the expression of fat synthesis genes in the liver (e.g., SCD, GR, and FAS), adipose (SCD and GR), and hypothalamus (GR).

Key words: fasting and refeeding, lipid metabolism, newly-hatched chick, development, AMPK

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INTRODUCTION

The growth rate of broilers is accelerating with the improvement in genetic breeding methods and feeding technology. However, rapid growth introduces a series of problems into the production of broilers. These problems include the increased incidence of ascites syndrome, sudden death syndrome, and leg disease and the elevated rate of abdominal fat deposition (Isabelle et al., 2013; Khan et al., 2021). Feeding restriction technology has been increasingly applied in broiler production. A large number of studies on broiler feeding restriction showed that limiting the early nutrient intake of broilers can control the growth and development of broilers and the deposition of body fat, thus improving the carcass quality of broilers (Fondevila et al., 2020; Bordin et al., 2021) and promoting the balanced development of organs and bones (Zhang et al., 2020). Moreover, this approach can improve the feed conversion efficiency and disease resistance of broilers (Zukiwsky et al., 2021) and reduce the incidence of sudden death syndrome (Bowes et al., 1988). Later growth can compensate for the weight loss that occurs during the feeding restriction period (Afsharmanesh et al., 2016). However, given that excessive feeding restriction would affect compensatory growth and reduce market weight, the degree of feeding restriction should be controlled well (Bordin et al., 2021).

Restricted feeding mainly influences fat metabolism in the body. Fat metabolism involves several key proteins containing fatty acid synthase (**FAS**), adenosine phospho-activated protein kinase (**AMPK**), stearoyl CoA desaturase (**SCD**), and glucocorticoid receptor (**GR**). FAS is a basic metabolic enzyme that catalyzes the synthesis of palmitic acid from acetyl CoA and monoyl malonate (160:0). The amount and activity of FAS play an important role in animal body fat deposition. AMPK is

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[1]Corresponding author: marsjy@163.com

a multifunctional serine/threonine protein kinase that consists of one catalytic subunit (α) and 2 regulatory subunits (β and γ). AMPK executes an important role in the regulation of liver lipid homeostasis and energy balance through the rapid and direct modification (phosphorylation) of metabolic enzymes. Phosphorylation at THR 172 activates AMPK, including the AMPKα subunit, into the active pAMPK form, which affects energy metabolism by regulating downstream protein kinases or gene expression. SCD, an endoplasmic reticulum binding protein (Lengi and Corl, 2008), is a key enzyme in the biosynthesis of monounsaturated fatty acids (**MUFAs**) in organisms (Kapil et al., 2021) and can introduce unsaturated double bonds at the C9 position of fatty acids. Palmitoyl CoA (C16:0) and stearoyl CoA (C18:0) undergo catalytic conversion into palmitoleic acid CoA (C16:1) and oleic acid CoA (C18:1), respectively. These unsaturated fatty acids are important substrates for the synthesis of phospholipids, cholesterol, wax lipids, and triglycerides (Ravaut et al., 2020). In addition, SCD participates in the differentiation of preadipocytes and plays an important role in the dynamic regulation of the balance between liver fat generation and fat oxidation (Zhang et al., 2021). GR is associated with energy metabolism in muscle tissue, gluconeogenesis in the liver, and the negative feedback regulation of the hypothalamo−pituitary axis in the hippocampus. The above proteases play important roles in the fat metabolism of organs.

Nutrition and hormone signaling could influence the transcription and expression of the fat synthase gene in mammals, and the same results were also observed in poultry (Hadinia et al., 2020; Xu et al., 2022). Feeding restriction significantly changes the expression levels of some key enzymes in the process of fat synthesis in the liver and skeletal muscle of poultry (Towle et al., 1996). However, only a few studies on the effects of feeding restriction on the expression of the key factors of fat metabolism in multiple organs of newly hatched chicks exist. The present experiment was conducted to investigate the effects of fasting−refeeding on the liver fat content and FAS, AMPKα, AMPKβ, AMPKγ, SCD, and GR expression levels in newly hatched chicks. It provides a theoretical basis for the reasonable application of feeding restriction in broiler production. At the same time, studying the expression levels of the key enzymes of fat metabolism in multiple organs of the chicken, a model animal, can provide interspecies comparison for further exploring the potential mechanism of energy restriction to improve animal health and prolong life span.

MATERIALS AND METHODS

Administration, Animals, Experimental Protocol, and Sample Collection

The fertile Ross broiler eggs (60−62 g) were purchased from a commercial hatchery (Dayong chicken breeding farm, Henan Province, China) and incubated in an intelligent automatic incubator (Dezhou Ruike incubation equipment factory, Shandong Province, China) at 37.5 ±0.2°C and 55.0 ± 2.0% relative humidity. To clarify the effect of fasting-refeeding on the levels of AMPK and genes associated with lipid metabolism of the newly-hatched chicks, 200 newly hatched male chicks with similar body weight (39−41 g) were randomly divided into 2 groups [Control group (Group C), n = 100; Fasting-refeeding group (Group T), n = 100]. No significant difference was observed in the average body weight between the two groups ($P > 0.05$). Ad libitum feeding and drinking water were provided to the chicks in Group C after they came out of the shell. The basal diet (Liuhe Feed Co., LTD, Shandong Province, China) was formulated according to the National Research Council (1994) standards (Table 1). Moreover, after hatching, chicks in Group T drank water freely. In terms of diet, the chicks in Group T first fasted for 72 h and then began to feed the same basal diet of chicks as Group C for another 48 h. During the experiment, 6 chickens in Group T were collected at fasting for 0 (just out of their shells, F0), 24 (F24), 48 (F48), 72 h (F72) and refeeding for 4 (FR4), 12 (FR12), 24 (FR24), and 48 h after fasting for 72 h (FR48), respectively. The sampling time (F0, F24, F48, F72, FR4, FR12, FR24, and FR48) and quantity (6 chickens) of Group C were consistent with those of Group T. The birds were euthanized with cervical dislocation under deep Nembutal anesthesia (45 μg/g of BW, intraperitonealin jection; Shanghai Chemical Factory, Shanghai, China) in another laboratory. The abdominal cavity was cut open to obtain the liver and adipose samples (intraperitoneal). Subsequently, the hypothalamus was obtained. All the samples were frozen in liquid nitrogen and stored at −80°C for RNA expression analysis. In addition, a part of the liver tissue obtained from 2 groups was taken and fixed in 4%

Table 1. Ingredient and nutrient composition of basal diet (as fed).

Item	Composition		Composition
Ingredients (%)		Calculated nutrient level[2]	
Corn	60.80	Metabolizable energy (MJ/kg)	12.55
Soybean meal	30.00	Crude protein	21.00
Corn gluten meal	3.00	Ether extract	4.50
Soybean oil	2.00	Crude fiber	2.20
$CaHPO_4$	1.30	Calcium	1.02
Limestone	1.60	Tryptophan	0.24
NaCl	0.30	Lysine	1.27
Methionine (98%)	0.20	Methionine	0.52
Lysine (98%)	0.35	Threonine	0.78
Premix[1]	0.23		
Mildew preventive	0.08		
Antioxidant	0.06		
Choline chloride (50%)	0.08		
Total	100.00		

[1]The premix provided the following per kg of diet: VA 6,500 IU, VD_3 3,000 IU, VE 80 IU, VK 5 mg, VB_1 4 mg, VB_2 5.5 mg, VB_6 5 mg, niacin 30 mg, pantothenic acid 12 mg, folic acid 1 mg, Mn (as manganese sulfate) 80 mg, Fe (as ferrous sulfate) 110 mg, Cu (as copper sulfate) 12 mg, Zn (as zinc sulfate) 70 mg, I (as potassium iodide) 0.4 mg, Se (as sodium selenite) 0.2 mg.

[2]ME was a calculated value, while the others were measured values.

Table 2. Sequence of the primers used in real-time PCR.

Gene name	Primer name	Sequence	Length	Reference sequence
FAS	F	5'- GAATGTGTATCGTGATGGAAAGTGG -3'	168	NM_205155
	R	5'- GGATTGGTTGTTTGGAAGTGTCG -3'	168	NM_205155
AMPKα	F	5'-CGGCAGATAAACAGAAGCACGAG-3'	148	NM_001039603
	R	5'-CGATTCAGGATCTTCACTGCAAC-3'	148	NM_001039603
AMPKβ	F	5'- TTTGTGGATGGGCAGTG-3'	239	NM_001039912.1
	R	5'-GGAAGAATGGGTGGAGA-3'	239	NM_001039912.1
AMPKγ	F	5'- F: AGAAGGCTTTCTTTGCACTGGTC -3'	186	NM_001034827
	R	5'-CGTCTCGATTTTGTGCTCCTCC -3'	186	NM_001034827
SCD	F	5'-ACTACCACCATACATTCCCCTACG-3'	165	NM_204890
	R	5'-CATCTCCAGTCCGCATTTTCC-3'	165	NM_204890
GR	F	5'-CTTCCATCCGCCCTTCA-3'	203	NM_001037826.1
	R	5'-TCGCATCTGTTTCACCC-3'	203	NM_001037826.1
β-actin	F	5'-CACCACAGCCGAGAGAGAAAT-3'	135	L08165.1
	R	5'-TGACCATCAGGGAGTTCATAGC-3'	135	L08165.1

Abbreviations: AMPKα, adenosine monophosphate-activated protein kinase alpha; AMPKβ, adenosine monophosphate-activated protein kinase beta; AMPKγ, adenosine monophosphate-activated protein kinase gamma; FAS, fatty acid synthase; GR, glucocorticoid receptor; SCD, stearoyl CoA desaturase; β-actin, beta actin.

paraformaldehyde for 2 h. Then, frozen sections were made directly and Sudan III staining was used to observe the changes in fat content. Another part of the liver tissue of the 2 groups was taken and fixed in 4% paraformaldehyde. After the experiment, the sections were made and stained with hematoxylin-eosin (**HE**) staining to observe the changes in hollow bubbles. Moreover, during the experiment, one chicken died at F48, and one chicken died at FR24 in Group C. Two chickens died at F72 and one chicken died at FR24 in Group T. The experiment was repeated three times. All procedures were approved by the Animal Care and Use Committee of China.

Quantitative RT-PCR Analysis of mRNA Expression of AMPK and Genes Associated With Lipid Metabolism

Total RNAs were extracted from proventriculi with TRIzol reagent (Life Technologies, 15596018, Carlsbad, CA) according to the manufacturer's instructions. Next, Prime Script TMRT reagent kits with gDNA eraser (Taraka, RR047A, Dalian, China) were introduced to eliminate possible genomic DNA contamination and to perform first-strand cDNA synthesis. The primer pairs for AMPK, genes associated with lipid metabolism, and β-actin are shown in Table 2. qRT-PCR was performed with SYBRR Premix Ex Taq TMII (Takara, RR820A, Dalian, China) according to manufacturer's instructions. Experiments on each sample were performed in triplicate with the above primers. Real-time PCR was performed as previously described (Xu et al., 2019). After PCR, data were analyzed with an ABI 7300 instrument (Applied Biosystems, Marsiling, Singapore) by using the $2^{-\Delta\Delta CT}$ method.

Paraffin Sectioning and Vacuole Counting in Hepatocytes

Liver tissue was fixed, dehydrated, embedded, sliced into sections approximately 5-μm thick, dewaxed, hydrated, and stained with hematoxylin for 5 min. After differentiation in 1% hydrochloric acid−ethanol solution, cells were cyanated in water for 30 min, then stained with 1% eosin for 3 min. Subsequently, the slices are dehydrated, transparented, and finally sealed. The tissue sections of 6 samples were observed under a 400 × light microscope, and the number of vacuoles in liver cells was counted. The determination criteria included vacuoles in the cytoplasm and the perinusial space of liver cells that were visible to the naked eye under a counting microscope.

Frozen Section Preparation and Fat Content Statistics

The fresh liver tissue of 2 groups was fixed with 10% neutral formalin solution for 2 h. The fixed liver tissue was first placed in 20% sucrose solution to sink, and then in 30% sucrose solution to sink. A specimen was placed on the specimen freezing tray of a frozen slicer and covered with OCT embedding agent. After freezing, the specimen was sliced into sections with the thickness of approximately 10 μm and washed with distilled water. Nuclei were dyed with hematoxylin for 1 to 2 min, washed with water, and cyanated. The sections were soaked in 70% ethanol for 5 min and then immersed in Sudan III dye for 10 min. Excess dye was washed away with 70% ethanol. The sections were sealed with glycerin gelatin. The gray value is a measure that reflects the fat content. The mean gray value of the fat content was analyzed by using Image-Pro Plus software, version 6.0 (Media Cybernetics, Inc., Rockville, MD), in which a high gray value indicated a low fat content (Xu et al., 2019).

Statistical Analysis

Data were analyzed using the software package SPSS 19.0 (IBM Corp., Armonk, NY) for Windows. Results were expressed as mean± standard error. Two-tailed 1-way ANOVA was used for the comparison of data between the 2 groups. Pared-sample t test was utilized for comparisons at different time points within the

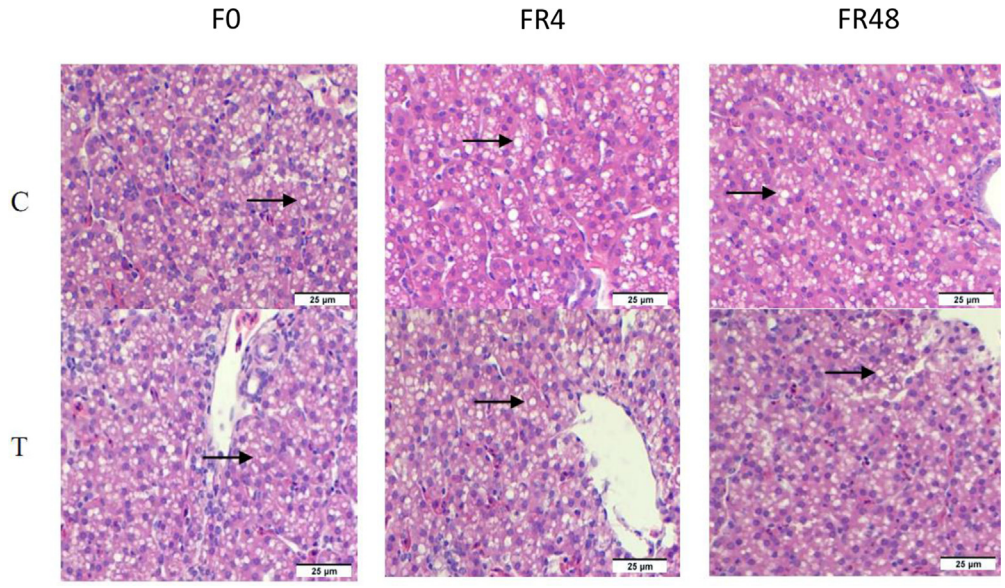

Photo 1. Liver structures in groups C and T at F0, FR4, and FR48, respectively (hematoxylin eosin staining; scale bar = 25 μm). "⟶" represents fat vacuole. Abbreviations: F, fasting; FR, refeeding after fasting.

group. $P < 0.05$, $P < 0.01$, and $P > 0.05$ indicate that the difference is significant, highly significant, and not significant, respectively. Graphs were generated using Origin 8.5 (OriginLab, Northampton, MA).

RESULTS AND DISCUSSION

Fasting and feeding are two completely different nutritional statuses. During fasting, the body's fat synthesis is weakened, fatty acid oxidation is promoted, and gluconeogenesis is enhanced to improve the concentration of glucose in the body and meet the body's energy needs. Therefore, when the body's energy status changes, the fat synthesis pathway is considerably inhibited, and the body controls carbon flow through gluconeogenesis and inhibits the fat synthesis pathway, thus mainly restricting the expression of the fat synthase gene at the transcriptional and translational levels (Desvergne et al., 2006). Refeeding has the opposite effect and can cause a dramatic increase in nutrition and energy, leading to the rapid elevation of blood glucose and an increment in fat synthesis and storage. Previous studies have indicated that fasting, even for a short period, can dramatically reduce the capacity for lipogenesis in the chicken liver, whereas refeeding can restore the reduced capacity for lipogenesis for 1 h (Leveille et al., 1975). Thus, identifying the drastic changes in nutritional and energy levels caused by fasting and refeeding can provide a theoretical basis for rational application of feed restriction in broiler production.

Changes in Fat Content in the Liver

The results of this experiment showed that the distribution and content of liver lipid droplets in Group C were normal. Compared with those in Group C, the hepatic sinus space gradually narrowed, and the number of lipid droplets increased with the extension of fasting time in Group T. With the prolongation of time after refeeding, the liver structure of Group T gradually recovered to the control state (Photo 1). The statistical results of vacuole number showed that the number of the liver lipid droplets of Group C gradually decreased ($P < 0.05$) at F0-FR48 (Figure 1). The number of the liver lipid droplets of Group T was not significantly different at F0 and F24 ($P > 0.05$) but significantly increased at F48 and FR48 ($P < 0.05$) and highly significantly increased at F72, FR4, FR12, and FR24 ($P < 0.01$; Figure 1) relative to that of Group C. This finding indicated that the liver fat content of the chicks in Group C gradually decreased with the increase of age, but fasting and refeeding increased the liver fat content of the chicks in Group T. The increased fat content in the liver caused by fasting and refeeding may be due to the low apolipoprotein content after fasting. The newly hatched chicks need to synthesize sugars and fats to maintain energy. During fasting, the chicks still rely on

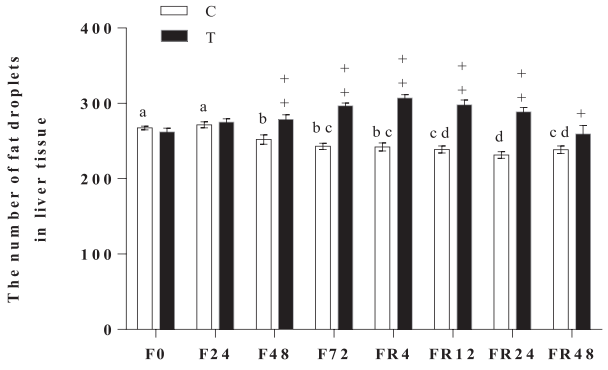

Figure 1. Effects of fasting-refeeding on the number of fat droplets in liver tissue of two groups. $^{+}P < 0.05$ vs C, $^{++}P < 0.01$ vs C. Significant difference ($P < 0.05$) within group C is indicated by different lowercase letters (a, b, c, d). The data are expressed as mean ± SD (n = 6). Abbreviations: F, fasting; FR, refeeding after fasting.

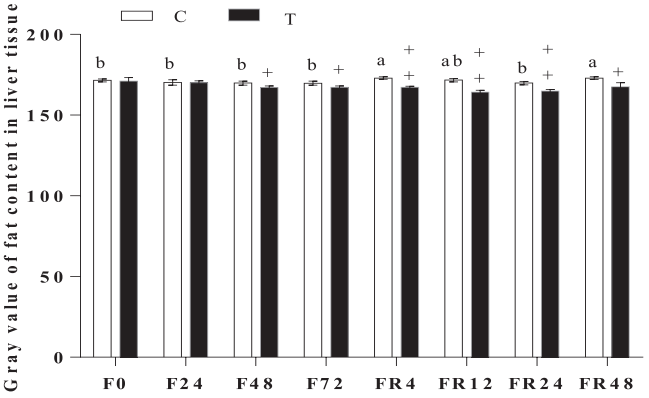

Figure 2. Effects of fasting-refeeding on the gray value of fat content in liver tissue of two groups. $^{+}P < 0.05$ vs C, $^{++}P < 0.01$ vs. C. Significant difference ($P < 0.05$) within group C is indicated by different lowercase letters (a, b). The data are expressed as mean ± SD (n = 6). Abbreviations: F, fasting; FR, refeeding after fasting.

the yolk sacs in their abdominal cavities to obtain some nutrients. The yolk sac mainly contains fatty parts, especially triglycerides. The liver is the main organ of fat metabolism. Triglycerides are broken down into unsaturated fatty acids after being transported to the liver, and the liver needs to synthesize water-soluble plasma lipoprotein with apolipoprotein for transport in the plasma (Bolsoni-Lopes and Alonso-Vale, 2015). The synthesis of plasma lipoprotein requires apolipoprotein, phospholipids, choline, and other substances. When fat from the yolk sac enters the liver during fasting, the amount of apolipoprotein is inadequate for the timely transport of fat from the liver. Thus, fat accumulates in the liver. This situation leads to an increase in fat content. After supplementation, the fat synthesis pathway is activated and enhanced. With the increase in dietary protein content, the apolipoprotein content in the plasma increases, the excess fatty acids in the liver are synthesized into water-soluble lipoproteins and enter the blood circulation, and the fat content in the liver decreases gradually.

The vacuoles in the liver tissue were identified as lipid droplets by using frozen sections and Sudan III staining. In this experiment, the gray value of chicken liver slices from Group C did not change significantly at F0-F72, FR12, and FR24. However, the gray value significantly increased ($P < 0.05$) at FR4 and FR48 (Figure 2; Photo 2). Compared with Group C, the gray value of chicken liver slices from Group T decreased gradually with the prolongation of fasting time and decreased significantly at F48 and F72 ($P < 0.05$). After refeeding, the gray values of Group T were lower than those of Group C at FR4, FR12, and FR24 ($P < 0.01$) and at FR48 ($P < 0.05$; Figure 2; Photo 2). A low gray value indicates high fat content. Therefore, these results further indicated that the liver fat content of the chicks in Group C gradually decreased with the increase in age. Moreover, the liver fat content did not change significantly in the early period of fasting but gradually increased in the middle and late periods of fasting. In the early and middle periods after refeeding, the liver fat content in Group T was highly significantly greater than that in Group C. In the later period of this experiment, the liver fat content in Group T was still higher than that in Group C. However, the difference between the 2 groups had decreased. The variation trend of fat content represented by the gray value was consistent with the variation trend of the number of vacuoles observed in HE sections. Both methods showed that fasting and refeeding led to an increase in the liver fat content of newly hatched chicks.

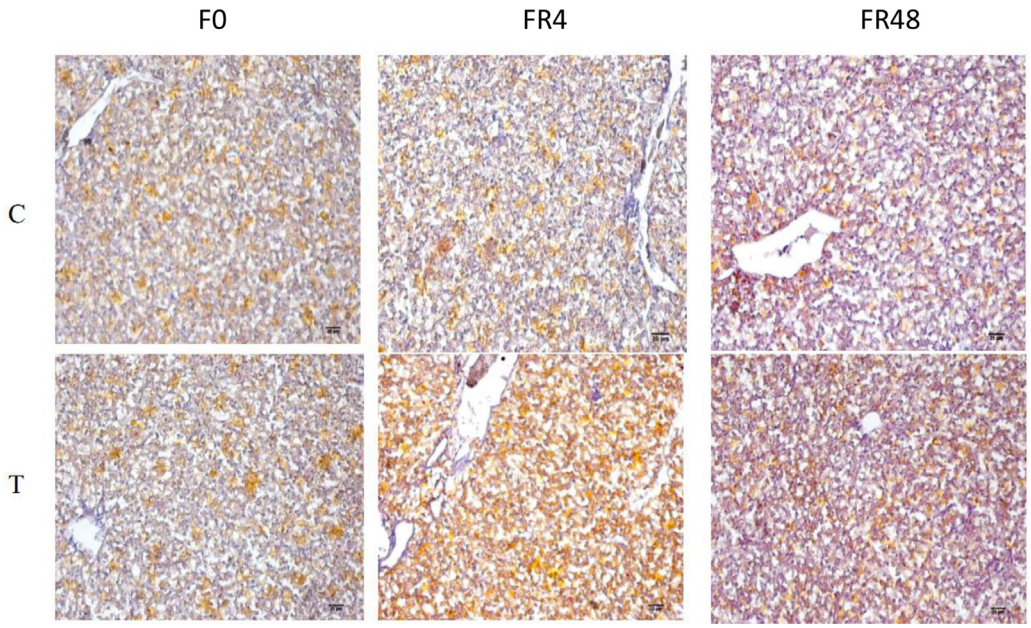

Photo 2. Liver tissue stained with fat in groups C and T at F0, FR4, and FR48, respectively (sudan III staining; scale bar = 25 μm). The yellow areas represent fat particles. Abbreviations: F, fasting; FR, refeeding after fasting.

Expression of SCD mRNA

SCD is a rate-limiting enzyme that catalyzes the formation of saturated fatty acids into MUFAs, which are essential fatty acids for the synthesis of body fat. SCD plays an important regulatory role in lipid metabolism. The results of this experiment showed that the expression of SCD in the liver tissue of Group C was gradually increased ($P < 0.05$) at F0−F72 and gradually decreased ($P < 0.05$) at FR4−FR12. However, the expression was significantly increased at FR24 ($P < 0.05$) (Figure 3A). The expression level was the highest at F72, indicating that the expression trend of SCD in liver would change greatly with the increase in age and dietary demand. In addition, liver SCD mRNA expression in Group T was significantly lower than that in Group C ($P < 0.05$ or $P < 0.01$) at 24 to 72 h after fasting and 4−24 h after refeeding (Figure 3A), suggesting that fasting and refeeding decreased SCD expression in liver at the middle and late stages of fasting and early stage of refeeding. Moreover, the SCD expression in adipose tissue of Group C was significantly increased ($P < 0.05$) at F24 and F72, and then decreased ($P < 0.05$) at FR12 and FR48 (Figure 3B). The expression level was the highest at

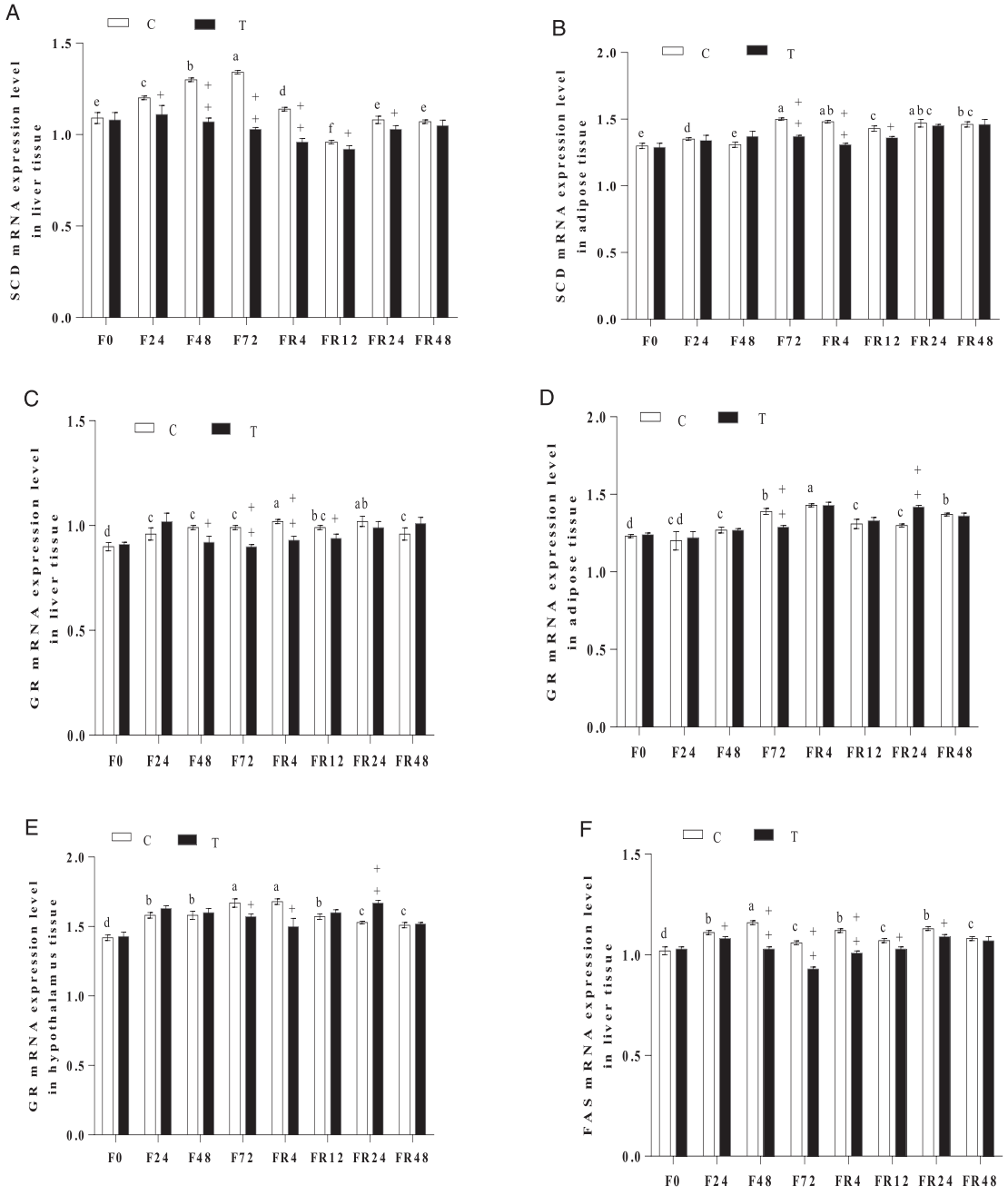

Figure 3. Effects of fasting-refeeding on the expression levels of SCD, GR, FAS, AMPKα, AMPKβ, and AMPKγ in liver, adipose, and hypothalamus of groups C and T at F0, F24, F48, F72, FR4, FR12, FR24, and FR48, respectively. $^{+}P < 0.05$ vs C, $^{++}P < 0.01$ vs C. Significant difference ($P < 0.05$) within group C is indicated by different lowercase letters (a, b, c, d, e). The data are expressed as mean ± SD (n = 6). Abbreviations: AMPKα, adenosine monophosphate-activated protein kinase alpha; AMPKβ, adenosine monophosphate-activated protein kinase beta; AMPKγ, adenosine monophosphate-activated protein kinase gamma; F, fasting; FAS, fatty acid synthase; FR, refeeding after fasting; GR, glucocorticoid receptor; SCD, stearoyl CoA desaturase.

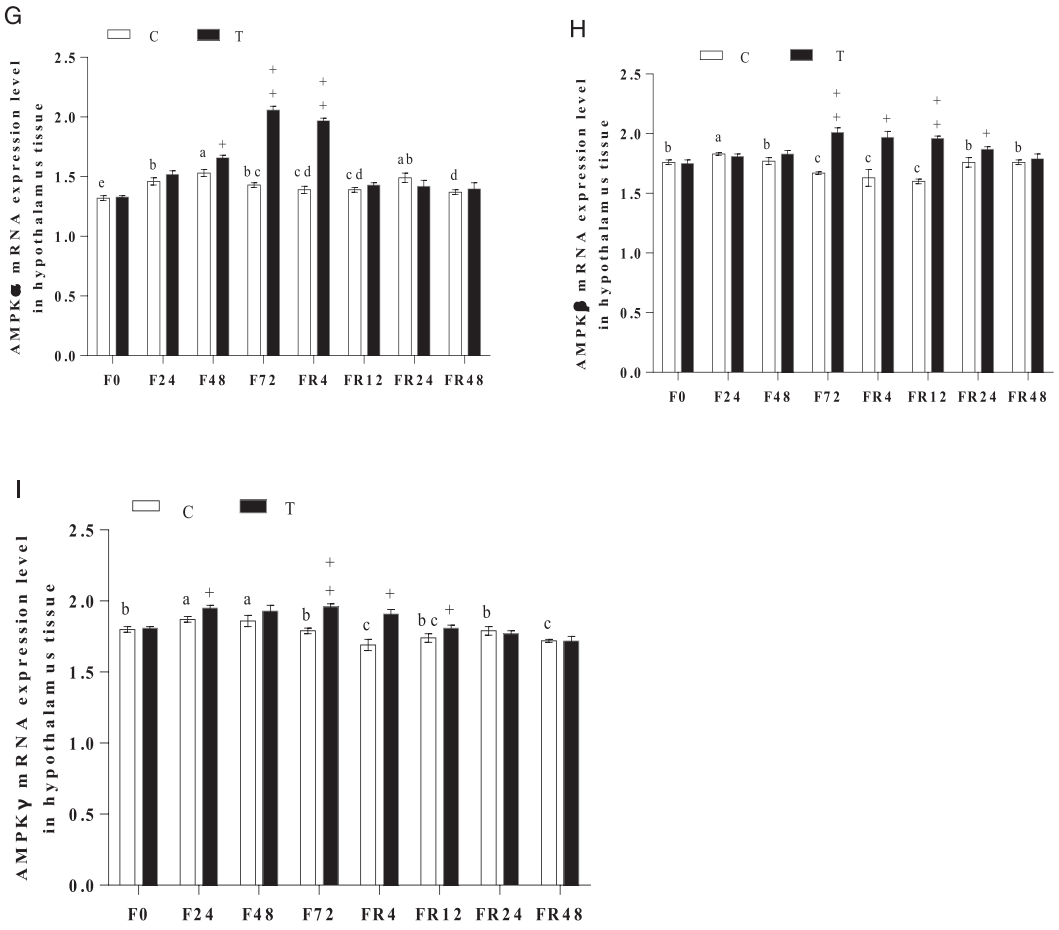

Figure 3 Continued.

F72, indicating that with the increase in age, the expression of SCD in adipose tissue increased first and then decreased. In addition, SCD mRNA expression in adipose tissue in Group T was significantly lower than that in Group C ($P < 0.05$ or $P < 0.01$) at F72 and FR4 −FR12 (Figure 3B), thereby indicating that fasting and refeeding decreased SCD expression in adipose tissue during the late fasting period and early refeeding period. Studies have shown that the reduction in zinc levels leads to iron overload, which directly activates the transformation activity of SCD, the main target of SREBP, and promotes lipid biosynthesis and accumulation (Zhang et al., 2017). In chickens, restricted feeding can decrease the expression of SCD in subcutaneous adipose tissue, and dietary *Lactobacillus acidophilus* and mannan oligosaccharide can upregulate the expression of SCD-1, thereby improving health indicators (Schübel et al., 2019). Decreased androgen levels upregulate the expression of genes that are related to lipid metabolism (APOA1, SCD, FABP7, RXRG, and FADS2), thus leading to increased fat accumulation (Cui et al., 2018). SCD is acutely inhibited after fasting and acutely increased after refeeding (Cogburn et al., 2020). The above data indicated that SCD can promote fat synthesis. The chicks used in this experiment were newborn chicks with residual yolk sacs in their abdominal cavities. The liver is the major organ of fat synthesis. The fat synthesis pathway stimulated by nutritional factors in Group T chicks was restrained relative to that in normally fed chicks. This condition resulted in the decrease in liver fat synthesis in Group T. In this experiment, the liver and adipose tissue of Group T had lower SCD gene expression than that of Group C, and the fat synthesis ability decreased. With the extension of nutrient acquisition time after refeeding, the fat synthesis pathway was activated, the ability of lipid synthesis was enhanced, and the expression of SCD gradually returned to normal levels.

Expression of GR mRNA

GR mediates most biological effects of glucocorticoids (**GCs**) (Motavalli et al., 2021; Zeyen et al., 2022). After binding to GCs, GR is separated from its chaperone complex and translocated to the nucleus, where it regulates gene transcription by binding to GC response elements in the control region of the target gene (Wang et al., 2012a). GR is an important gene in fat anabolism, and its genetic variation may cause the overstorage of triacylglycerols. In the liver tissue used in this work, the GR expression of Group C was gradually increased ($P < 0.05$) at F24−FR4 and then decreased ($P < 0.05$) at FR12 and FR48 (Figure 3C). The expression level was the highest at FR4, indicating that the trend of liver GR expression increased first and then decreased

with the increment of age. In addition, the GR mRNA expression in Group T was significantly lower in than that in Group C at F48−F72 and FR4−FR12 ($P < 0.05$ or $P < 0.01$) (Figure 3C). Such result suggests that fasting and refeeding decreased the expression of GR in liver tissues at the middle and late stages of fasting and early stage of refeeding. In the adipose tissue of this work, the GR expression of Group C was increased ($P < 0.05$) at F24−FR4 and decreased ($P < 0.05$) at FR12−FR24, but the expression was increased ($P < 0.05$) at FR48 (Figure 3D). The expression level was the highest at SF4. This finding indicated that the trend of GR expression in adipose tissue changed greatly with the increase in age and dietary demand. In addition, GR mRNA expression in Group T was significantly lower than that in Group C at F72 ($P < 0.01$) and significantly higher than that in Group C at FR24 ($P < 0.01$; Figure 3D). These results indicated that fasting and refeeding inhibited GR expression in adipose tissue at the late stage of fasting and promoted GR expression at the middle stage after refeeding. Moreover, in the hypothalamus tissue used in this work, the GR expression of Group C was gradually increased ($P < 0.05$) at F24−FR4 and gradually decreased ($P < 0.05$) at FR12−FR48 (Figure 3E). The expression level was the highest at FR4, indicating that GR expression showed a trend of increasing first and then decreasing with the increment of age. In addition, GR mRNA expression of Group T was significantly lower than that of Group C at F72 and at FR4 ($P < 0.05$) and higher than that of Group C at FR24 ($P < 0.01$; Figure 3E). These results indicated that fasting and refeeding inhibited the GR expression in the late fasting period and early refeeding period, and promoted the GR expression in the middle period after refeeding. Studies have shown that muscles can secrete MSTN to inhibit GR levels in adipose cells, thereby reducing adipose cell proliferation and affecting intramuscular fat deposition (Chu et al., 2017). GC promotes heterotopic fat deposition in muscle by upregulating the mRNA expression levels of GR and AMPKα2 (Wang et al., 2012b). The GR-mediated activation of FTO reverse transcription and the demethylation of m6A into adipogenic mRNA promote the activation of adipogenic genes in the Cort-induced chicken fatty liver and the accumulation of lipid in OA/DEX-induced chicken primary hepatocytes (Hu et al., 2020). The above data suggested that GR can promote fat synthesis. In this experiment, the activation of the fat synthesis pathway in the liver of the normal feeding group was enhanced by exogenous nutrition's due to dietary intake (Fu et al., 2020). However, the demand for fat decomposition increased and GR expression was significantly lower in Group T than in Group C given that in the former group, energy maintenance mainly depended on the abdominal yolk sac and body fat. Even in the early stage after refeeding, the liver GR expression in Group T was still lower than that in Group C. With the extension of refeeding time, liver GR expression gradually returned to normal levels. Fat is an energy storage tissue. Fasting resulted in decreased GR expression and enhanced adipose decomposition ability. With diet intake, the consumption of excessive fat was no longer necessary to meet the body's needs. At this point, the adipose tissue of Group T had a greater energy storage potential than that of Group C, GR expression increased, and excess fatty acids were synthesized into fat by the body to reserve energy. The hypothalamus is an important part of the body, and an excessively high or low fat content affects the normal operation of its function (Miller and Spencer, 2014; van Opstal, 2021). The hypothalamus is also an organ that burns fat relatively late. In the late fasting period, GR expression was decreased and lipolysis was strengthened. With feed intake after refeeding, GR expression increased, fat deposition in the hypothalamus began to supplement the portion of fat consumed during fasting, and fat was maintained at normal proportions in hypothalamus tissue. Therefore, fasting and refeeding inhibited the GR expression in liver, adipose, and hypothalamus. Moreover, the expression level of GR gradually returned to the control level with the prolongation of refeeding time.

Expression of FAS mRNA

FAS is a key enzyme in fatty acid synthesis. In the chick liver, FAS activity and expression level are related to the rate of fatty acid synthesis, and the change in FAS mRNA expression is positively correlated with the rate of FAS protein synthesis (Back et al., 1986). In this experiment, the FAS expression in liver tissues of Group C was increased ($P < 0.05$) at F24 and F48. However, the expression trend of FAS was not obvious at F72−FR48 (Figure 3F). Moreover, the expression level was the highest at FR48. These results indicated that FAS expression in liver tissue would change greatly with the increase in age and dietary demand. In addition, at F24−F72 and FR4−FR24, the FAS mRNA expression of the chick liver in Group T was significantly lower than that in Group C ($P < 0.05$ or $P < 0.01$; Figure 3F). This finding indicated that FAS expression was inhibited by fasting and refeeding in the middle and late periods of fasting and early and middle periods of refeeding. Studies have shown that hypoxic exposure (1% O_2 concentration) can inhibit the expression of srebp-1c, downregulate the expression of FAS mRNA, and reduce the synthesis of FAS, thus inhibiting fat production in cells (Krzystek-Korpacka et al., 2011). (−)-Hydroxycitric acid decreases FAS mRNA levels, increases PPARα mRNA levels, and ultimately reduces lipid droplet accumulation (Peng et al., 2018). Epigallocatechin gallate decreases FAS mRNA expression and lipid accumulation in 3T3-L1 preadipocytes but does not significantly reduce abdominal fat (Chen et al., 2021). Infection by Marek's disease virus activates FAS and increases the number of lipid droplets (Boodhoo et al., 2019). RoSebrough (Rosebrough, 2000) pointed out that the gene expression of FAS in skeletal muscle and liver decreases at 24 h after fasting. The significantly lower FAS expression level in Group T than in Group C found in this study was consistent with a previously reported result

(Rosebrough, 2000). With dietary intake, FAS expression gradually increased and lipid synthesis ability gradually enhanced. At the end of the experiment, liver FAS expression in Group T was restored to control levels.

Expression of AMPK mRNA

AMPK is a heterotrimer protein that is composed of α, β, and γ subunits. The α subunit plays a catalytic role, while the β and γ subunits play a regulatory role (Yan et al., 2018). The α subunit contains an N-terminal kinase domain and a C-terminal domain, which are approximately equal in size. The N-terminal is the catalytic core, and the C-terminal is responsible for binding to the β and γ subunits. AMPK, an important protein kinase, is involved in various metabolic processes, and its activity is regulated by the AMP/ATP ratio. AMPK is known as the metabolic receptor of cells or the fuel switch that regulates cellular ATP and AMP levels. When the AMP/ATP ratio increases, AMPK is activated through the effects of phosphorylated proteins or the regulation of gene expression in energy metabolism, thereby inhibiting anabolic pathways, such as cholesterol and fat synthesis, and enhancing catabolic pathways, such as fatty acid oxidation. In the hypothalamic tissues used in this experiment, the AMPKα expression of Group C was increased at F24−F48 ($P < 0.05$), and then decreased at F72−FR12 ($P < 0.05$). Moreover, the expression was increased at FR24 ($P < 0.05$), but decreased again at FR48 ($P < 0.05$; Figure 3G). The expression level was the highest at F48. These results indicated that the AMPKα expression would change greatly with the increase in age and dietary demand. In addition, at F48−F72 and FR4, hypothalamic AMPKα mRNA expression in Group T was significantly higher than that in Group C ($P < 0.05$ or $P < 0.01$; Figure 3G). This finding indicated that fasting and refeeding promoted the AMPKα expression in the middle and late fasting and early refeeding stages. The AMPKβ expression of Group C was increased ($P < 0.05$) at F24 and decreased ($P < 0.05$) at F72−FR12, and then increased ($P < 0.05$) at FR24−FR48 (Figure 3H). The expression level was the highest at F24. These results indicated that with the increase in age, the AMPKβ expression increased first and then decreased and finally stabilized after increasing again. In addition, at F72 and FR4 −FR24, the AMPKβ mRNA expression in Group T was significantly higher than that in Group C ($P < 0.05$ or $P < 0.01$; Figure 3H). Such finding indicated that fasting and refeeding promoted the AMPKβ expression in the late fasting period and early-middle refeeding period. For the AMPKγ mRNA expression, Group C increased at F24−F48 ($P < 0.05$) and gradually decreased at F72 −FR48 ($P < 0.05$; Figure 3I), suggesting that the AMPKγ expression increased first and then decreased with the increment of age. In addition, compared with Group C, at F24, F72, and FR4−FR12, the AMPKγ expression in Group T significantly increased ($P < 0.05$ or $P < 0.01$) (Figure 3I), indicating that fasting and refeeding promoted AMPKγ expression in the middle and late fasting and early refeeding stages. Studies have shown that mice with kidney tubular epithelial cell-specific AMPKα deletion exhibited exacerbated kidney impairment after ischemia/reperfusion accompanied by the accumulation of lipid droplets in the renal tubules. Moreover, the activation of AMPK restores fatty acid oxidation in renal tubular cells (Ma et al., 2022). The activation of AMPK by metformin causes an augmentation of Insig stability and reduction of lipogenic gene expression, and leads to the attenuation of hepatic steatosis in HFHS diet-fed mice (Han et al., 2019). AMPK activator C24 increased the phosphorylation of AMPKα and acetyl-CoA carboxylase (**ACC**), and inhibited lipogenesis in HepG2 cells (Sun et al., 2021). The pretreatment with compound C (inhibitor of AMPK) inhibited the effect of pinolenic acid on promoting the expression of p-AMPK, SIRT1, and PPARα for lipolysis (Zhang et al., 2019). The above data indicated that AMPK could enhance the catabolic pathway of fat. These experimental results indicated that in the early fasting phase, energy consumption was insignificant and did not cause significant changes in AMPK gene expression. With the prolongation of fasting time, the depletion of ATP in hypothalamus tissue promoted the mRNA expression of AMPK α, β, and γ. AMPK α, β, and γ were still highly expressed even in the early refeeding stage because at this point, the body still needed to consume adipose tissue to increase ATP expression. With the extension of refeeding time, the intake of protein and the synthesis of fat increased; the ATP content of the hypothalamus increased gradually; and the mRNA expression levels of AMPK α, β, and γ gradually returned to control levels.

Relationship Between Liver Fat Content and Lipid Metabolism Genes

In newly hatched chicks receiving delayed feeding, nutrition mainly originated from the yolk sac in the abdominal cavity. As the blood circulated, triglycerides moved from the yolk sac to the liver. Given the increased expression of lipolytic genes and the decreased expression of synthetic genes in the liver, fat was broken down into energy, resulting in the production of a large number of free fatty acids. The production of low-density lipoprotein was hindered as a result of insufficient apolipoprotein levels, leading to the interruption of hepatic triglyceride transport and ultimately resulting in the accumulation of fat in the liver. The intake of nutrients after refeeding activated the fat synthesis pathway of exogenous nutrients. With the increment in the expression of fat synthesis genes in the liver and the decrement in the expression of decomposition genes, excess fatty acids in the liver were transported out of the liver, and the fat content in the liver gradually returned to the control level.

Collectively, our results confirmed that fasting decreased the expression of fat synthesis genes in the liver (SCD, GR, and FAS), adipose (SCD and GR), and

hypothalamus tissue (GR) and increased the expression of lipolytic genes in the hypothalamus (AMPKα, AMPKβ, and AMPKγ). After refeeding, GR expression in adipose and hypothalamus tissues was upregulated and gradually returned to the control level with the extension of feeding time. Fatty acids from the yolk sac and body tissues accumulated in the liver of newly hatched chicks because of the down-regulated expression of fat synthesis genes in the liver. This effect increased in lipid droplets in the liver, with the most significant increase observed at 4 h after refeeding.

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DISCLOSURES

We declare that we have no conflict of interest.

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