Adiponectin/adiponectin receptors mRNA expression profiles in chickens and their response to feed restriction

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ABSTRACT Adiponectin (**ADPN**) is related to fatty acid synthesis and oxidation in mammals. In chickens, the lipid metabolism, structure and sequence of ADPN are different from that in mammals. The aim of this study was to determine the role of ADPN in broilers lipid metabolism by investigating the temporal and spatial expression profiles of *ADPN* and its receptors, as well as their response to feed restriction. The results showed that the abdominal fat has the highest expression level, followed by the duodenum, glandular stomach, heart, hypothalamus, liver, and skeletal muscle. Broilers have high energy mobilization during their early stage of growth, in which the fat demand in the liver and muscles is high, thus the expression of *ADPN* and its receptor are also increased. To study the effects of feed restriction on ADPN and lipid metabolism, broilers were fasted for 12 h and refeed for 2 h. The results showed that fasting decreased the concentration of triglyceride (**TG**) ($P < 0.05$) and total cholesterol (**TCHO**) ($P < 0.05$) in plasma. The mRNA expression of *ADPN* in the liver ($P < 0.05$), breast ($P < 0.05$) and thigh ($P < 0.05$), and the mRNA expression of *ADPNR1* in the liver ($P < 0.05$) and duodenum ($P < 0.05$) were significantly increased in the Fasted group. All above phenomena were recovered after refeeding, suggesting that feed restriction may promote the utilization of fatty acids in active metabolism tissues through ADPN, to guarantee the energy homeostasis of the body. However, the AMP-activated protein kinase (**AMPK**) signaling pathway and hepatic lipid metabolism were not necessary to cause the above changes under this experimental condition.

Key words: broiler, lipid metabolism, adiponectin, fasting

INTRODUCTION

Genetic improvement in growth rate and feed efficiency has allowed modern broilers to reach market weight in a shorter period of time, which fully reflects the characteristics of fast growth and large weight of broilers, with huge economic benefits for farmers. However, selection for these economically important traits has been accompanied by an increase in the number of problems encountered during production, including obesity, ascites, sudden death syndrome, and leg abnormalities (Alrubaye, 2013). The increased fat deposition of broilers will reduce feed conversion efficiency and the body's resistance to disease, impact on carcass quality, which will reduce meat production and causing economic losses to processors and consumers. Previous studies showed that feed restriction could decrease the fat deposition and improve the carcass quality of broilers (Santoso, 2001; Nielsen et al., 2003), since it is accompanied by a decrease in growth rate. The negative impact on broiler growth and carcass weight could be counterbalanced partly by appropriate intensity of feed restriction and compensatory growth (Yagoub and Babiker, 2008). Similar findings have been reported as well in mammals (Keogh et al., 2015).

Adiponectin (**ADPN**) is a protein produced and released primarily by adipocytes into the peripheral blood stream (Sreenivasa et al., 2005). It plays a dominant role in lipid metabolism in mammals (Lee and Shao, 2012; Sena et al., 2017). In chickens, ADPN genes has been isolated, cloned and sequenced (Sreenivasa et al., 2005). Compared with mammals, Sreenivasa et al. (2005) reported that there are 50 to 65% similarities of ADPN cDNA sequences between chickens and mammals. In mammalian adipocytes, the ADPN monomers are organized as oligomers and multimers, they are released in 3 isoforms with distinct molecular weights, denoted as low, medium and heavy isoforms (Tsao et al., 2002, 2003; Waki et al., 2003). Unlike the mammalian ADPN, chicken ADPN expressed in adipose tissue or in plasma is predominantly a heavy molecular weight multimeric

(Hendricks et al., 2009). Adiponectin receptor1 (**ADPNR1**) and adiponectin receptor2 (**ADPNR2**) are 2 main receptors of ADPN (Yamauchi et al., 2003), they are more conserved in mammals (Ramachandran et al., 2007).

The lipid metabolism in chickens is different from mammals. Modern broilers have the characteristics of high fat deposition and high feed conversion efficiency (Halevy et al., 2000), suggesting chickens have a more refractory insulin cascade and lipid metabolism in skeletal muscle tissues, the study of the chicken model would be beneficial to our understanding of ADPN. Previous studies reported that chicken *ADPN* mRNA is expressed in various tissues (Sreenivasa et al., 2005; Yuan et al., 2006; Zhang et al., 2017). Overfeeding and obesity decreases blood ADPN concentrations, whereas calorie restriction results in increased *ADPN* levels and insulin sensitization in rats (Min et al., 2004). Ventricular injection of ADPN can regulate energy metabolism and body weight in mice (Yong et al., 2004). Recent research has suggested that calorie restriction may increase circulating ADPN levels (Rusinov et al., 2018). It's been revealed that nutritional deficiency can trigger increased ATP consumption, resulting in activation of the AMP-activated protein kinase (**AMPK**) signaling pathway (Choi and Lee, 2013; Wierman et al., 2017). This connotes that AMPK plays a crucial role in the regulation of feed intake during calorie restriction. Several studies have demonstrated the significance of AMPK in the control of glucose and fatty acid metabolism in birds, pointing to its essential role in regulating whole body energy utilization (Andersson et al., 2004; Arsenault et al., 2013, Hu et al., 2015). Also, studies in chickens have found that ADPN regulates fatty acid-induced mitochondrial biosynthesis through AMPK/acetyl CoA carboxylase (**ACC**) pathway (Gan et al., 2015). Therefore, in the present study, we investigated the role of ADPN in broilers lipid metabolism by examining the temporal and spatial expression profiles of *ADPN* and its receptors, as well as their response to feed restriction. The involvement of AMPK pathway was further highlighted.

MATERIALS AND METHODS

Animals

One-day-old healthy male broiler chicks (Arbor Acres, *Gallus gallus domesticus*) were obtained from a local hatchery (Dabao Breeding Technology Co., Ltd., Taian, China). The chicks were managed in environmentally controlled chambers. The brooding temperature was maintained at 32°C for the first 5 d and then gradually reduced 1°C every 3 d until the final temperature was 25°C and this was maintained throughout the experiment. The humidity was maintained at 60 to 70% for the first 2 wk of brooding and then at 50 to 60%. Chicks were fed starter diet (apparent metabolizable energy: 2,897 Kcal/kg; 21% crude protein) until 21 d of age; thereafter, they received a grower diet (apparent

metabolizable energy: 3,002 Kcal/kg; 18% crude protein) until the end of the experiment.

The experimental design and setup was reviewed and approved by the Institutional Animal Care and Use Committee of Shandong Agricultural University (No. 2001002) and performed in accordance with the "Guidelines for Experimental Animals" of the Ministry of Science and Technology (Beijing, China).

Experiment 1: Spatial and Temporal Expression Profiles of ADPN and Its Receptors

The Spatial Expression Profiles of ADPN and Its Receptors Eight, 42-day-old broilers with similar body weight (**BW**, 2.42 ± 0.09 kg) were sacrificed by cervical dislocation, followed by exsanguination (Close et al., 1997). The abdominal fat, liver, breast muscle, thigh muscle, heart, glandular stomach, duodenum, and hypothalamus were sampled, snap frozen in liquid nitrogen, and then stored at $-80°C$ for further analysis.

The Temporal Expression Profiles of ADPN and Its Receptors

- Embryonic age: The hatching broilers' embryos were collected at the 11th, 13th, 15th, 17th, 19th day of embryonic age. Six chicken embryos were collected every time. The fertile eggs were purchased from the same hatchery as the broiler chicks (Dabao Breeding Technology Co., Ltd., Taian, China) and incubated at 37°C under a relative humidity of 60 to 70%. On the sampling day, all live embryos were euthanized. The liver, breast muscle, and thigh muscle were sampled, snap frozen in liquid nitrogen and then stored at $-80°C$ for further analysis.
- Before and after hatch: Ten chickens were selected per week (18E, 0W, 1W, 2W, 3W, 4W, 5W, and 6W) and they were sacrificed by cervical dislocation, followed by exsanguination (Close et al., 1997). The abdominal fat, liver, breast muscle, thigh muscle, heart, glandular stomach, duodenum, and hypothalamus were sampled, snap frozen in liquid nitrogen and then stored at $-80°C$ for further analysis.

Experiment 2: Effect of Feed Restriction on ADPN Expression and Lipid Metabolism

One hundred twenty six, 41-day-old broilers were randomly divided into 3 groups: fed ad libitum as control (Feed), fasted for 12 h (Fast), 2 h re-fed after 10 h feed withdrawal (Refeed), with 6 replicates and 7 chickens each. Chickens had free access to water during the experimental period. Before being sacrificed, a blood sample was drawn from a wing vein and collected in blood vessel of heparin sodium. Plasma samples were obtained after centrifugation at 4,000 rpm for 10 min at 4°C and were stored at $-20°C$ for further analysis. Tissue samples were obtained by the same method as Experiment 1.

Table 1. Primers for the targeted and reference transcripts.

Gene	Primer sequence	Accession NO.	Product size (bp)
GAPDH	F:ACATGGCATCCAAGGAGTGAG	NM-204305.1	244
	R:GGGGAGACAGAAGGGAACAGA		
ADPN	F:ACCCAGACACAGATGACCGTT	NM-206991	238
	R:GAGCAAGAGCAGAGGTAGGAGT		
ADPNR1	F:GGAGAAGGTTGTGTTTGGGATGT	NM-001031027	218
	R:TGGAGAGGTAGATGAGTCTTGGC		
ADPNR2	F:ACACACAGAGACTGGCAACATC	NM-001007854	144
	R:CCCAAGAAGAACAATCCAACAACC		

Abbreviation: ADPN, adiponectin.

Blood Metabolites and ADPN Measurement Plasma concentration of glucose (**GLU**, CH0101101, Maccura Biotechnology Co., Sichuan, China), triglycerides (**TG**, CH0101151, Maccura Biotechnology Co.), and total cholesterol (**TCHO**, CH010140, Maccura Biotechnology Co.) were measured with automatic biochemistry analyzer (Maccura Biotechnology Co.). Plasma concentration of very low density lipoprotein (**VLDL**) was measured according to Whitehead and Griffin (1982). The concentration of plasma ADPN was measured with a commercial diagnostic ELISA kit (H179, Jiancheng Bioengineering Institute, Nanjing, China).

Tissue TG, TCHO, and CPT-1 Enzyme Activity Measurement TG (F001) and TCHO (F002) were measured with commercial diagnostic kits (Jiancheng Bioengineering Institute). The enzyme activity of carnitine palmitoyl transferase-1 (**CPT-1**) was measured with a commercial diagnostic ELISA kit (H230, Jiancheng Bioengineering Institute).

RNA Isolation and Analysis The mRNA expression of ADPN and its receptors were determined by real-time PCR. Total RNA of the collected tissues was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA). The quantity and quality of the isolated RNA were determined using a bio-photometer (Eppendorf, Hamburg, Germany) and agarose-gelelectrophoresis, respectively. RT-qPCR analysis was conducted using 20 μL mix consisting of 2 μL the cDNA template (diluted 5 or 10 times), 0.8 μL of each primer, 0.4 μL ROX Reference Dye (50X) and 10 μL TB Green Premix Ex Taq II (Tli RNaseH Plus) (2X) (TaKaRa, Dalian, China) on ABI 7500 Real-Time PCR System (Q5, Applied Biosystems Inc., Carlsbad, CA) using the following parameters: 95°C for 30 s of predenaturation, followed by 40 cycles consisting of denaturation at 95°C for 5 s, annealing and extension at 60°C for 34 s. The expression levels of target genes were normalized with the expression of chicken GAPDH mRNA. The relative amount of mRNA of target gene was calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Therefore, all gene transcription results are reported as the n-fold difference relative to the calibrator. All of the samples were run in duplicate, the primers were designed to span an intron to avoid genomic DNA contamination. Primers used in this study were designed using Primer 5.0 software and synthesized by Sangon Biotech (Shanghai, P. R. China, Table 1). The specificity of the amplification product was verified by the standard curve and dissolution curve.

Western Blotting Analysis Tissue samples (0.1 g) were homogenized using 1 mL lysis buffer (Beyotime, Shanghai, China), centrifuged at 4°C, 12,000 g for 10 min. The supernatant (500 uL aliquot) was collected, the protein content was determined using the BCA protein assay kit (Beyotime) according to the manufacturer's protocol. Protein samples (18 μg) were separated using 7.5 to 10% SDS polyacrylamide gels (Bio-Rad, Richmond, CA), transferred onto polyvinylidene fluoride membrane (Millipore, Bilrica, MA) at 200 mA for 2 h. After western transfer, the membranes were incubated with western blocking buffer (Beyotime) for 1 h at room temperature, thereafter membranes were incubated with the primary antibodies at 4°C, with gentle shaking overnight. The primary antibodies used were anti-AMPKα (Cell Signaling Technology, Danvers, MA), anti-CPT-1 (Cell Signaling Technology) and anti-β-actin (Beyotime). Membrane was washed with Tris-buffered saline/Tween buffer for 3 times at 10-min interval, and then incubated with secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG, 1:1,000; Beyotime) for 4 h at 4°C. Afterward, washing was carried out and membranes were visualized by exposing to Hyperfilm ECL (Beyotime). Western blots were developed and quantified using BioSpectrum 810 with VisionWorks LS 7.1 software (UVP LLC, Upland, CA). The band intensity was normalized to the β-actin band within the same sample.

Statistical Analysis All the values were expressed as the means $\pm$ standard error (**SEM**). All statistical analyses were performed by one-way ANOVA using SAS statistical software (SAS version 8e, SAS Institute, Cary, NC). When the main effect of the treatment was significant, the differences between means were assessed by Student's t test. $P < 0.05$ was considered as statistically significant. GraphPad Prism software (version 6, GraphPad Software Inc., San Diego, CA) was used for regression analysis between plasma ADPN concentration and abdominal fat rate of broilers.

RESULTS

Spatial Expression Profiles of ADPN and Its Receptors in Broilers

ADPN and its receptors mRNA were highly expressed in the abdominal fat in broilers, followed by the glandular stomach, duodenum, heart, liver, hypothalamus, and skeletal muscle ($P < 0.0001$; Figure 1).
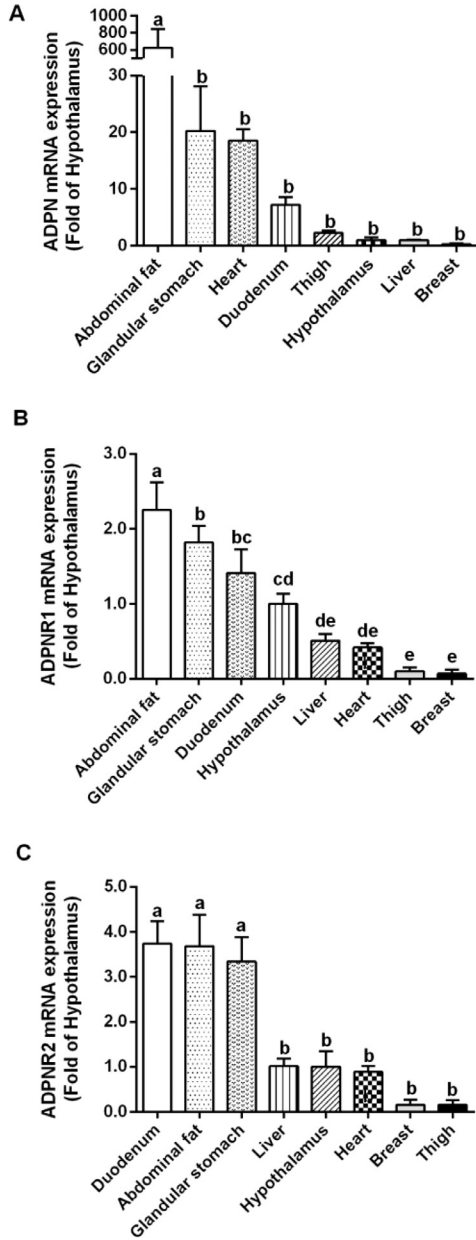
Figure 1. The mRNA expression levels of target genes in different tissues of broilers at 42 d. (A) Adiponectin (ADPN), (B) adiponectin receptor 1 (ADPNR1), (C) adiponectin receptor 2 (ADPNR2). Data were presented as mean ± SEM (n = 8). Different letters mean significant difference, $P < 0.05$.

Temporal Expression Profiles of ADPN and Its Receptors and Lipid Metabolites in Broilers

With the growth of broilers, plasma ADPN concentration was significantly increased ($P < 0.0001$; Figure 2A); plasma VLDL concentration was significantly declined ($P < 0.0001$; Figure 2B); TG concentration was at an unstable state, there were significant difference ($P < 0.01$) between 3 W and 4 W, while other time point were no difference ($P > 0.05$; Figure 2C). Abdominal fat rate was significantly increased ($P < 0.0001$; Figure 2D). Linear regression analysis showed that there was a positive correlation between plasma ADPN and abdominal fat rate in broilers (r = 0.5799; $P < 0.0001$; Figure 2E).

The target genes expressions were evaluated before and after hatch. In the abdominal fat, the mRNAs expression of *ADPN* and *ADPNR1* were not different ($P > 0.05$), but *ADPNR2* was significantly increased ($P < 0.01$), with the growth of broilers (Figure 3A). The expression levels of *ADPN, ADPNR1,* and *ADPNR2* were unstable ($P < 0.0001$) in the liver (Figure 3B). These genes increased before hatch, whereas, they decreased after hatch in the skeletal muscles ($P < 0.0001$; Figures 3C and 3D). In the duodenum, the mRNA expression of *ADPN* was significantly higher ($P < 0.01$) before 1 W, it was stable thereafter; The *ADPNR1* ($P < 0.001$) and *ADPNR2* ($P < 0.001$) were lower until hatch and showed no significant difference ($P > 0.05$) after 1 W (Figure 3E). In the glandular stomach, *ADPN* expression level was highest at 2 W ($P < 0.0001$), *ADPNR1* ($P < 0.0001$) and *ADPNR2* ($P < 0.0001$) were significantly increased at 0 W, *ADPNR1* was stable after hatch, and *ADPNR2* was gradually reduced with the growth of broilers (Figure 3F). In the hypothalamus, the mRNAs expression of *ADPN* and *ADPNR2* were highest at 4 W, it was lowest before hatch ($P < 0.01$) and there were no differences ($P > 0.05$) at other age; *ADPNR1* did not altered ($P > 0.05$) with the growth of broilers (Figure 3G).

The mRNA expression of *ADPN, ADPNR1,* and *ADPNR2* were determined in liver and skeletal muscle during embryonic age. The expression of *ADPN* was significantly increased in the liver ($P < 0.0001$), breast ($P < 0.01$), and thigh ($P < 0.0001$) along with the development (Figures 4A−4C). The expression of *ADPNR1* and *ADPNR2* were not significantly different ($P > 0.05$) in the liver and breast muscle (Figures 4A and 4B), however, the levels of *ADPNR1* ($P < 0.0001$) and *ADPNR2* ($P < 0.0001$) were significantly decreased along with the development of thigh muscle (Figure 4C).

We further measured the content of TG and TCHO in the liver, breast, and thigh muscle before and after hatch. TG and TCHO content in liver were highest at 0W ($P < 0.01$) and showed a downward trend overall after hatch (Figure 5A). In breast muscle, the concentration of TG was highest ($P < 0.0001$) at 0 W, there was no significant difference ($P > 0.05$) after 1 W; the concentration of TCHO was significantly decreased ($P < 0.0001$) with age, it had no significant difference ($P > 0.05$) after 2 W (Figure 5B). In thigh muscle, the concentration of TG was significantly higher at 0 W, it was decreased ($P < 0.0001$) with the growth of broilers afterward (Figure 5C); the concentration of TCHO was significantly higher ($P < 0.0001$) until 0 W, it was gradually declined afterward (Figure 5C).

The Effect of Feed Restriction on ADPN and Its Receptors in Broilers

We first investigated the influence of feed restriction on plasma metabolites in broilers. ADPN concentration
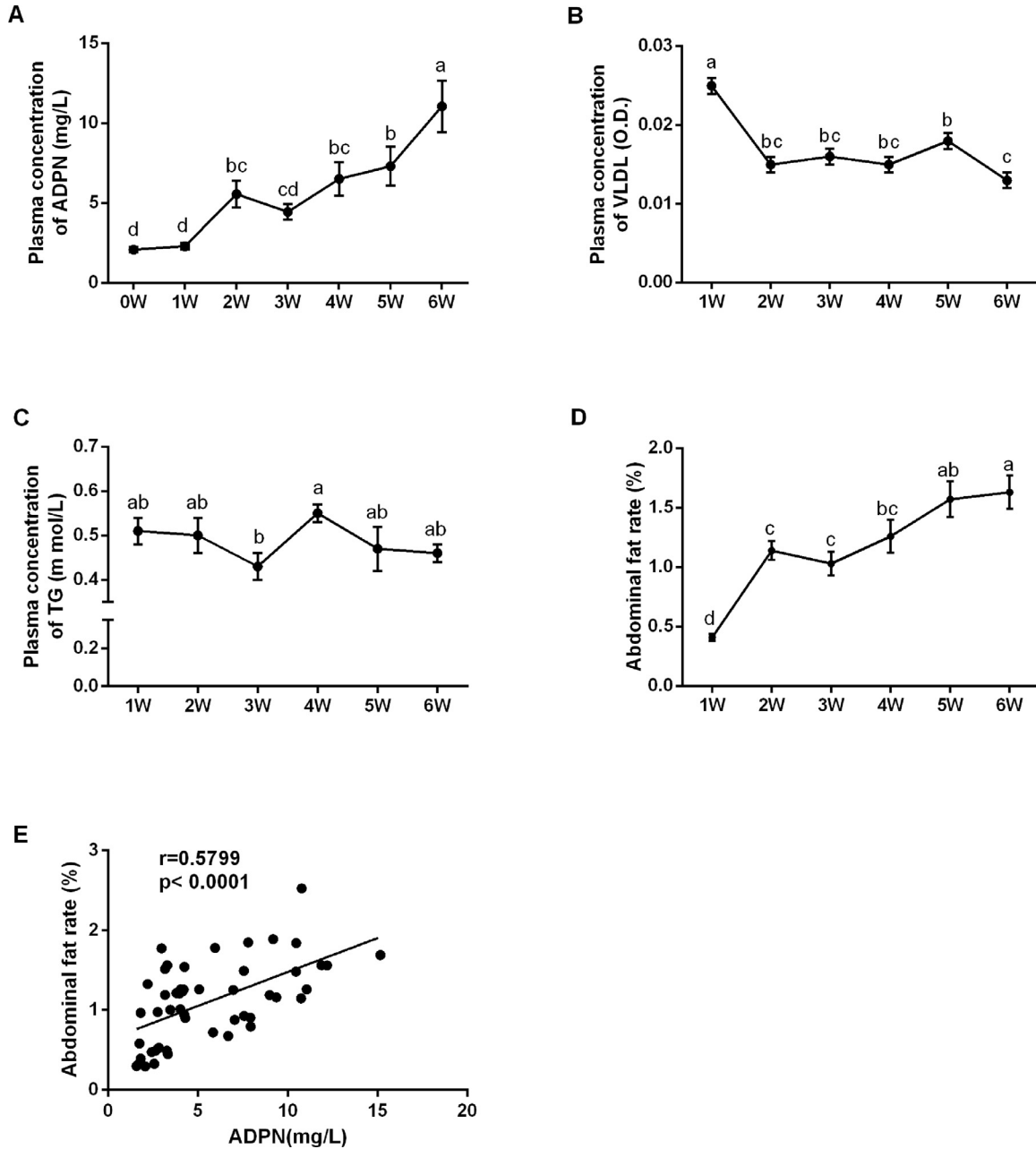
Figure 2. The blood metabolities and abdominal fat rate during the development of broilers. The contents of (A) adiponectin (ADPN, mg/L), (B) very low density lipoprotein (VLDL, O. D.), and (C) triglyceride (TG, m mol/L), (D) abdominal fat rate (%). Data were presented as mean ± SEM (n = 10). Different letters mean significant difference, $P < 0.05$. (E) The relationship between abdominal fat rate (%) and plasma concentration of ADPN (n = 50).

of Refeed group was significantly higher ($P < 0.01$) than other groups (Figure 6A). Feed restriction had no effect ($P > 0.05$) on plasma VLDL content (Figure 6B). Plasma concentration of TG ($P < 0.05$) and TCHO ($P < 0.0001$) were decreased after fasting for 12 h and they were recovered after refeeding (Figures 6C and 6D). GLU concentration of Refeed group was higher ($P < 0.05$) than Fast group, but not changed compared with Feed group ($P > 0.05$; Figure 6E).

We thereafter examined the effect of feed restriction on mRNA expression of *ADPN, ADPNR1*, and *ADPNR2* in different tissues. In the abdominal fat, the *ADPN* level was decreased ($P < 0.01$) in the Refeed group, *ADPNR2* level was highest ($P < 0.01$) in the Fast

group (Figure 7A). Fasted chickens had significantly higher ($P < 0.01$) *ADPN* mRNA level in the liver. The *ADPNR1* levels were significantly upregulated ($P < 0.05$) in the Fast group compared to the Feed group in the liver and duodenum (Figures 7B and 7E). *ADPN* expressions in the Fast group were significantly increased ($P < 0.05$) in muscle tissues (Figures 7C and 7D). In the glandular stomach, *ADPN* level was significantly decreased ($P < 0.05$) at the Fast group (Figure 7F). Feed restriction had no effect ($P > 0.05$) on above 3 genes expression in the hypothalamus (Figure 7G), neither on the mRNAs expressions of *ADPNR1* and *ADPNR2* in the skeletal muscle and glandular stomach.
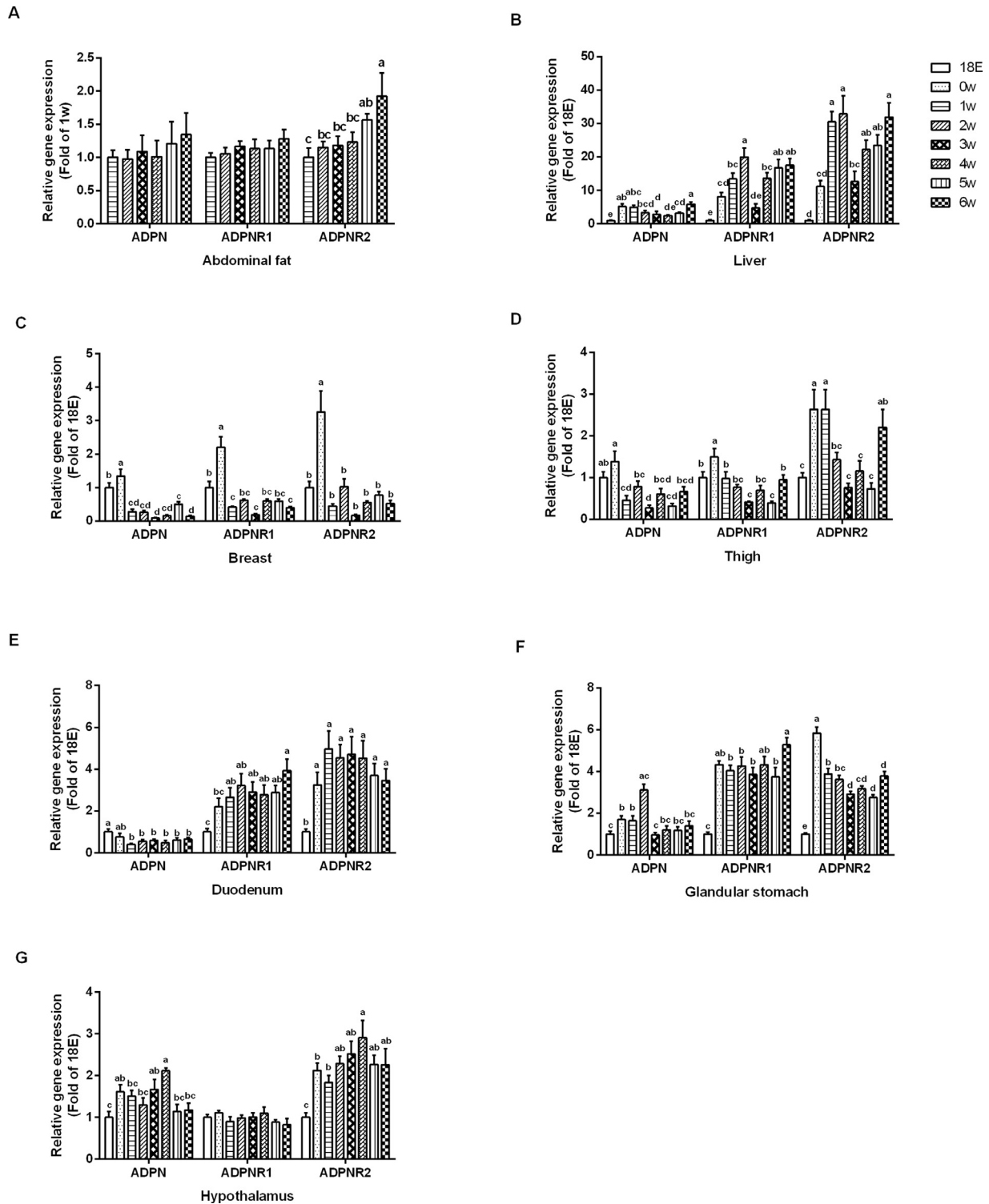
Figure 3. The mRNA expression levels of adiponectin (ADPN) and adiponectin receptors (ADPNR1 and ADPNR2) during the development of broilers. (A) Abdominal fat, (B) liver, (C) breast, (D) thigh, (E) duodenum, (F) glandular stomach, and (G) hypothalamus. Data were presented as mean ± SEM (n = 8). Different letters mean significant difference, $P < 0.05$.

The Effect of Feed Restriction on AMPK Signaling Pathway

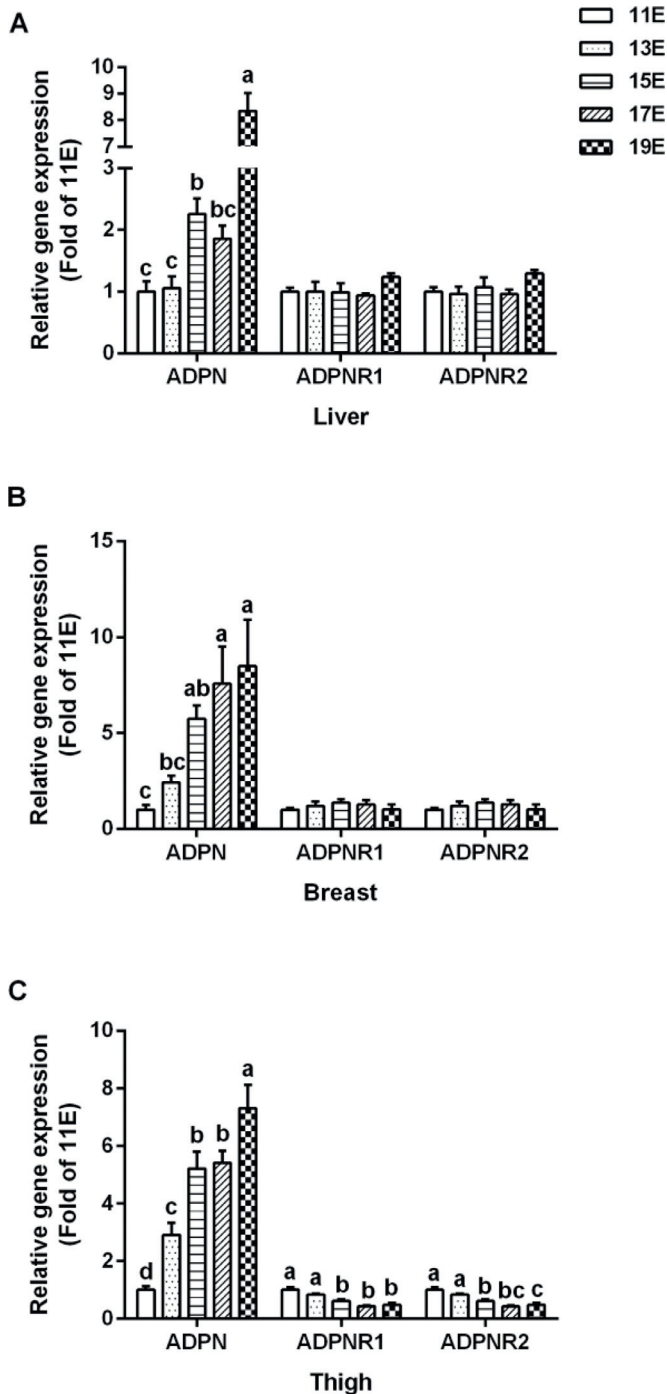As Figure 8 A shown, among the seven tissue indexes examined, only liver index decreased significantly ($P < 0.05$) caused by fasting. The duodenal index also shows a decrease, which largely due to intestinal emptying, however. Therefore, we focused on the liver and further explored the effect of feed restriction on lipid metabolism as well as the involvement AMPK pathway. We found that fasting and refeeding did not cause significant changes ($P > 0.05$) in TG concentration in the liver

Figure 4. The mRNA expression levels of adiponectin (ADPN) and adiponectin receptors (ADPNR1 and ADPNR2) during the development of broilers' embryo. (A) Liver, (B) breast, and (C) thigh. Data were presented as mean ± SEM (n = 6). Different letters mean significant difference, $P < 0.05$.

(Figure 8B), neither in the enzyme activity and protein of CPT-1 (Figures 8C and 8E). Compared with feed group, the phosphorylation level of AMPK in fasting group increased by 40.65%, but the difference was not significant ($P > 0.05$; Figure 8D). The absence of significant difference indicated that 12-h feed restriction did not cause significant changes in AMPK signaling pathways.

DISCUSSION

ADPN Secretion is Positively Correlated With Fat Deposition in Broilers
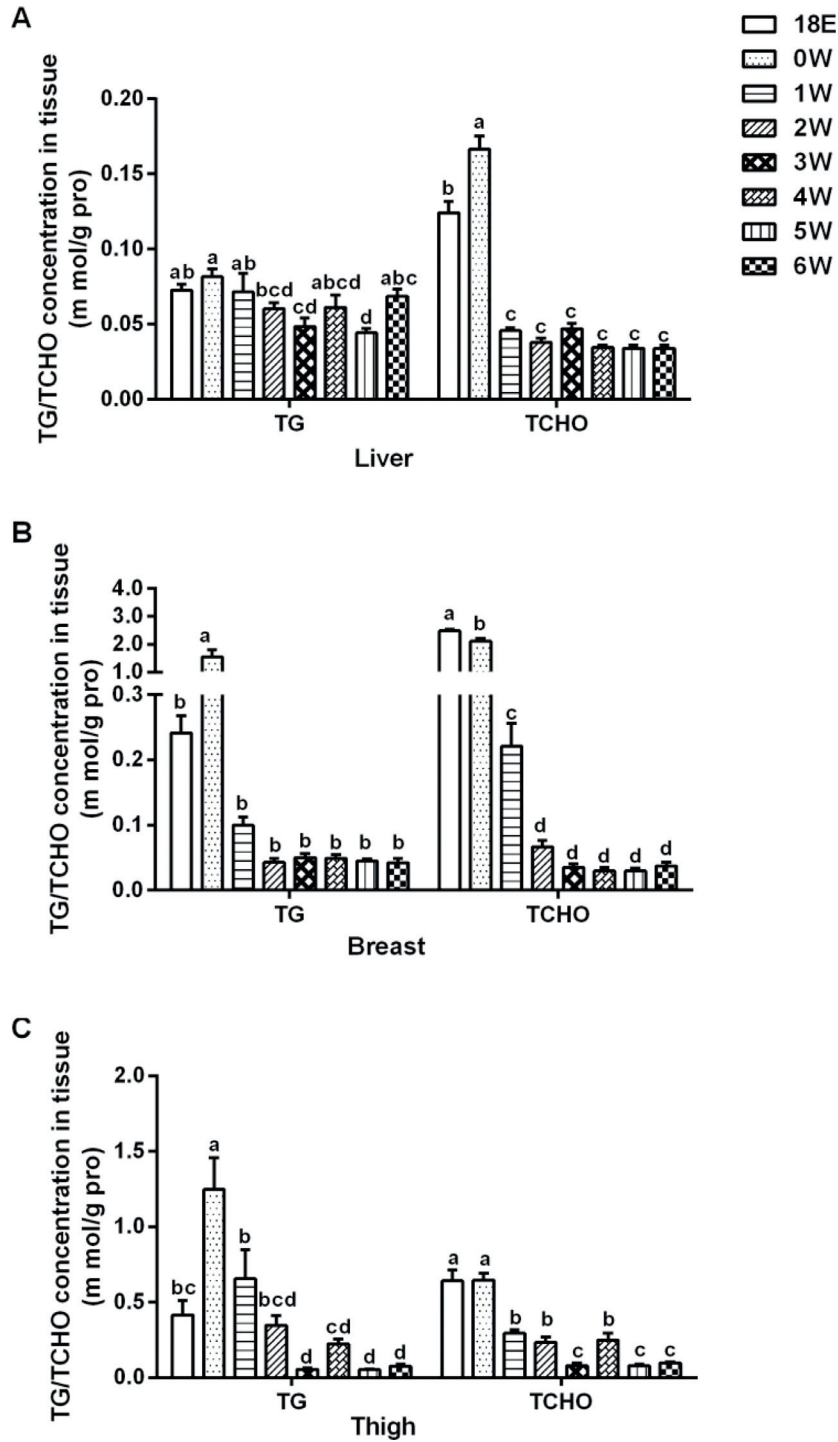
ADPN is mainly secreted from adipose tissue in mammals (Hu et al., 1996; Maeda et al., 1996; Yasuko et al., 1996). In the present study in chickens, the mRNAs level of *ADPN* and its receptors were highest in the abdominal fat, followed by the duodenum and glandular stomach, they were also expressed at the liver and skeletal muscle, as previously reported in chickens (Zhang et al., 2017) and in mammals (Sreenivasa et al., 2005). These results clearly indicated that the *ADPN* mRNA in broilers was mainly secreted by adipose tissue, acting on other tissues and playing its biological role.

It's well known that fat deposition mainly depends on the available VLDL transported TG (Hermier, 1997; Nielsen et al., 2003; Musa et al., 2006). TG is the major form of stored fat and a principal energy source for vertebrates. When the body has excess lipid content, the lipid will be transformed into TG by a series of enzymes and stored in the adipose tissue (Yang et al., 2009). The blood VLDL concentration was found to be associated with an increased hepatic lipogenesis (Hermier, 1997; Rezaei and Hajati, 2010). The balance between VLDL synthesis and VLDL secretion may be the determinant to regulate fattening in broilers. In the present study, with the growth of broilers, plasma VLDL concentration was significantly decreased and TG concentration was at an unstable state, while the plasma ADPN concentration significantly increased. In accordance with the previous reports (Li et al., 2015), linear regression analysis showed that there was a positive correlation between ADPN in plasma and abdominal fat rate from 3 to 6 wk old in broilers (r = 0.5799; $P < 0.0001$), but it was oppositely correlated with VLDL. Thus, plasma ADPN is positively related to the fat deposition of broilers and is involved in body lipid metabolism. Adipocytes are divided into preadipocytes and mature adipocytes, the preadipocytes can transform into mature adipocytes (Gregoire et al., 1998). It can be seen that the rate of abdominal fat deposition significantly increased from 1 to 6 wk of age as shown in Figure 2D, indicating that the precursor fat cells differentiate into mature adipocytes. It might be possible that the fat deposition inhibit ADPN by impairing adipocyte differentiation when it reached a certain extent, and after, *ADPN* mRNA expression and plasma ADPN concentration may decrease. Previous studies showed that *ADPN* gene may be associated with the initiation and growth processes of adipose tissue deposition in chickens (Hendricks et al., 2009; Tahmoorespur et al., 2010). But Li et al. (2015) showed that *ADPN* mRNA level was negatively correlated with abdominal fat deposition from 7 to 8 wk old. Zhang et al. (2017) showed *ADPN* mRNA was decreased in adipose tissue after 81 d in Tibetan chicken. Combined with the above results, we speculated that the secretion of ADPN might be related to age. However in this study, we didn't measure the abdominal fat after 6

A

B

C

Figure 5. The concentration of triglyceride (TG, m mol/g protein) and total cholesterol (TCHO, m mol/g protein) in tissues during the development of broiler. (A) Liver, (B) breast, and (C) thigh. Data were presented as mean $\pm$ SEM (n = 8). Different letters mean significant difference, $P < 0.05$.

wk of age, so more studies are needed to confirm this hypothesis in future. In mammals, abdominal fat rate was negatively correlated with plasma ADPN levels, possibly because they did cross-sectional studies and all experimental animals were under the same growth state (Yong et al., 2004; Vendrell et al., 2012; Mohammadpour et al., 2020). We conducted a longitudinal study on the relationship of ADPN and abdominal fat rate with growing.

ADPN Plays a More Important Role in Regulating Lipid Metabolism at the Early Stage of Broilers

In the present study, *ADPNR2* expression in abdominal fat increased with age, while in other tissues, mRNAs expressions of *ADPN* and its receptors were higher at the early stage after birth. We then examined the expressions of *ADPN* and its receptors in tissues with
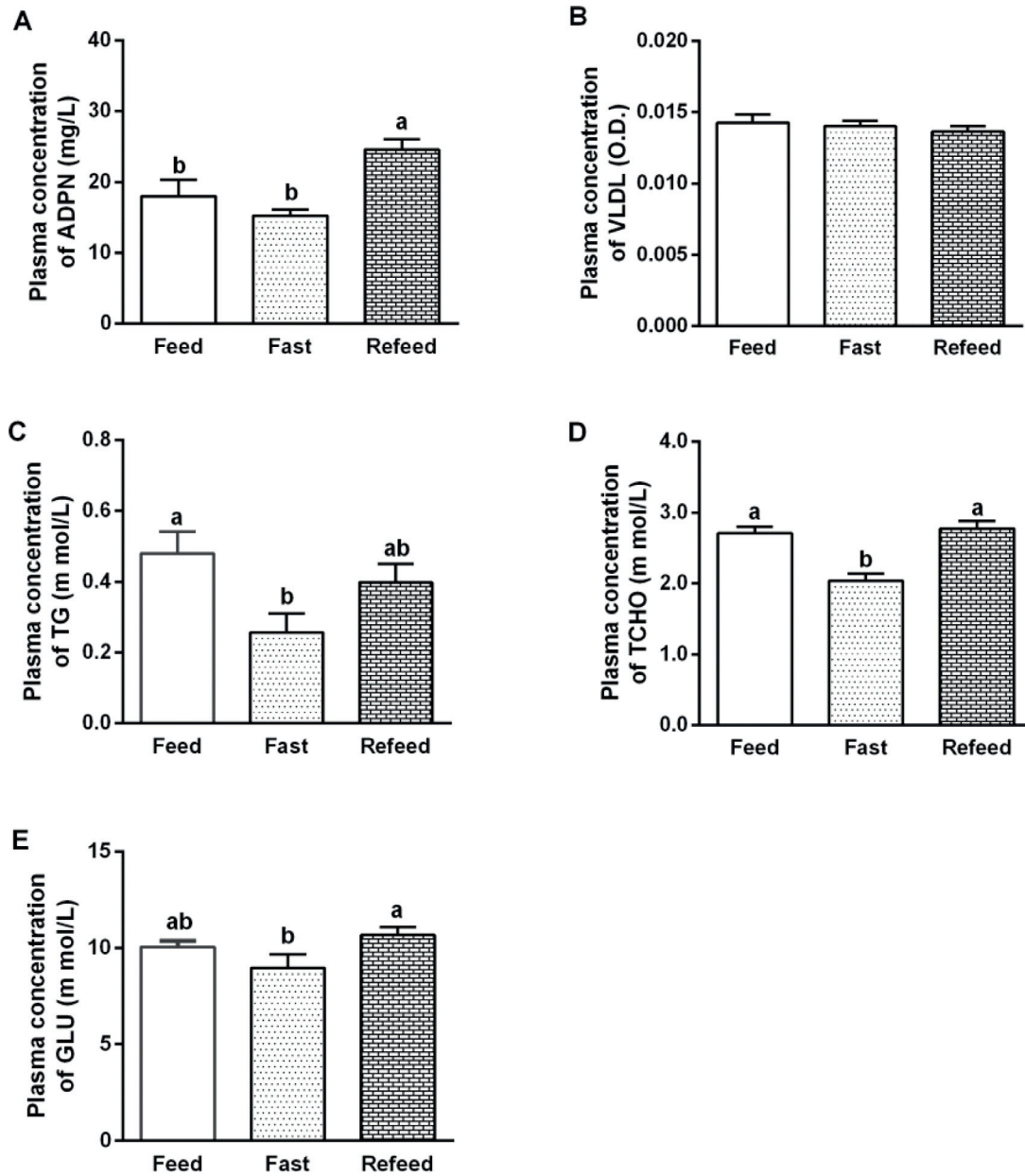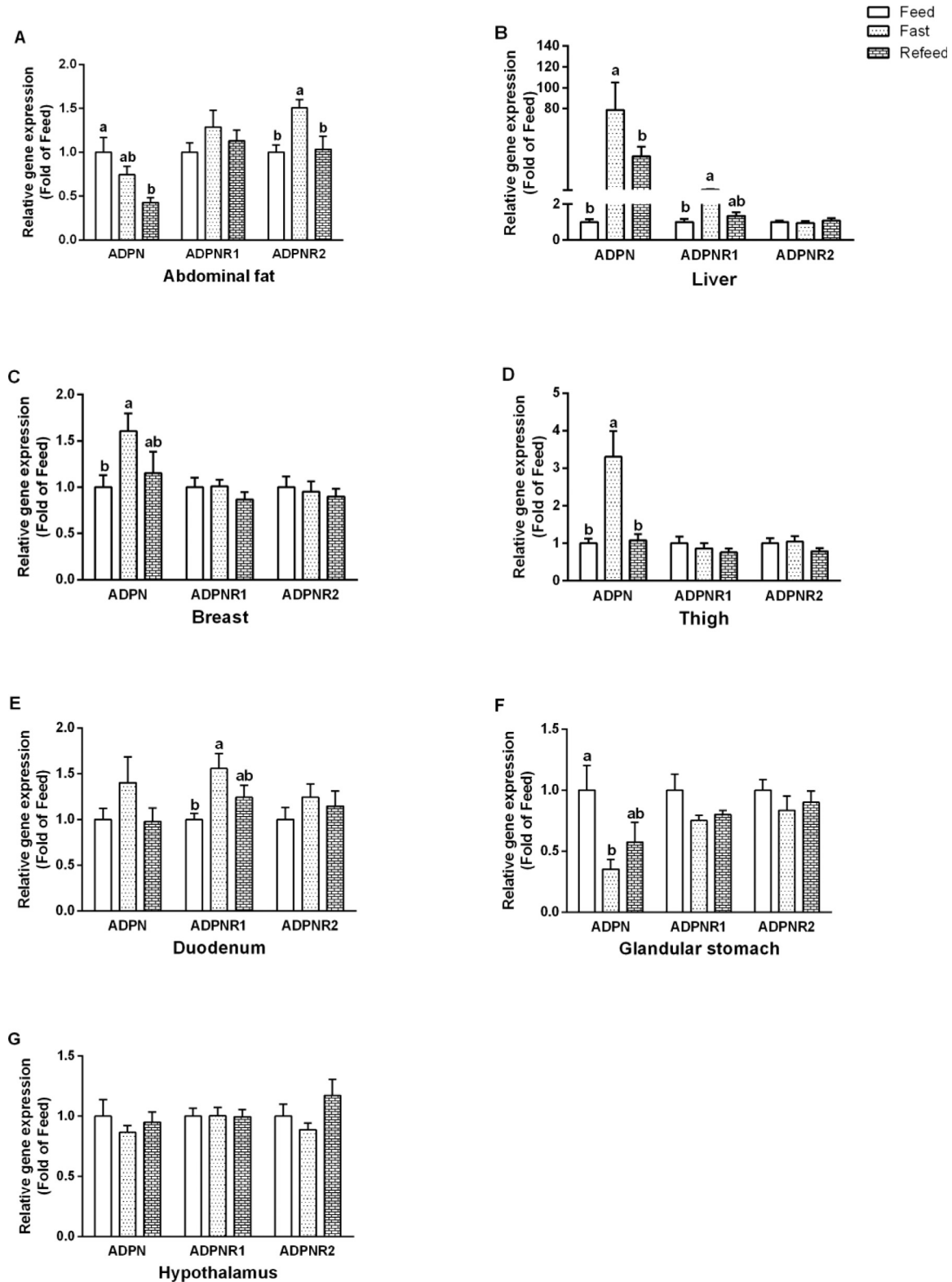
Figure 6. The effects of feed restriction on blood metabolites. The contents of (A) adiponectin (ADPN, mg/L), (B) very low density lipoprotein (VLDL, O.D.), (C) triglyceride (TG, m mol/L), (D) total cholesterol (TCHO, m mol/L), and (E) glucose (GLU, m mol/L). Data were presented as mean ± SEM (n = 8). Different letters mean significant difference, $P < 0.05$.

high fat metabolism (liver and muscles) during the embryonic period and found that *ADPN* expression was increased with the growth of the embryo. These results were in line with previous work (Li et al., 2010), which showed that intramuscular fat deposition was fastest around hatch. We thus speculate that the expressions of *ADPN* and its receptors were gradually increased during the embryonic stage; they were significantly higher at the early growth stages after hatching. During the late embryonic age and early birth age, energy mobilization, and consumption increase and fatty acid oxidation enhances gradually, so ADPN may play an important role in promoting fatty acid oxidation.

Fatty acids are mainly formed in adipose tissue in mammals; however, the main site of fatty acid synthesis is the liver in chickens (Bedu et al., 2002). In the present study, the concentrations of TG and TCHO in the liver were significantly higher around hatch, and there were no differences after 2 W. In tissues with active lipid metabolism including breast and thigh muscles, TG, and TCHO patterns were largely consistent with liver. Previous work of mammals showed that adipocytes in the skeletal muscle of cattle began to proliferate in the middle of embryonic age, and completed until the early stage of growth (Du and Dreyfus, 2010). The changes in intramuscular fat during embryonic age to early stage of growth have great influence on the deposition ability of intramuscular fat in later stage (Du and Dreyfus, 2010). Some studies have found that intramuscular fat is highest at 1 d of age in poultry ducks (Chartrin and

Figure 7. The effects of feed restriction on the mRNA expression levels of adiponectin (ADPN) and adiponectin receptors (ADPNR1 and ADPNR2) in broilers' tissues. (A) Abdominal fat, (B) liver, (C) breast, (D) thigh, (E) duodenum, (F) glandular stomach, and (G) hypothalamus. Data were presented as mean $\pm$ SEM (n = 8). Different letters mean significant difference, $P < 0.05$.

Baéza, 2007), which is in line with the present study. Also, we found that the expressions of $ADPN$ and its receptors in the liver and skeletal muscle were largely consistent with the TG and TCHO content in these tissues. The results suggested that at the early growth stage of broilers, the body has a higher energy mobilization; the liver and skeletal muscle have a greater demand for fat, which requires higher expression levels of ADPN and its receptors. ADPN can not only promote fatty acid oxidation, but also inhibit fat synthesis (Lee and
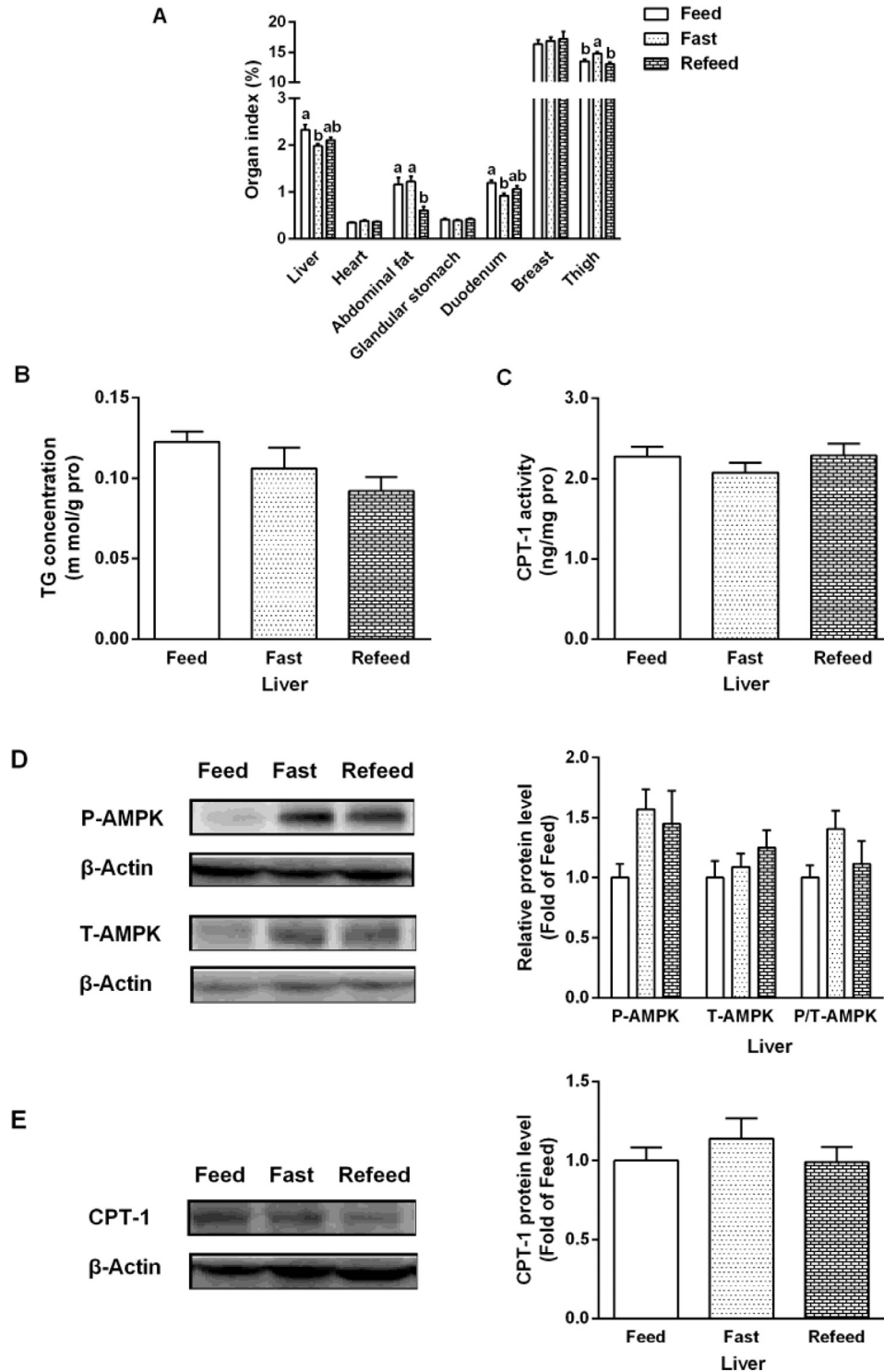
Figure 8. The effects of feed restriction on (A) organ indexes of tissues, (B) TG concentration in liver, (C) CPT-1 activity in liver, the protein levels of (D) AMPK and (E) CPT-1 in liver. Data were presented as mean ± SEM (n = 8). Different letters mean significant difference, $P < 0.05$.

Shao, 2012). Previous studies showed that during chickens' embryonic period, lipid serves as the primary energy source, whereas at hatch, the main metabolic adaptations is carbohydrate utilization over the first 2 wk posthatch (Noy and Sklan, 1999; Sklan and Noy, 2000; Zhang and Hillgartner, 2004). Thus, ANPN and its receptors will promote energy homeostasis and they will mobilize or deposit as needed.

Feed Restriction Affects ADPN mRNA Expression and Lipid Metabolism

Changing birds' diet by altering the energy levels or feeding time can modify the endocrine and blood metabolite patterns (Hornick et al., 2000). Fasting is usually employed to limit fat deposition in broilers and to improve feed conversion (Savory et al., 2006;

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Boostani et al., 2010; Rezaeipor et al., 2010). Herein, we investigated the changes in energy and lipid metabolites of birds' plasma subjected to 12-h feed restriction followed by 2-h refeeding. Results showed that VLDL concentration was not changed by feed restriction. However, the content of TG, TCHO, and GLU were decreased after fasting, and restored after refeeding, which were in accordance with the results of previous studies (Song et al., 2012; Hu et al., 2015; Song et al., 2018). This can be attributed to the acute feed restriction enhanced lipid oxidation in an attempt to meet energy needs, eliciting negative effects on the carcass fat content (Jahanpour et al., 2013). In the present study, the plasma concentration of ADPN showed no significant change after fasting for 12 h. Similar findings had also been reported in mammals, which showed that acute food deprivation for 48 h had no effect on serum concentration of ADPN in rats (Zhang et al., 2002).

Consistent with the studies in rats fasted for 24 h (Bertile and Raclot, 2004) and in laying hens fasted for 48 h (Sreenivasa et al., 2005), we observed that the *ADPN* mRNA expression was downregulated by feed restriction in the abdominal fat of broilers. However, *ADPN* in the liver and skeletal muscle, and *ADPNR1* in liver and duodenum were all upregulated after fasting, indicating that 12-h fasting resulted in energy deficiency, which caused the response of ADPN. Moreover, the above genes were restored after refeeding. In line with our findings, it has been reported that circulating ADPN was increased after 60% calorie restriction for 4 mo in mice, and the fat will be reduced to maintain the body's energy balance during calorie restriction (Berg et al., 2001). Contrasting with our findings, it has been reported that *ADPN* mRNA expression decreased after 48-h fasting in the adipose tissue, liver, and the anterior pituitary gland but not in the diencephalon (Sreenivasa et al., 2005). The decrease of *ADPN* mRNA expression in the white adipose tissue caused by short-term fasting and the increase of serum ADPN with long-term calorie restriction may be due to the duration and severity of the dietary restriction. The energy utilization during fasting is mainly divided into 3 stages, first, the depletion of carbohydrate reserves; second, mobilization of lipids; finally, breakdown of protein (Cuendet et al., 1975; Goodman et al., 1980). The 12-h fasting in the present study maybe at the second stage of energy utilization, lipids are mobilized to provide the energy needed for body, ADPN and its receptors are involved in this stage, fatty acid oxidation function was enhanced in the internal organs (liver, muscle, and duodenum) leading to increase the *ADPN* mRNA expression. In the second stage, the digestive function of the glandular stomach is low, which resulted in a decline in *ADPN* mRNA expression. After refeeding, the glandular stomach began to accelerate digestion and tissue oxidation, which may lead to increase of *ADPN* mRNA expression. It's not obvious that there is any impact of fasting on the abdominal fat *ADPN* mRNA expression, maybe because fatty acid oxidation in the second stage mainly occurs in visceral fat than abdominal fat. It is also possible that a decrease in *ADPN* mRNA expression need not necessarily mean a decrease in ADPN protein concentration in abdominal fat or other tissues. Previously, such an unparalleled gene expression and protein expression level have been reported for leptin (Ranganathan et al., 1998). Further research is needed to understand whether ADPN protein has changed in chickens by fasting and refeeding.

Among the seven tissue indexes examined in Experiment 2, only liver index decreased significantly caused by fasting. The duodenal index also shows a decrease, which largely due to intestinal emptying, however. As the main site of fatty acid synthesis in poultry, liver plays an important role in lipid metabolism and energy regulation of body (Bedu et al., 2002). Therefore, we focused on the liver and further explored the effect of feed restriction on lipid metabolism as well as the involvement AMPK pathway. The results showed that the TG concentration in liver did not change significantly with feed restriction, and although AMPK phosphorylation increased by 40.65% in the Fast group compared with Feed chickens, there was no statistical difference in protein phosphorylation of AMPK and CPT-1, or in CPT-1 activity. These results indicated that fasting did not obviously affect AMPK signaling pathway. Likewise, in others reports, fasting for 24 h and calorie restriction for 4 mo did not activate AMPK pathway in the heart, liver, and muscle in rats (Gonzalez et al., 2004). This results indicated that activation of AMPK is not a necessary pathway for the liver to respond to the complex metabolic caused by 12-h fasting. The most likely explanation for this result is the reduction of GLU, TG, TCHO induced by 12-h fasting was not sufficient to cause a decrease in intracellular ATP/AMP ratios and initiate an increase in AMPK activity.

In conclusion, ADPN is closely related to lipid metabolism and energy regulation of broilers. Twelve-hour feed restriction causes energy deficiency in the body, leading to the upregulation of *ADPN* and its receptors expression in liver, muscle, and gut. AMPK is not involved in this short-time feed restriction.

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DISCLOSURES

The authors have declared no conflicts of interest.

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