

## Effects of sex and age on chicken *TBC1D1* gene mRNA expression

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**ABSTRACT.** The objective of this study was to investigate the effects of sex and slaughter age of chickens on fatty acid composition and *TBC1D1* gene expression in 4 different tissues: breast muscle, thigh muscle, abdominal fat, and subcutaneous fat. Sixty Erlang mountainous chickens (hybrid SD02 x SD03) were raised under the same conditions and slaughtered at 8, 10, and 13 weeks of age. The results showed that the sex of the animal significantly affected the content of arachidic acid (C20:0), sinapic (C22:1), linoleic (C18:2n-6), eicosapentaenoic (C20:5n-3), and docosahexaenoic acids (C22:6n-3), whereas other fatty acid contents were not affected. Age had a significant effect on most monounsaturated fatty acids, except for octadecenoic acid (C18:1). *TBC1D1* mRNA was abundant in all tissues at all 3 ages of slaughter. Cocks exhibited higher *TBC1D1*

mRNA levels than hens in the thigh muscle and abdominal fat at 10 and 13 weeks, respectively.

**Key words:** Age; Chicken; Fatty acids; Sex; TBC1D1

## INTRODUCTION

In recent years, the composition of fat and fatty acid (FA) in meat has been widely studied because whether in adipose tissue or muscle, fat plays an important role in several aspects of meat quality (Wood et al., 2008; Webb and O'Neill, 2008) and in human health. In developed countries, the high content of saturated FA (SFA) in the diet has been correlated with various cancers, and particularly with coronary heart disease (Pascual et al., 2007). In contrast, other FA such as monounsaturated (MUFA) and polyunsaturated FA have been shown to be beneficial to human health (Luciano, 2009). Previous studies have shown that several factors, such as breed, genotype, age, sex, diet, and anatomical location, can influence the fatty acid composition of meat (Lawrie and Ledward, 2006; Wood et al., 2008).

In a study of the effects of sex and age on meat FA composition, Kazala et al. (1999) found that the MUFA/SFA ratio in heifers was slightly higher than that in steers, but no significant difference was found in linoleic acid content. Similarly, Warnants et al. (1999) reported no difference in phosphatide FA composition in muscle between primiparous sows and emasculated boars, while total lipids and triglyceride polyunsaturated FA contents in primiparous sows were higher than in emasculated boars. Bartoň et al. (2011) found that the total amount of FA in muscle increased markedly in older animals, with higher contents of monounsaturated FA in heifers than in bulls. However, these studies were limited to pigs and cattle; additional studies are needed to examine other meat animals and birds.

TBC1D1 is not only a Rab-GTPase-activating related protein, but also an obesity candidate gene regulating glucose transporter type 4 translocation in adipocytes (Sakamoto and Holman, 2008; Park et al., 2011). Some researchers reported that the variations in TBC1D1 in mammals were likely related to physiological variables such as insulin resistance, adiposity, obesity, and type 2 diabetes (Fontanesi and Bertolini, 2013). It has also been reported that a missense mutation (R125W) in *TBC1D1* was associated with obesity in women (Stone et al., 2006). Furthermore, TBC1D1 is expressed preferentially in tissues that are important depots for both glucose and FA. However, little is known about the effect of sex on *TBC1D1* mRNA in different chicken tissues.

Therefore, the aim of this study was to determine the FA composition of breast muscle in Erlang mountainous chickens under similar management conditions and slaughtered at different developmental stages at 8, 10, and 13 weeks of age. Moreover, we investigated the expression profile of the *TBC1D1* gene during ontogenesis in the breast muscle, thigh muscle, and subcutaneous adipose tissue of Erlang mountainous chickens. This information is essential for understanding the molecular mechanism of the regulation of FA metabolism.

## MATERIAL AND METHODS

### Animals, rearing conditions, and diets

The Erlang mountainous chicken of the hybrid breed SD02 x SD03 was selected as the experimental bird. The Erlang mountainous chicken is indigenous to Tianquan in Sichuan Prov-

ince, with good performance traits and fleshy characteristics. Sixty (female:male = 1:1) SD02 x SD03 chickens were wing-banded at 1 day old and reared under the same conditions in conventional cages (1.9 x 1.0 x 0.7 m; 0.21 m<sup>2</sup> per bird). All cages were placed in a house equipped with windows and thus had natural lighting. Birds were given *ad libitum* access to water and an identical diet consisting of maize, wheat, soybean meal, and a vitamin/mineral mixture. The chickens were slaughtered at 3 ages (8, 10, and 13 weeks) for both sexes. At weeks 8, 10, and 13, 20 chickens were weighed and slaughtered after overnight fasting. Chickens were killed by cutting the carotid artery and then bled immediately. The carcasses were scalded, defeathered, and eviscerated according to standard procedures. The major muscles were collected from the left side of the breast muscle and frozen at -20°C until FA analyses were performed. All procedures were conducted according to the guidelines of the Ethics Committee of Sichuan Agricultural University for the protection of animals used for experimental and other scientific purposes.

### FA analysis

The FA content in the breast muscle was determined using duplicate samples from a 0.5-1 g meat sample using 2 mol chloroform-methanol (2:1, v/v), following the extraction method of Folch et al. (1957), with some modifications. The lipids were methylated in a solution of diazomethane in diethyl ether, and then the methylated lipid samples were analyzed using a gas chromatograph (SP6890, Beijing Jingke Ruida Science and Technology, Ltd., Beijing, China) equipped with a flame ionization detector and a J&W DB-23 capillary column (65 m x 250 µm with a film thickness of 0.25 µm) (Agilent Technologies, Santa Clara, CA, USA) with helium as the carrier gas at 30 cm/s. The temperature of the injector and detector was 250°C and the initial temperature of the oven was 180°C for 10 min, increasing to 200°C at a rate of 4°C/min over 5 min, and then finally increasing to 230°C at a rate of 10°C/min over 3 min. FA were quantified using the internal standard (C19:0) after adjusting for the response as determined using Sigma-Aldrich standard mixtures (St. Louis, MO, USA). Proportions of different FA were obtained from individual FA percentages.

### RNA isolation and reverse transcription

Total RNA was extracted from the breast muscle, thigh muscle, abdominal fat, and subcutaneous fat of the chicken at different ages (8, 10, and 13 weeks) using Trizol reagent (Takara, Shiga, Japan) according to manufacturer instructions. The concentration and purity of RNA were measured using a Nucleic Acid Protein Analyzer (DU800; Beckman Coulter, Brea, CA, USA).

Reverse transcription was carried out using a total volume of 10 µL containing 1 µg total RNA, 2 µL 5X reverse transcription buffer, 0.5 µL reverse transcription enzyme mix, 0.5 µL primer mix, and 6 µL nuclease-free water using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Reverse transcription was maintained at 37°C for 15 min and ended with incubation at 98°C for 5 min.

### Gene expression quantification

Real-time polymerase chain reaction (PCR) was performed with the Bio-Rad CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The gene expression level was

detected for *TBC1D1*, while  $\beta$ -actin was used as an internal reference gene. Primer sequences for *TBC1D1* and  $\beta$ -actin were designed using Primer 5.0 (Premier Biosoft, Palo Alto, CA, USA) and Oligo 6.0 (Molecular Biology Insights, Inc. (Colorado Springs, CO, USA) softwares and were synthesized by Takara. The *TBC1D1* sequence (XM\_423827) was as follows: forward, 5'-TCTG CCGTGCGATGTAAG-3'; reverse, 5'-AAAGAATGGTGCCCTAAT-3';  $\beta$ -actin sequence (AF047874) was as follows: forward, 5'-GAGAAATTGTGCGTGACATCA-3'; reverse, 5'-CCT GAACCTCTCATTGCCA-3'. The PCR was performed in triplicate in a total volume of 10  $\mu$ L, containing 5  $\mu$ L SsoFast EvaGreen Supermix (Bio-Rad), 1  $\mu$ L 50 ng/ $\mu$ L cDNA, 0.8  $\mu$ L 10  $\mu$ M of each primer, and 2.4  $\mu$ L nuclease-free water. The PCR program was as follows: 98°C for 2 min, 39 cycles at 98°C for 5 s, and 55.7°C for 10 s. The relative transcript levels of the target gene, *TBC1D1*, were calculated for each sample as  $2^{-\Delta\Delta Ct}$ , where  $\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$ .

### Statistical analyses

FA data and *TBC1D1* transcript levels were analyzed using the general linear model procedure of SAS (SAS Institute Inc., Cary, NC, USA). The following model was used:

$$Y_{ij} = \mu + A_i + S_j + (A \times S)_{ij} + e_{ij}$$

where  $Y_{ij}$  is the observation of dependent variables,  $\mu$  is the general mean,  $A_i$  is the effect of age of slaughter,  $S_j$  is the effect of sex,  $(A \times S)_{ij}$  is the interaction between age and sex, and  $e_{ij}$  is the random error.

When the interaction was significant ( $P < 0.05$ ), the differences between means were tested by Tukey's procedure. Pearson's correlation coefficients were calculated to evaluate the association between *TBC1D1* transcript levels in the breast muscle, thigh muscle, abdominal fat, and subcutaneous fat and FA composition using the CORR procedure of SAS (SAS Institute Inc.).

## RESULTS

### Influence of sex and slaughter age on FA composition

Sex had significant effects on the concentrations of arachidic acid (C20:0), sinapic acid (C22:1), linoleic acid (C18:2n-6), eicosapentaenoic acid (C20:5n-3), and docosahexaenoic acid (C22:6n-3) ( $P < 0.01$ ), whereas the contents of other FA were not affected ( $P > 0.05$ ). The contents of C20:0, C22:1, C18:2n-6, and C20:5n-3 were found to be significantly higher in female than in male chickens, but female chickens contained a lower concentration of C22:6n-3 than did male chickens (Table 1).

Age had a significant effect on most MUFAs ( $P < 0.01$  and  $P < 0.05$ , respectively) except for octadecenoic acid (C18:1). Chickens slaughtered at 13 weeks had higher values of C22:1 and C18:2n-6 FA ( $P < 0.01$  and  $P < 0.05$ , respectively) compared with those slaughtered at 8 and 10 weeks). In contrast, myristoleic acid (C14:1) content was significantly lower at 13 weeks than that at 8 and 10 weeks ( $P < 0.01$ ), whereas the palmitoleic acid (C16:1) concentration at 10 weeks in chicken breast muscle was higher than that at other slaughter ages ( $P < 0.05$ ). For SFAs, the concentrations of lignoceric (C24:0) and cerotic acids (C26:0) were significantly higher in chickens slaughtered at 13 weeks than in those slaughtered at 8 and 10 weeks ( $P < 0.01$ ), whereas the C24:0 and C26:0 contents at 8 and 10 weeks showed no signifi-

cant differences. Other SFAs were not affected by age at slaughter ( $P > 0.05$ ). For polyunsaturated FA, only C20:5n-3 and C22:6n-3 were affected by slaughter age: C20:5n-3 at 8 weeks was significantly higher than that at 10 weeks ( $P < 0.01$ ), but not at 13 weeks. C22:6n-3 at 13 weeks was significantly higher than that at 10 weeks ( $P < 0.05$ ), but not at 8 weeks.

### **mRNA abundance of *TBC1D1* in chicken**

The *TBC1D1* mRNA, present in all collected tissues (breast muscle, thigh muscle, abdominal fat, and subcutaneous fat) at all 3 slaughter ages (8, 10, and 13 weeks) in the chicken populations were analyzed for interactions between sex, age, and tissue type. The total relative expression data, including the main effects and interactions, are shown in Table 2, with separate rows for tissue, age, and sex. We observed a main effect of tissue on *TBC1D1* mRNA, with the greatest expression in abdominal fat compared to in other tissues ( $P < 0.01$ ). The *TBC1D1* mRNA expression level was nearly 1.8-fold greater in abdominal fat than in subcutaneous fat. In addition, we observed intermediate expression in the breast muscle and thigh muscle, with 1.4- and 1.3-fold greater expression in these tissues than in subcutaneous fat ( $P < 0.05$ ).

### **Effect of age on *TBC1D1* expression**

Table 2 shows that age influenced *TBC1D1* expression, with greater mRNA abundance at 13 weeks than at 8 and 10 weeks ( $P < 0.05$ ). Additionally, the interaction between age and tissue was significant ( $P < 0.05$ ; Table 2), indicating a tissue-specific pattern of *TBC1D1* expression between ages 8 and 13 weeks. Therefore, we further analyzed the influence of age on *TBC1D1* gene expression in cocks and hens. As shown in Figure 1a, for cocks, the expression of *TBC1D1* mRNA in abdominal fat increased from 8 to 13 weeks, with greater expression at 10 and 13 weeks compared with at 8 weeks ( $P < 0.05$ ). In the thigh muscle, an increase ( $P < 0.05$ ) in *TBC1D1* mRNA expression was observed between 8 and 10 weeks, but there were no difference between 10 and 13 weeks. However, *TBC1D1* mRNA expression in subcutaneous fat was high at 8 weeks and decreased substantially at 10 weeks ( $P < 0.05$ ), and then increased at 13 weeks. In contrast, the expression of *TBC1D1* mRNA in the breast muscle was not influenced by age. As shown in Figure 1b, for hens, *TBC1D1* mRNA levels were similar in thigh muscle and subcutaneous fat. Expression levels were higher at 8 and 13 weeks than at 10 weeks ( $P < 0.05$ ). However, in breast muscle and abdominal fat, age had no effect on *TBC1D1* expression ( $P > 0.05$ ).

### **Effect of sex on *TBC1D1* expression**

Sex had a significant effect on *TBC1D1* expression ( $P = 0.0033$ ; Table 2), indicating tissue-specific *TBC1D1* expression related to sex. As shown in Figure 2a, *TBC1D1* expression in the thigh muscle of hens was significantly higher than that of cocks at 8 weeks ( $P < 0.05$ ). Although *TBC1D1* expression in the abdominal fat was higher than that of cocks at 8 weeks, the difference was not significant ( $P > 0.05$ ). As shown in Figure 2b, *TBC1D1* expression in the thigh muscle of cocks was significantly higher than that of hens at 10 weeks ( $P < 0.05$ ), while in other tissues, *TBC1D1* expression of cocks was higher than that of hens, but not significant ( $P > 0.05$ ). At 13 weeks, *TBC1D1* expression in the abdominal fat of cocks was significantly higher than that of hens ( $P < 0.05$ ). Although not significant, hens generally had higher *TBC1D1* mRNA levels in the thigh muscle and subcutaneous fat than did cocks ( $P > 0.05$ ; Figure 2c).

**Table 1.** Fatty acid composition of breast muscle (mg/100 g tissue).

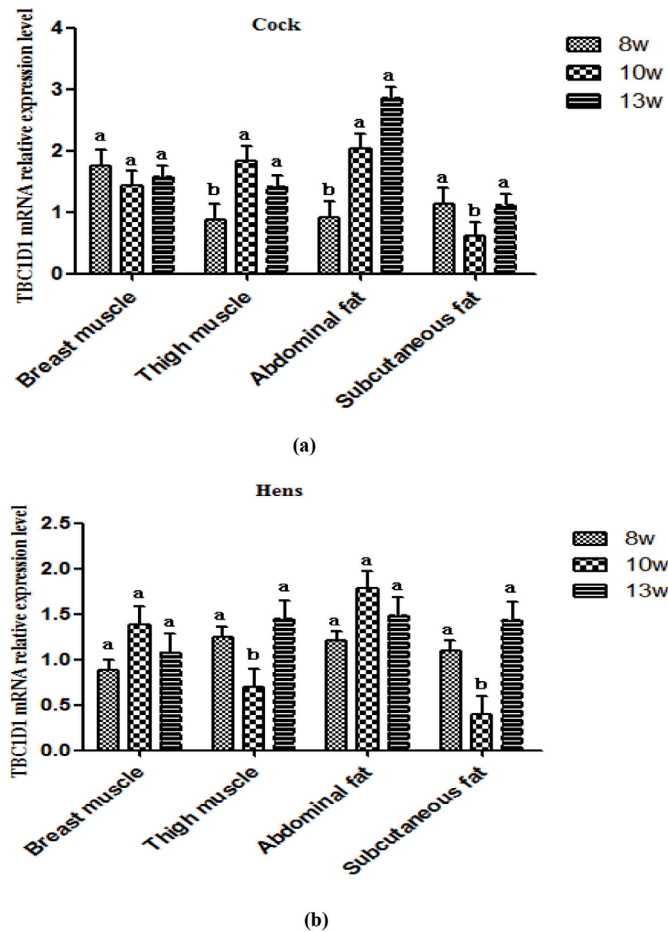
Fatty acid	Trait	Abbreviation	Sex		Slaughter age (week)			Significance <sup>a</sup>		
			Female	Male	8	10	13	Sex	Age	Sex x Age
Saturated fatty acid (SFAs)	Myristic acid	C14:0	5.47 ± 0.37	5.37 ± 0.37	5.15 ± 0.46	5.30 ± 0.44	5.80 ± 0.46	ns	ns	ns
	Palmitic acid	C16:0	14.08 ± 0.96	14.03 ± 0.95	14.28 ± 1.19	13.85 ± 1.13	14.05 ± 1.20	ns	ns	ns
	Stearic acid	C18:0	10.74 ± 0.74	10.68 ± 0.75	9.51 ± 0.94	10.55 ± 0.88	12.06 ± 0.93	ns	ns	ns
	Arachic acid	C20:0	138.01 ± 6.49	78.11 ± 6.48	98.51 ± 8.09	105.06 ± 7.64	120.60 ± 8.09	**	ns	**
	Behenic acid	C22:0	160.17 ± 10.21	155.45 ± 10.21	144.95 ± 12.74	159.10 ± 12.02	169.38 ± 12.75	ns	ns	ns
	Lignoceric acid	C24:0	206.75 ± 14.23	233.32 ± 14.23	187.89 ± 17.76	186.35 ± 16.75	285.89 ± 17.76	ns	**	ns
Monounsaturated fatty acid (MUFAs)	Cerotic acid	C26:0	22.91 ± 1.59	25.09 ± 1.62	14.73 ± 1.99	27.47 ± 1.93	29.83 ± 1.99	ns	**	ns
	Myristoleic acid	C14:1	10.14 ± 0.82	9.28 ± 0.81	11.47 ± 1.01	11.15 ± 0.96	6.53 ± 1.02	ns	**	ns
	Palmitoleic acid	C16:1	51.43 ± 2.96	56.88 ± 2.96	43.24 ± 3.69	74.55 ± 3.48	44.68 ± 3.70	ns	**	ns
	Octadecenoic acid	C18:1	540.41 ± 21.42	497.63 ± 21.42	536.26 ± 26.74	526.80 ± 25.21	494.00 ± 26.74	ns	ns	ns
	Sinapic acid	C22:1	193.92 ± 6.12	168.18 ± 6.11	178.18 ± 7.64	136.35 ± 7.19	228.61 ± 7.63	**	**	**
	Linoleic acid	C18:2n-6	335.89 ± 14.53	259.90 ± 14.53	261.34 ± 18.13	308.00 ± 17.10	324.35 ± 18.13	**	*	*
Polyunsaturated fatty acid (PUFAs)	Alpha linolenic acid	C18:3n-3	530.53 ± 34.57	491.08 ± 33.13	505.30 ± 42.95	564.85 ± 38.98	462.26 ± 42.36	ns	ns	ns
	Gamma-linolenic acid	C18:3n-6	878.75 ± 76.42	921.97 ± 76.42	859.78 ± 95.38	837.00 ± 89.93	1004.30 ± 95.38	ns	ns	ns
	Arachidonic acid	C20:4n-6	8.62 ± 0.32	9.18 ± 0.33	8.83 ± 0.41	9.10 ± 0.39	8.76 ± 0.41	ns	ns	ns
	Eicosapentaenoic acid	C20:5n-3	54.34 ± 1.20	49.38 ± 1.20	59.86 ± 1.49	37.65 ± 1.41	58.06 ± 1.50	**	**	**
	Docosahexaenoic acid	C22:6n-3	53.07 ± 2.44	63.45 ± 2.45	59.70 ± 3.05	51.25 ± 2.88	63.83 ± 3.06	**	*	**

<sup>a</sup>Significance of main effects (sex, age) and their interaction. \*P < 0.05; \*\*P < 0.01; ns = not significant (P > 0.05).

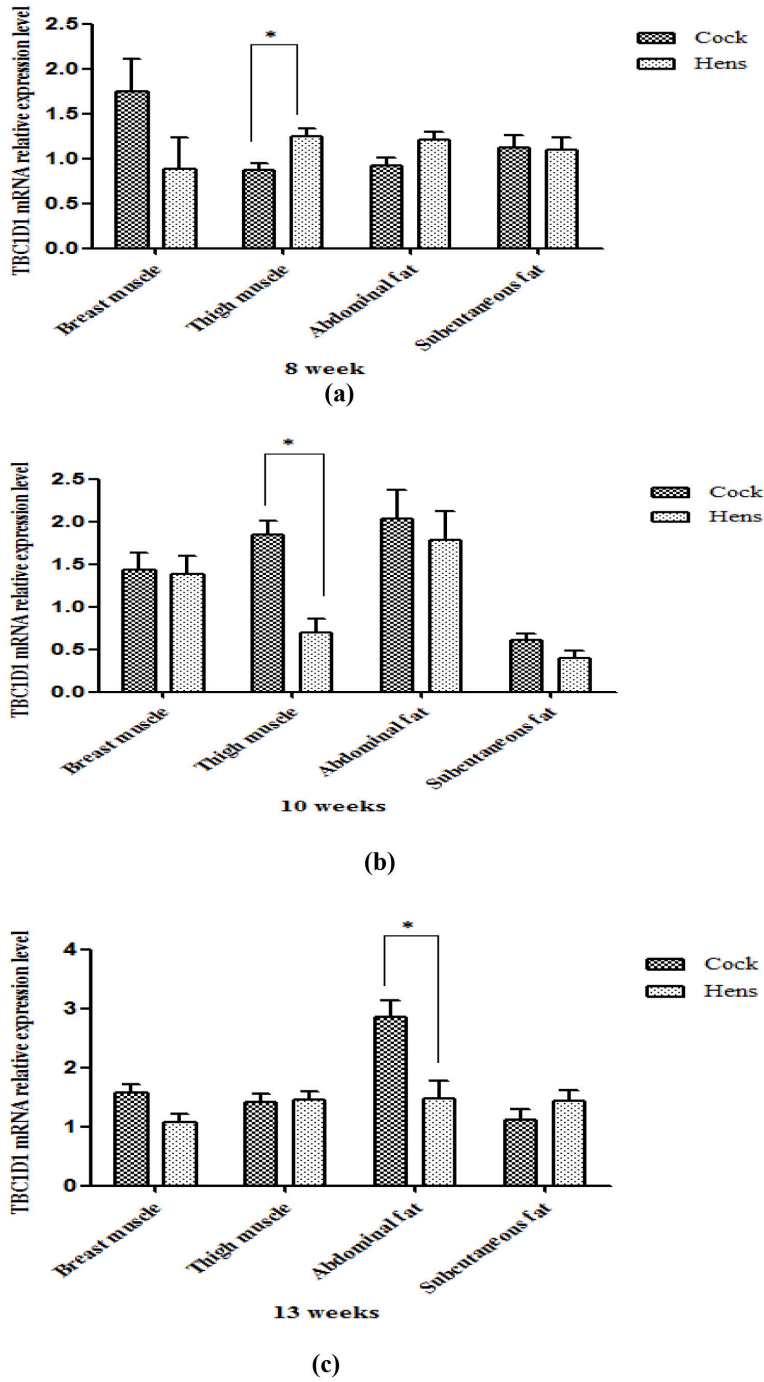
**Table 2.** Main effects and interactions between tissue, age, and sex on chicken *TBC1D1* gene expression.

Tissue	Breast muscle	Thigh muscle	Subcutaneous fat	Abdominal fat	SE	P value
	1.349 <sup>B</sup>	1.252 <sup>BC</sup>	0.961 <sup>C</sup>	1.712 <sup>A</sup>	0.092	0.0001
Age	8 weeks	10 weeks	13 weeks		0.082	0.0019
	1.139 <sup>B</sup>	1.271 <sup>AB</sup>	1.547 <sup>A</sup>			
Sex	Male	Female			0.068	0.0033
	1.460 <sup>A</sup>	1.177 <sup>B</sup>				
Interactions						P value
T x A						0.024
T x S						0.265
A x S						0.317

Data are reported as least squares means ± standard errors of the means. Mean values with different letters within a row differ significantly ( $P < 0.05$ ). For the interactions, T, A, and S represent the main effects of tissue, age, and sex, respectively.



**Figure 1.** *TBC1D1* mRNA abundance in different tissues of cocks and hens of Erlang mountainous chicken at 8, 10, and 13 weeks of age. (a) Cocks; (b) Hens. Bars represent means ± SE. a, b different letters within the same tissue indicate significant differences between ages ( $P < 0.05$ ). w = weeks.



**Figure 2.** Expression of the *TBCID1* gene of Erlang mountainous chicken at different time points. (a) 8 weeks; (b) 10 weeks; (c) 13 weeks. Bars represent means  $\pm$  SE. \*Significant differences between ages ( $P < 0.05$ ).



## Correlation of gene expression and FA composition

The correlation coefficients between relative *TBC1D1* mRNA levels measured in the breast muscle are shown in Table 3. The C16:1 levels were positively correlated with *TBC1D1* mRNA levels in the breast muscle ( $P < 0.01$ ). However, for other FA, no significant relationships were observed with *TBC1D1* gene expression in breast muscle.

**Table 3.** Pearson's correlation coefficients for the relationships between fatty acid composition and *TBC1D1* expression levels measured in breast muscle.

		Fatty acid composition					
		C14:0	C16:0	C18:0	C14:1	C16:1	C18:1
Breast muscle	r	0.130	0.064	0.044	-0.047	0.711**	-0.158
	P value	0.492	0.736	0.817	0.805	<0.001	0.403

\*\* $P < 0.01$ .

## DISCUSSION

The effects of age and sex on FA composition have been described in previous studies, but the results have been inconsistent. Chen et al. (2003) found that the essential FA and the linoleic acid contents in cocks were 2.5 and 5.31% higher than that in hens, while sinapic acid in cocks was 5.4% lower than that in hens. Based on the results of Chen et al. (2003), we found that sex affected FA composition. In contrast, another study reported that gender had no significant effect on FA composition and content (Zhu et al., 2012). In the present study, female chickens were found to have higher arachidic acid, sinapic acid, linoleic acid, and eicosapentaenoic acid contents than males, whereas other FA contents were not affected by sex. This agrees with the results of Pu et al. (2009), who found that MUFA content in the breast muscle of the Hongshan hen was significantly higher than that in the cock, but the content of other FAs showed no significant difference between cocks and hens.

However, Zhu et al. (2012) found that age had a significant effect on FA composition. In the present study, age had a significant effect on the content of most MUFAs, including C24:0, C26:0, C14:1, C16:1, C22:1, C18:2n-6, C20:5n-3, and C22:6n-3. Additionally, the content of C26:0, C16:1, and C18:2n-6 FA in the breast muscle tissue increased with age, while the content of C14:1 decreased. Other studies have also reported that MUFA increased or decreased in the breast muscle with age (Xu and Lei, 1999; Li and Chen, 2004).

Differences between tissues in relative *TBC1D1* mRNA abundance were observed in this study, with high values detected in the abdominal fat, breast muscle, and thigh muscle and a low value in subcutaneous fat tissue. In agreement with our results, high mRNA expression of long *TBC1D1* transcripts was observed in skeletal muscle and adipose tissue (Taylor et al., 2008). In addition, high expression levels of the *TBC1D1* gene in the skeletal muscle and low expression levels in the white and brown adipose tissues have been reported in New Zealand obese mice (Chadt et al., 2008).

The data from the present study indicate a pattern of sex- and tissue-specific *TBC1D1* mRNA expression. Cocks showed higher *TBC1D1* mRNA levels than hens in the thigh muscle and abdominal fat tissue at 10 and 13 weeks, respectively. Additionally, at 8 weeks, the *TBC1D1* expression level in the thigh muscle in hens was higher than that in cocks. However,

as there have been no similar studies on the influence of sex on *TBC1D1* expression, future investigation with more breeds and a larger sample size is necessary to confirm our results. In addition, to obtain a comprehensive understanding of the characteristics of *TBC1D1*, we examined its ontogeny in specific tissues of chickens during their development, observing that the expression level of *TBC1D1* changed in different ways in the tissues as the chickens aged. *TBC1D1* mRNA expression in the abdominal fat of cocks and hens increased with age, reaching a peak at 13 weeks (Figure 1a). However, in subcutaneous fat, the *TBC1D1* mRNA expression exhibited a “decline-rise” developmental change, and its expression at 8 and 13 weeks was significantly higher than at 10 weeks ( $P < 0.05$ ). In addition, in the breast and thigh muscles, *TBC1D1* mRNA expression exhibited a “decline-rise” or “rise-decline” developmental change, but these differences were not significant in the breast muscle. Similar to the present study, no differences in *TBC1D1* protein expression were found in muscles (soleus, gastrocnemius, and vastus lateralis) and before and after exercise (Frøsig et al., 2010; Jensen et al., 2012). This is the first study to describe the expression of *TBC1D1* in chicken tissues during development; these findings broaden our understanding of the mechanisms underlying normal fatty acid transportation in different tissues. This may assist future studies focusing on *TBC1D1* as a potential target for preventing or reversing obesity.

In summary, the results of the present study revealed sex-dependent differences in the FA composition of breast muscle tissue from chickens slaughtered at different ages. In addition, we showed that *TBC1D1* mRNA was most abundant in the muscle and abdominal fat and that variations partially contributed to sex and age differences.

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