



Variations in Tissue-Specific Expression of Adipose Differentiation-Related Protein Gene in Two Native Yunnan Chicken Breeds

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ABSTRACT

Adipose differentiation-related protein (ADFP) is a fatty acid-binding protein that can promote the absorption of long-chain fatty acids. However, few results have been published regarding its role in Yunnan Native chicken breeds. The aim of this study was to determine ADFP gene tissue-specific expression in Piao chickens (PC) and Wuliangshan black-bone chickens (WBC) by RT-qPCR. The ontogenetic expression levels of the ADFP gene were significantly different during growth and development phases in the subcutaneous fat, liver, and pectoralis muscle of PC, and in the subcutaneous fat, liver, and pectoralis muscle of WBC ($p < 0.05$). Individual tissue-differential expression levels were detected on d 91 and 112 in PC, with highest levels determined in abdominal fat and subcutaneous fat, respectively. However, in WBC, the highest levels were determined on d 49, 91 and 112 d in the pectoralis muscle and liver. Correlation analysis revealed ADFP expression level in liver of WBC was significantly related with LW and HC ($p < 0.05$), while no significant correlations with carcass fatness (CF) were found in PC ($p > 0.05$). The results suggest ADFP differential expression in the liver and pectoral muscles of PC and WBC during the growth and development phases ($p < 0.05$). The observed expression patterns indicate that the ADFP gene plays an important role in lipid metabolism of PC and WBC, and that these patterns are expressed differently in the tissues of different chicken genotypes.

INTRODUCTION

Fat content and distribution in the carcass are not only economically important traits in livestock, but also key factors affecting meat color, tenderness, taste, and other meat quality traits (Chen *et al.*, 2008; Mossab *et al.*, 2002). Therefore, it is widely accepted that carcass fatness (CF) is one of the most important carcass traits that determine the meat quality of chickens (Jiang *et al.*, 2000). According to Castellini *et al.* (2002), intramuscular fat (IMF) content influences meat tenderness and taste. Carcasses with appropriate fat content may obtain higher chicken broiler market prices. On the other hand, because fat deposition per unit requires three times more energy than lean meat accretion per unit, excessive fat deposition reduces feed utilization (Boekholt *et al.*, 1994). In addition, obesity is also associated with fatty-liver syndrome, increased mortality, and reduced egg production and fertility in laying chickens (Lee *et al.*, 1975). Consequently, it is important to study the genetic mechanism of fat synthesis and regulation in chickens.

Adipose differentiation-related protein (ADFP) is a fatty acid-binding protein that belongs to the perilipin-adipophilin-TIP47 protein family (Magra *et al.*, 2006) and can promote the absorption of long-chain fatty acids (Tobin *et al.*, 2006). The mRNA sequence of ADFP was isolated for the first time from the cDNA library of mouse adipocytes by differential



hybridization screening technique and it is shown to be expressed in almost all types of mammalian cells (Jiang & Serrero, 1992; Buechler *et al.*, 2001). It is reported that ADFP plays an important role in the process of lipid aggregation and nucleation to form new lipid droplets (Wang *et al.*, 2003). Chang *et al.* (2006) observed that the developmental expression of ADFP mRNA in mouse lung tissue was accompanied by the deposition of triglycerides in the lung tissue, and that triglyceride storage in the liver was reduced by 60% after the ADFP gene was inactivated. These results indicate that the ADFP gene is an important marker of lipid deposition in the adipocytes, and may be used as a potential candidate gene influencing fat deposition in livestock.

Although many research studies have been devoted to the regulation of the ADFP gene in mammals, to the best of our knowledge, no studies on the role of the ADFP gene in Yunnan Native chicken breeds have been published, except for the research of Decai *et al.* (2017). The objective of the present study was to detect the tissue-specific expression of the ADFP gene in Piao chickens (PC) and Wuliangshan black-bone chickens (WBC) in order elucidate its role in fat deposition in two Yunnan native chicken breeds.

MATERIALS AND METHODS

Experimental birds and sample collection

All experimental procedures were reviewed and approved by the Yunnan Animal Science and Veterinary Institute Ethics Committee.

Piao chickens (PC) and Wuliangshan black-bone chickens (WBC) are native breeds of the Yunnan province, China. Piao chickens are tailless and have muscular thighs, and are known for having low bone and high meat content, in addition of sweet and tender meat. Wuliangshan black-bone chickens have the “one green and three black” characteristics (green ear, and black skin, meat and bones) and are known for their tolerance to roughage, fast growth rate, high medicinal value and delicious meat. They are reared for meat and egg production.

Chickens were obtained from a farm located in Pu'er city, Yunnan Province, China. All birds had access to water and feed *ad libitum*. Feed was formulated to supply the National Research Council (NRC) requirements for broilers (Nick & Dale, 1994).

On five different days of the grow-out period, at days 28, 49, 70, 91, and 112 days of age, 12 individuals of each breed (six males and six females)

were slaughtered, and the pectoralis muscle, liver, abdominal fat and subcutaneous fat were collected according to the sampling method of Decai *et al.* (2017) and stored in liquid nitrogen at 80°C. Live weight (LW), backfat thickness (BFT) (on the back midline and anterior to the uropygial gland) and comb weight (CW) were determined (Table 1).

Total RNA extraction and cDNA synthesis

Total RNA was directly extracted from above tissues according to the manufacturer's instructions using TRIZOL Reagent kit (Tian'gen, Beijing, China) and its concentration (400 ng/μL) and purity (OD260/OD280=1.8 ~ 2.0) were detected using the Ultra Micro Nucleic Acid Protein Analyzer (AJ, Germany). Then, first strand of cDNA was synthesized using the protocol specified by the FastQuant cDNA first strand synthesis kit (Tian'gen, Beijing, China) and was subsequently used for quantitative real-time polymerase-chain reaction PCR (RT-qPCR) analysis.

Primer design

In this study, for the evaluation of relative ADFP gene expression, the β-actin gene was used as internal control. The primers used for ADFP mRNA (GenBank accession: NM_001031420.1) and β-actin mRNA (GenBank accession: NM_205518.1) amplification sequences are reported elsewhere (Decai *et al.*, 2017). All primer sequences were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China)

Real-time quantitative PCR (RT-qPCR) system and conditions

The optimal reaction system and conditions were determined by experimentation. The SuperRealPreMix Plus kit (Tian'gen, Beijing, China) was used to prepare the qPCR reaction system (20 μL) according to the manufacturer's instructions, including 2 x 10 μL SuperRealPreMix Plus, 0.6 μL (10 μmol/L) of forward and reverse primers, respectively, 1 μL (about 100 ng) cDNA, 50 x Δ 0.4 μL ROX Reference Dye, and 7.4 μL ddH₂O. Each sample was tested in duplicate at least thrice. Then, the qPCR reaction system was performed in a Real-Time PCR System (Applied Biosystems 7500, Thermo Fisher Scientific, Germany) with the following program: 1 cycle at 95°C for 30 s, followed by 40 cycles at 95°C for 3 s, 60°C for 30 s, and at 72°C for 20 s. The melting curve was built according to the default condition of the system.

Statistical analysis

The relative expression levels of ADFP gene in the above tissues of chickens were calculated



Table 1 – Partial fatness trait (CF) indicators of two Yunnan Native chicken breeds.

Breeds	Growth points	Gender	LW(g)	HC(g)	BFT(mm)
PC	28d	Male	336.00±33.94	0.43±0.09	2.17±0.27
		Female	296.00±20.01	0.18±0.01	1.19±0.06
	49d	Male	671.50±57.28	0.96±0.01	3.36±0.03
		Female	483.50±6.36	0.16±0.03	2.81±0.06
	70d	Male	675.00±84.85	0.44±0.06	2.83±0.04
		Female	525.00±8.49	0.13±0.07	1.77±0.09
	91d	Male	1246.00±8.49	2.82±0.96	3.59±0.12
		Female	918.50±31.22	0.26±0.04	2.20±0.11
	112d	Male	1537.00±50.91	5.61±1.80	3.01±0.14
		Female	1332.5±70.78	0.32±0.09	4.93±1.50
WBC	28d	Male	179.50±7.78	0.11±0.01	0.95±0.33
		Female	156.00±22.63	0.05±0.01	0.72±0.31
	49d	Male	491.50±21.92	0.53±0.00	2.32±0.88
		Female	308.50±6.36	0.07±0.01	1.13±0.01
	70d	Male	568.00±79.20	0.48±0.04	0.95±0.35
		Female	569.00±52.74	0.11±0.02	1.45±0.04
	91d	Male	787.00±9.90	0.95±0.21	3.69±0.55
		Female	1010.00±41.01	0.16±0.00	3.55±0.24
	112d	Male	1088.00±35.35	1.41±0.52	5.10±0.57
		Female	1133.50±92.84	0.20±0.08	6.44±0.71

Note: PC) Piao chickens, WBC) Wuliangshan black-bone chickens, LW) live weight, HC) heavy comb, BFT) backfat thickness.

using the $2^{-\Delta\Delta Ct}$ method (Livak *et al.*, 2001). And the data were presented as means \pm SD. Ontogenetic expression and tissue-differential expression of the ADFP gene were analyzed with Duncan's test of SPSS 18 software in two Yunnan Native chicken breeds. Correlation analysis between ADFP mRNA expression levels and CF were calculated using the Pearson method according to Decai *et al.* (2017). *P*-values lower than 0.05 were considered significant.

RESULTS

Amplification plots and melting curves of the ADFP gene and internal control gene

The total RNA concentration and the OD260/OD280 values of all samples tested were approximately 400ng/ μ L and 1.8-2.0, respectively, which were available for the subsequent experimental. In addition, the results show that the efficiency and individual repeatability of the amplification plots (Figure 1) of all genes were

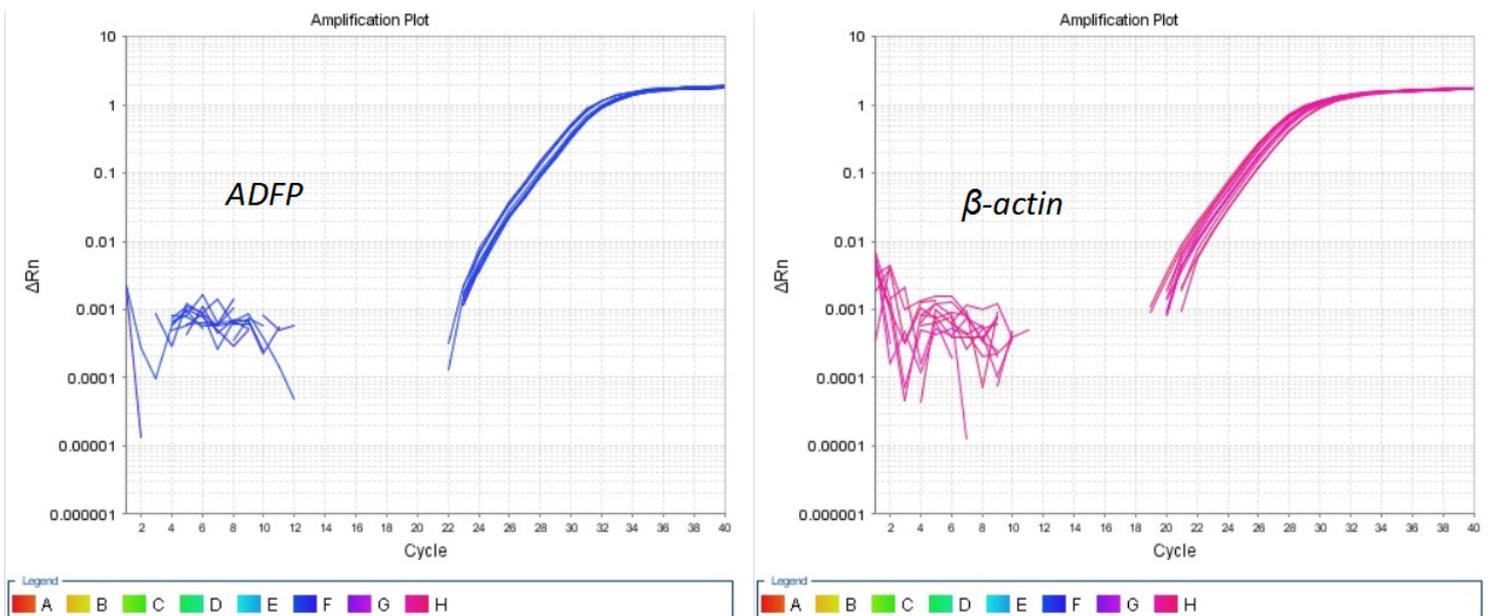


Figure 1 – Amplification plots of the ADFP and β -actin genes.



very good. No primer dimer or nonspecific peaks were found in any genes according to the melting curves (Figure 2), showing that the amplified products were

the specific products needed for this study, and the Ct values generated were reliable and could be used for statistical analysis.

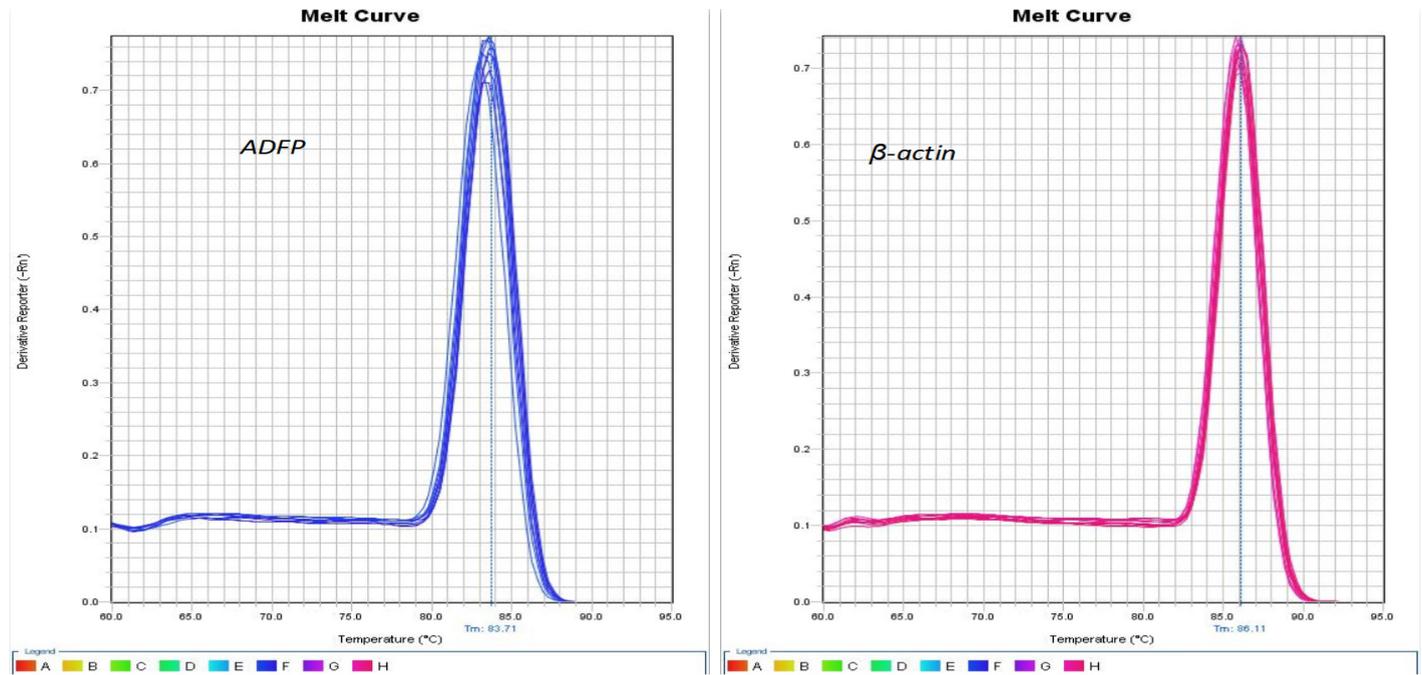


Figure 2 – Melting curves of the ADFP and β -actin genes.

ADFP temporal expression during chicken development

We used the Ct value obtained on d 28 in each tissue as the control to compute the ADFP ontogenetic expression level in PC and WBC at different points.

In PC birds, the ADFP expression level in subcutaneous fat was higher on d 70 d than on d 28, 49, and 112 ($p < 0.05$); not significantly different ($p > 0.05$) in abdominal fat among each growth points; higher in liver on d 70 and 91 compared with d 28, 49, and 112 ($p < 0.05$); and higher in the pectoralis muscle on d 70 than on d 28 and 49 ($p < 0.05$) (Figure 3A). Therefore, in PC birds, ADFP expression levels in the subcutaneous fat, liver, and pectoralis muscle were higher on d 70 and then declined on d 112.

In WBC birds, ADFP expression peaked on d 91, 112d, and 28 in the abdominal fat, liver, and pectoralis muscle, respectively (Figure 3B). ADFP expression levels in SF were not significantly different among growth points ($p > 0.05$), but were significantly higher ($p < 0.05$) in the abdominal fat on d 70, 91, and 112 compared with d 28, in the liver on d 112 compared with d 28, 49, 70, and 91, and in pectoralis muscle on d 28 compared with d 49, 70, and 112 ($p < 0.05$).

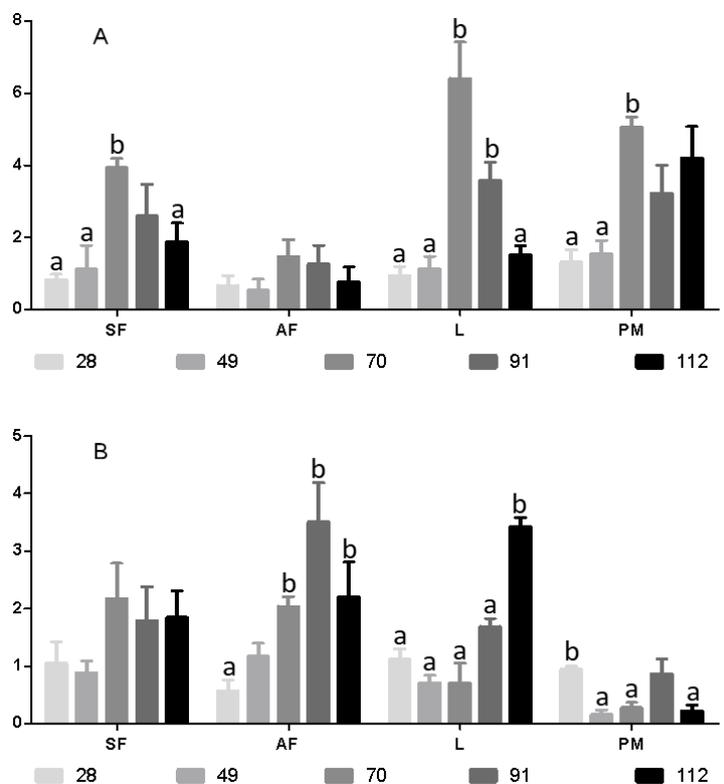


Figure 3 – ADFP temporal expression during chicken development phase. At each time point, Ct value was used as the control to compare ADFP expression level differences in different tissues of PC and WBC. Lower case letters indicate statistical significance ($p < 0.05$). PC: Piao chickens, WBC: Wuliangshan black-bone chickens, SF: subcutaneous fat, AF: abdominal fat, L: liver, PM: pectoralis muscle.



ADFP temporal expression in different chicken tissues

Figure 4 summarizes the ADFP tissue-differential expression among several tissues in PC and WBC. At each time point, the Ct value of subcutaneous fat was used as the control to compare the ADFP expression level differences in different tissues of PC and WBC.

In PC, ADFP expression levels were higher in abdominal fat than that in subcutaneous fat and liver on d 91 ($p < 0.05$), whereas on d 112, higher levels were determined in subcutaneous fat than that in abdominal fat and liver ($p < 0.05$). No significant differences in ADFP expression levels were detected among tissues on d 28, 49, or 70 ($p > 0.05$) (Figure 4A).

In WBC, higher ADFP expression level was determined in the pectoralis muscle compared with subcutaneous fat on d 49 ($p < 0.05$), as well as in the liver compared with subcutaneous fat on d 91 and 112 ($p < 0.05$). No significant ADFP expression level differences among tissues were determined on d 28 and 70 ($p > 0.05$) (Figure 4B). The results showed that the ADFP tissue-differential expression was not observed until d 91 in PC, while it was observed already on d 49 in WBC.

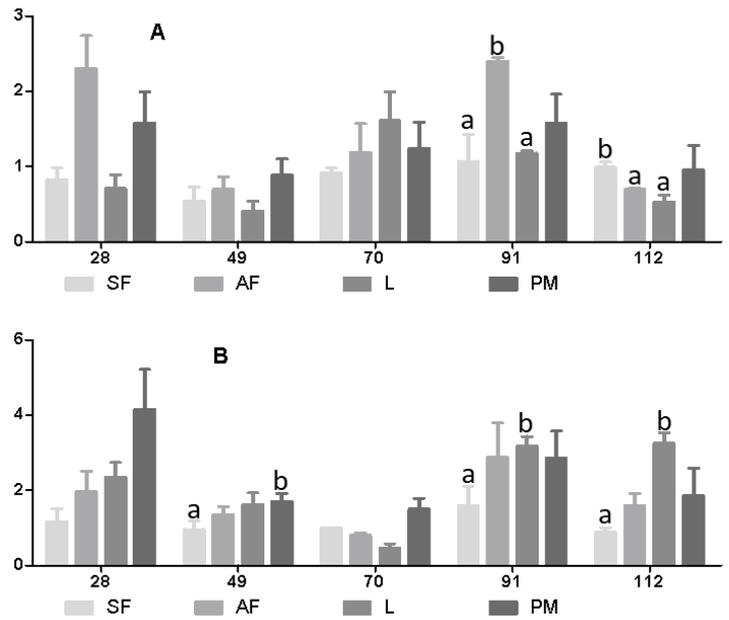


Figure 4 – ADFP temporal expression in different chicken tissues. At each time point, SF Ct value was used as the control to compare ADFP expression level differences in different tissues of PC and WBC. Lower case letters indicate statistical significance ($p < 0.05$). PC: Piao chickens, WBC: Wuliangshan black-bone chickens, SF: subcutaneous fat, AF: abdominal fat, L: liver, PM: pectoralis muscle.

Correlation analysis between ADFP gene with CF

The results of the correlation analysis of ADFP gene expression in different tissues with CF in PC and WBC are shown in Table 2. Although there was a weak

Table 2 – Correlation coefficients of the ADFP gene expression in different tissues with carcass fatness (CF) in PC and WBC.

Breeds	Traits	SF	AF	L	PM
PC	LW	0.212	0.130	-0.103	0.002
	HC	0.009	-0.174	-0.184	0.227
	BFT	-0.013	-0.092	-0.349	-0.193
WBC	LW	0.342	0.298	0.606**	-0.077
	HC	0.286	0.190	0.551*	-0.137
	BFT	0.273	0.396	0.421	0.034

Note: * $p < 0.05$; ** $p < 0.01$. PC) Piao chickens, WBC) Wuliangshan black-bone chickens, SF) subcutaneous fat, AF) abdominal fat, L) liver, PM) pectoralis muscle, LW) live weight, HC) heavy comb, BFT) sebum thickness.

correlation between the ADFP gene expression level in different tissues and CF in PC ($|R| > 0$), there was no significant difference between them ($p > 0.05$). However, significant positive correlations of ADFP gene expression level in the liver with LW and HC were obtained in WBC ($p < 0.05$). ADFP gene expression level in the liver was negatively correlated with CF in PC, and positively correlated with CF in WBC. The correlation between the ADFP gene expression level in pectoralis muscle and CF in PC was opposite to that found in WBC.

Tissue-differential expression of the ADFP gene between PC and WBC

In order to clarify the roles of the ADFP gene in fat development, we further analyzed its tissue-differential expression between PC and WBC. In each tissue, the Ct values determined on d 28 in PC were used as controls to compare the ADFP tissue-differential expression at different time points between PC and WBC. The results showed that the ADFP gene expression levels in the subcutaneous fat and abdominal fat were no



significantly different at each time point between PC and WBC ($p < 0.05$) (Figure 5). However, lower liver levels were obtained in PC than in WBC on d 28, 49, and 112 ($p < 0.05$), but higher in PC on d 70 ($p < 0.05$). In addition, lower ADFP gene expression levels were measured in the pectoralis muscle of PC compared with WBC on d 28 and 91 ($p < 0.05$).

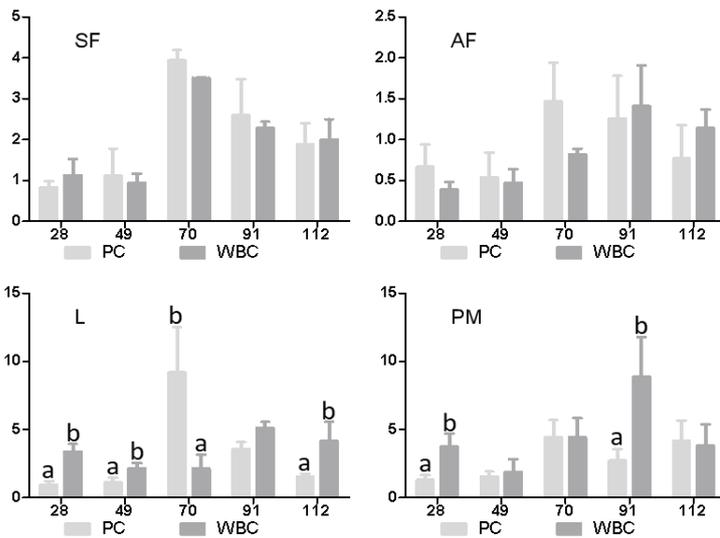


Figure 5 – ADFP gene tissue-differential expression between PC and WBC. In each tissue, the Ct value on d 28 in PC was used as the control to compare the ADFP tissue-differential expression at different time points between PC and WBC. Lower case letters indicate statistical significance ($p < 0.05$). PC: Piao chickens, WBC:Wuliangshan black-bone chickens, SF: subcutaneous fat, AF: abdominal fat, L: liver, PM: pectoralis muscle.

DISCUSSION

Several studies have shown that ADFP is involved in the lipid metabolism of macrophages (Larigauderie *et al.*, 2006), stimulating lipid accumulation and lipid droplet formation in fibroblasts (Imamura *et al.*, 2002), transfer of lipids between lipofibroblasts and EPII cells, and maintenance of triglyceride reserves (Chang *et al.*, 2006; Schultz *et al.*, 2002) through physiological processes. Further, previous studies found that the ADFP gene was associated not only with the production traits of dairy goats (Li *et al.*, 2014), but also with carotid atherosclerosis (Nuotio *et al.*, 2007), colorectal cancer (Matsubara *et al.*, 2011) and other prevalent human diseases. The reason may be that ADFP, as a phospholipid protein covered by lipid droplets, may promote the absorption and storage of fatty acids and regulate the synthesis and decomposition of lipids (Imamura *et al.*, 2002; Robenek *et al.*, 2006). Consequently, these findings suggest that the ADFP gene may influence fat deposition in livestock.

The results of the present study showed that the ADFP gene is expressed in the abdominal fat, subcutaneous fat, liver and pectoralis muscle of both PC and WBC,

which is consistent with previous research (Decai *et al.*, 2017; Brasaemle *et al.*, 1997; Zhao *et al.*, 2010). In addition, ADFP gene expression level peaked in PC on d 70 in the liver, subcutaneous fat and pectoralis muscle, whereas in WBC, the highest ADFP expression levels in abdominal fat, liver, and pectoralis muscle were determined on d 91, 112, and 28, respectively. These results show that, compared with Sichuan Mountainous Black-bone chicken (Zhao *et al.*, 2010) and Daweishan Mini chicken (Decai *et al.*, 2017), liver lipid synthesis, fatty-acid absorption and storage in the subcutaneous fat and the pectoralis muscle of PC were relatively faster, with peak on d 70, whereas in WBC, peaks were observed on d 112, 91, and 28 in the liver, abdominal fat, and pectoralis muscle, respectively. This indicates that PC are most likely to gain fat at 10 weeks of age, while WBC are more likely to gain fat at 4, 13 and 16 weeks of age, respectively.

Previous studies reported that ADFP gene is expressed in muscle, lung, liver, kidney, brain and other tissues of adult rats, with highest expression in the tissue with the most neutral lipid (Yan *et al.*, 2006). In the present study, ADFP gene expression level of PC in the abdominal fat on d 91 was higher than that in the liver and subcutaneous fat ($p < 0.05$), and higher in the subcutaneous fat on d 112 than that in the abdominal fat and liver ($p < 0.05$). However, in WBC, higher ADFP gene expression level was found in the pectoralis muscle compared with subcutaneous fat on 49 d ($p < 0.05$), and in the liver compared with subcutaneous fat on d 91 and 112 ($p < 0.05$). These results suggest that, in PC, on d 91 and 112 d, lipids were mainly absorbed and stored in abdominal fat and subcutaneous fat, respectively, which may be one of the reasons why PC has more tasty meat after 112 d. However, the ADFP gene expression in adipocytes of WBC was the same as that in Daweishan Mini chicken, i.e., its level in pectoralis muscle was, in general, higher than that in adipose tissue during the period of fast muscle growth (d 49 to 70) (Decai *et al.*, 2017). In addition, lipid synthesis in WBC between d 91 and 112 occurred in the liver, which is the main tissue of poultry triglyceride synthesis (Hermier, 1997).

The correlation analysis showed that ADFP gene expression levels in different tissues of PC birds were not significantly correlated with CF, while its level in liver of WBC was significantly related to LW and HC. Further analysis showed ADFP gene expression in the liver and pectoral muscles of PC and WBC birds were different during the growth and development process, demonstrating the tissue-differential expression of the ADFP gene between PC and WBC.



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