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# PPARγ, FAS, HSL mRNA and protein expression during Tan sheep fat-tail development

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ABSTRACT

*Background:* The objective of this study was to investigate proliferator-activated receptor (PPARγ), fatty acid synthase (FAS) and hormone-sensitive lipase (HSL) mRNA and protein expression in fat tails of Tan sheep. Rams from different developmental stages (aged 3, 6, 9, 12, 15 and 18 months) were selected, and their tail measurements including length (L), width (W) and girth (G) were recorded. The mRNA and protein expressions of PPARγ, FAS and HSL were examined by quantitative real-time polymerase chain reaction (PCR) and Western blot.
*Results:* The tail measurements increased with age. We observed no significant differences ($P > 0.05$) of PPARγ mRNA expression between ages 9 and 15 months, and between 12 and 15 months; FAS mRNA expression levels at each developmental stage were observed significantly in Tan sheep ($P < 0.05$); HSL mRNA expression with no significant differences were only observed between 6 and 15 months ($P > 0.05$). Significant differences ($P < 0.05$) of PPARγ, FAS and HSL protein expressions at each developmental stage were observed in Tan sheep.
*Conclusion:* We observed that the mRNA expression patterns of PPARγ and FAS decreased first before they increased again and then this process repeated. Conversely, the mRNA expression patterns of HSL increased first before they decreased and then this process repeated. The protein expression patterns of PPARγ and FAS decreased first before they increased again and then this process repeated. Conversely, the protein expression pattern of HSL increased first before it decreased again and then increased again.



## 1. Introduction

This is always a hotspot about fat deposition of livestock for several years [1,2,3,4]. While most studies have concentrated on the fat deposition in subcutaneous [5], intramuscular [6] and visceral adipose tissues [7], relatively little has been done on the tails of sheep. Fat-tailed sheep anatomy and morphological characteristics have been thoroughly reviewed by Pourlis [8]. Briefly, fat-tailed sheep features the deposition of fat at the level of the hindquarters, and the shape and size of the fat-tail vary considerably between and within breeds. Fat-tailed sheep are an ideal breed for extensive production systems in desert or semi-desert areas of the world. The sheep are characterized by the deposition of reserve fat in the tail [9]. One of the most famous Chinese indigenous fat-tailed sheep breed is the Tan sheep in Ningxia. The tail is moderately long (about 30–40 cm), the bone reaching to the hock joint or below. Fat tail is the most common characteristic that differentiates from other breeds caused by large fat deposits in the tail.

Fat deposition is closely related to the regulation of genetic factors. Peroxisome proliferator-activated receptor (PPARγ), fatty acid synthase (FAS) and hormone-sensitive lipase (HSL) are the most important transcription factors and key enzyme genes in this process [10,11,12]. PPARγ, a nuclear receptor, is highly expressed in adipose and now recognized to be a master regulator of adipogenesis [13]. FAS is a key multifunctional enzyme contributing to the synthesis of fatty acids [14]. HSL is the key and rate-limiting enzyme that initiates the decomposition of triglyceride in the fatty tissue; it also has an effect on the deposition of animal fat [15]. Tail measurements of sheep were significantly correlated with lipocytes in tails, carcass fat, and total body fat. Some researches have calculated the tail length (9.5 ± 0.7 cm in Mehraban; 18.4 ± 0.7 cm in Ghezel), the width (9.4 ± 0.4 cm in Mehraban; 13.1 ± 0.4 cm in Ghezel), and the circumference (16.1 ± 1.5 cm in Mehraban; 33.0 ± 2.2 cm in Ghezel) in various sheep [8]. In order to better compare the role of fat deposition in tail development in a Chinese fat-tailed sheep breed (Tan sheep), we examined the mRNA and protein expression patterns of three major candidate genes involved in fat deposition during sheep tail development.

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## 2. Materials and Methods

### 2.1. Reagents

All reagents were of analytical grade of the highest purity commercially available. Prime Script RT reagent Kit with gDNA Eraser (Perfect Real Time) and SYBR Premix Ex Taq were purchased from Takara Biotechnology (Dalian). Proteo JETTM Mammalian Cell Lysis Reagent was purchased from Fermentas Scientific Molecular Biology Corporation (Fermentas, EU). Western Lightning ECL kit (Perkin Elmer) was purchased from Perkin Elmer Corporation (Perkin Elmer, USA).

### 2.2. Animal treatment

Animal experiments were carried out according to the guidelines for animal experiments at the National Institute of Animal Health. All experimental sheep were under the same forage and feeding management conditions. Adipose tissues (about 300 g) were collected from the tails of 24 Tan sheep for each group (12 males and 12 females) at the ages of 3, 6, 9, 12, 15 and 18 months by operative. The tissues were collected and immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Measurements included length (L), width (W) and girth (G) of the fat-tails. Length and girth were measured using an ordinary flexible tape measure. Width was measured by calipers designed for this purpose [16,17]. All measurements were made while the animal was held in a standing position. No significant differences were found between males and females at the same age. Measurements included the following:

Length: the distance from the leading edge of the first coccygeal vertebra to the end of the tail.

Width: the straight-line distance of the widest point of the tail.

Girth: the perimeter at the widest point of the tail.

### 2.3. RNA and protein extraction

Total RNA was isolated from the frozen tissues using Trizol reagent according to the manufacturer's protocol (Takara, Dalian) and previous research [18]. Proteins were isolated from the frozen tissues using a ProteoJETTM Mammalian Cell Lysis Reagent (EU). PMSF was added in the right proportions (1:100) before using the reagent. The RNA and protein quantity were both determined with a Maestro Nanomicro-spectrophotometer (MaestroGEN, USA). The protein products were analyzed by SDS-PAGE.

### 2.4. RT-polymerase chain reaction (PCR)

Total RNA was reverse-transcribed in a total volume of 20 μL using the PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, Dalian) containing gDNA Eraser and PrimeScript RT Enzyme Mix, and RT Primer Mix. Reactions were incubated for 15 min at 37°C, 5 s at 85°C, and a final 10 min extension at 4°C. The transcribed cDNA was amplified with TaqDNA polymerase (Takara, Dalian) by polymerase chain reaction (PCR) in a thermo cycler using paired sense and antisense primers (Table 1). Specific primers were designed from GenBank sequences using Primer Premier 5.0. Optimal PCR conditions consisted of an initial 5 min denaturation at 94°C; 35 cycles of 30 s at 94°C, 30 s annealing at 58°C, and 45 s extension at 72°C; and a final 10 min extension at 72°C.

### 2.5. Quantitative PCR

The expression levels of the ovine PPARγ, FAS and HSL genes were examined by RT-PCR using SYBR Premix Ex Taq (Takara, Dalian). β-Actin was chosen as the internal reference gene. The primer sequences used are shown in Table 1. The thermal cycling program starts with an initial denaturation step at 95°C for 10 min, and is followed by 40 PCR cycles (dissociation for 15 s at 95°C, annealing for 30 s at 60°C, and elongation for 45 s at 60°C), one melting cycle (consisting of 15 s at 95°C, 30 s at 55°C, and a step cycle up to 72°C for 45 s, and finally a cooling down cycle at 72°C for 45 s).

These raw CT values were analyzed with a modified delta-Ct method using a PCR data analysis program, (iQ5 Optical System software) to obtain relative quantification values [19]. We chose β-actin as the internal control gene, because the expression level of β-actin is basically consistent in each tissue of the animal body. For the treated samples, evaluation of $2^{-\Delta\Delta Ct}$ indicates the fold change in gene expression relative to the untreated control.

$$\Delta\Delta Ct = \left(Ct,_{\text{Target}} - Ct,_{\text{Actin}}\right)\text{Time x} - \left(Ct,_{\text{Target}} - Ct,_{\text{Actin}}\right)\text{Time 0}$$

### 2.6. Western blot analysis

The protein concentration of the extracts was analyzed by Western blot using commercially available antibodies from Bioworld Technology, Inc. for PPARγ (MB0080); from Abcam (Hong Kong) Ltd. for FAS (ab22759); from Santa Cruz Biotechnology, Inc. for HSL (sc-25843); and from Beijing Biosynthesis Biotechnology Co., LED for GAPDH (bsm-0978M). The second antibody was provided by CWBIOTECH for PPARγ (CW0102), FAS (CW0105), HSL (CW0103) and GAPDH (CW0102).

Protein extracts (80 μg) were mixed with sample buffer (80 μg), boiled, separated by sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes. The blots were saturated with 5% non-fat milk in PBS containing 0.1% (v/v) Tween-20 (PBS-T). After three washes with PBS-T, the blots were incubated with primary antibodies, washed, and incubated with secondary antibody. All antibodies were diluted in 5% (w/v) non-fat milk powder in Tris-buffered saline. The peroxidase signal was detected by enhanced chemiluminescence with the Western Lightning ECL kit (Perkin Elmer) [20]. Immunoblots were quantified by densitometric analysis, and the protein values were normalized to a loading control (GAPDH).

**Table 1**
Primer sequences used for RT-PCR.

| Gene | GenBank accession number | Oligos sequences | Product size (bp) | Tm (°C) |
|---|---|---|---|---|
| PPARγ | NM001100921.1 | F: 5′-ACGGGAAAGACGACAGACAAA-3′<br>R: 5′-AAACTGACACCCCTGGAAGATG-3′ | 150 | 62 |
| FAS | NM001012669.1 | F: 5′-CCCAGCAGCATTATCCAGTGT-3′<br>R: 5′-ATTCATCCGCCATCCAGTTC-3′ | 87 | 62 |
| HSL | NM001128154.1 | F: 5′-CTTTCGCACCAGCCACAAC-3′<br>R: 5′-CTCGTCGCCCTCAAAGAAGA-3′ | 136 | 62 |
| β-actin | NM001009784.1 | F: 5′-TGAACCCCAAAGCCAACC-3′<br>R: 5′-AGAGGCGTACAGGGACAGCA-3′ | 107 | 61 |

Densitometric analysis of the images was performed using ImageJ software (Toronto Western Research Institute University Health Network).

$$\text{Relative quantitative expression level } (\%) = \left(\text{Mean gray value}_{,\,\text{Target}} / \text{Mean gray value}_{,\,\text{GAPDH}}\right) \times 100$$

### 2.7. Statistical analyses

Tail measurement values expressed on a unit of length (cm). The mRNA and protein were expressed on a relative percentage basis. The data are expressed as means ± SEM and statistically analyzed using SPSS Statistics 21.0 for Windows Software. Statistical differences between experimental groups were assessed by one-way ANOVA. Differences were considered statistically significant at $P < 0.05$.

## 3. Results

### 3.1. Tail measurement changes during development

Tail measurements were significantly different ($P < 0.05$) at each developmental stage, but no such differences ($P > 0.05$) were observed in 9 months and 12 months in tail length; 12 months and 15 months in tail width; and in 6 months and 9 months in tail girth. The results showed that the tail measurements increased continuously with sheep growth. The most significant changes occurred from 3 months to 9 months in tail length; from 3 months to 6 months in tail width; and from 15 months to 18 months in tail girth (Fig. 1). All experimental sheep were developed and fat was metabolized normally.

### 3.2. mRNA expression of PPARγ, FAS and HSL during development

All investigated genes were expressed throughout the selected developmental stages from the age of 3 months to the age of 18 months. PPARγ mRNA expression levels with no significant differences ($P > 0.05$) were observed in 9 months and 15 months, in 12 months and 15 months; FAS mRNA expression levels were significantly different ($P < 0.05$) at each developmental stage; HSL mRNA expression levels with no significant differences ($P > 0.05$) were observed in 6 months and 15 months. The results indicated that the PPARγ and FAS mRNA levels were very low at 6 months; but they were very high at 18 months. The HSL mRNA levels were very low at 9 months; but they were very high at 12 months. The expression patterns of PPARγ and FAS decreased first before they increased again and then this process repeated. Conversely, the expression patterns of HSL increased first before they decreased and then this process repeated (Fig. 2).

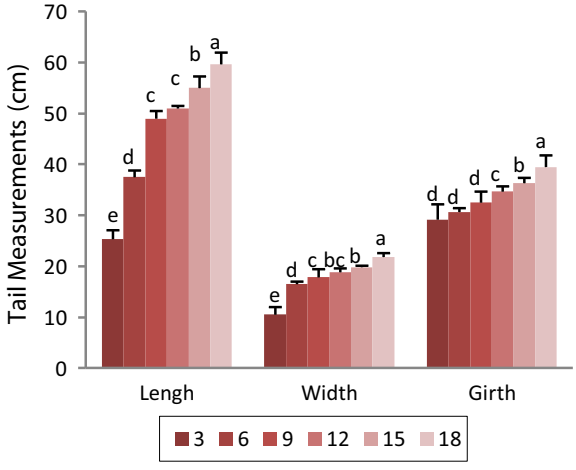


**Fig. 1.** Six ages: 3, 6, 9, 12, 15 and 18 months old. Data are measurements of tail length, width and girth of different ages (cm). Each bar represents means ± SEM. Lowercases $P < 0.05$.

### 3.3. Protein expression of PPARγ, FAS and HSL during development

The results showed that PPARγ, FAS and HSL protein expression levels were significantly different ($P < 0.05$) in Tan sheep at every developmental stage. The results indicated that PPARγ and FAS protein levels were very low at 6 months; but they were higher at 3 and 18 months than the other months. The HSL mRNA level was very low at 15 months; but it was very high at 9 months. The expression patterns of PPARγ and FAS decreased first before they increased again and then this process repeated. Conversely, the expression pattern of HSL increased first before it decreased again and then increased again (Fig. 3, Fig. 4).

## 4. Discussion

Fat deposition is a steady equilibrium control mechanism in animal nutrition metabolic processes. The energy obtained from feed, beyond maintaining growth, is stored in the body as fat. It is important for maintaining energy balance [21]. Fat deposition is an important factor in the process of meat production. In the natural grazing and extensive management of animal husbandry production conditions, animals store a lot of fat instinctively on plentiful fodder. Indeed, the appraisal of body fat content in living farm animals is based largely on the evaluation of the fat depots using body condition scores.

Tan sheep's tail fat in a certain sense, is on behalf of the whole level of body fat deposition. The process of fat deposition in animals is a dynamic process involving fat synthesis and catabolism. There are many factors affecting fat deposition, such as breed, age, nutrition and environmental factors [22]. From a molecular perspective, the animal's growth, development, senescence and disease are created by the differential expression of some functional genes in different stages and positions. Differences in gene expression can control the whole process by regulating cell activation, proliferation, differentiation, diseases and apoptosis to some extent. Therefore, investigation of changes in gene expression is a very important factor in understanding the regulation of growth and development and mechanism of diseases [23].

PPARγ is a transcription factor (TF) belonging to the superfamily of nuclear receptors. PPARγ has a well-established central role in differentiation and function of mature adipocytes [24]. FAS and HSL are important enzymes in adipocyte metabolism. They play an important role in the homeostasis of adipocyte accumulation and metabolism, which include both the fatty acid synthesis and hydrolysis [25]. As key enzymes in catabolic and anabolic processes of triglycerides, the expression of FAS and HSL may have some effect on fat deposits in animals. FAS/HSL expression establishes a dynamic-balanced system of key enzymes involved in fat metabolism. It is proposed that the relative growth of fat is related to the relative growth of muscles. Adipose tissue growth is a combination of cell proliferation coupled with differentiation and cell hypertrophy. Proliferation is a property of the undifferentiated preadipocyte and is predominant in neonatal development. Preadipocytes differentiate into adipocytes with subsequent growth of the adipocyte, increase in mass of the tissue being the result of accumulation of triacylglycerol in a large central intracellular lipid droplet [26]. Fat-tailed sheep store a lot of fat in their tails at birth. Therefore, it can be considered that fat deposition in tails occurs prior to that subcutaneously. The tail fat in fat-tailed Tan sheep accounts for most of the overall body fat. Therefore, the changes in tail fat would reveal energy ratio and flow.

Fat-tail is a major concern of livestock keepers. It is satisfactory to breed against a large fat-tail, because it is a carcass waste and

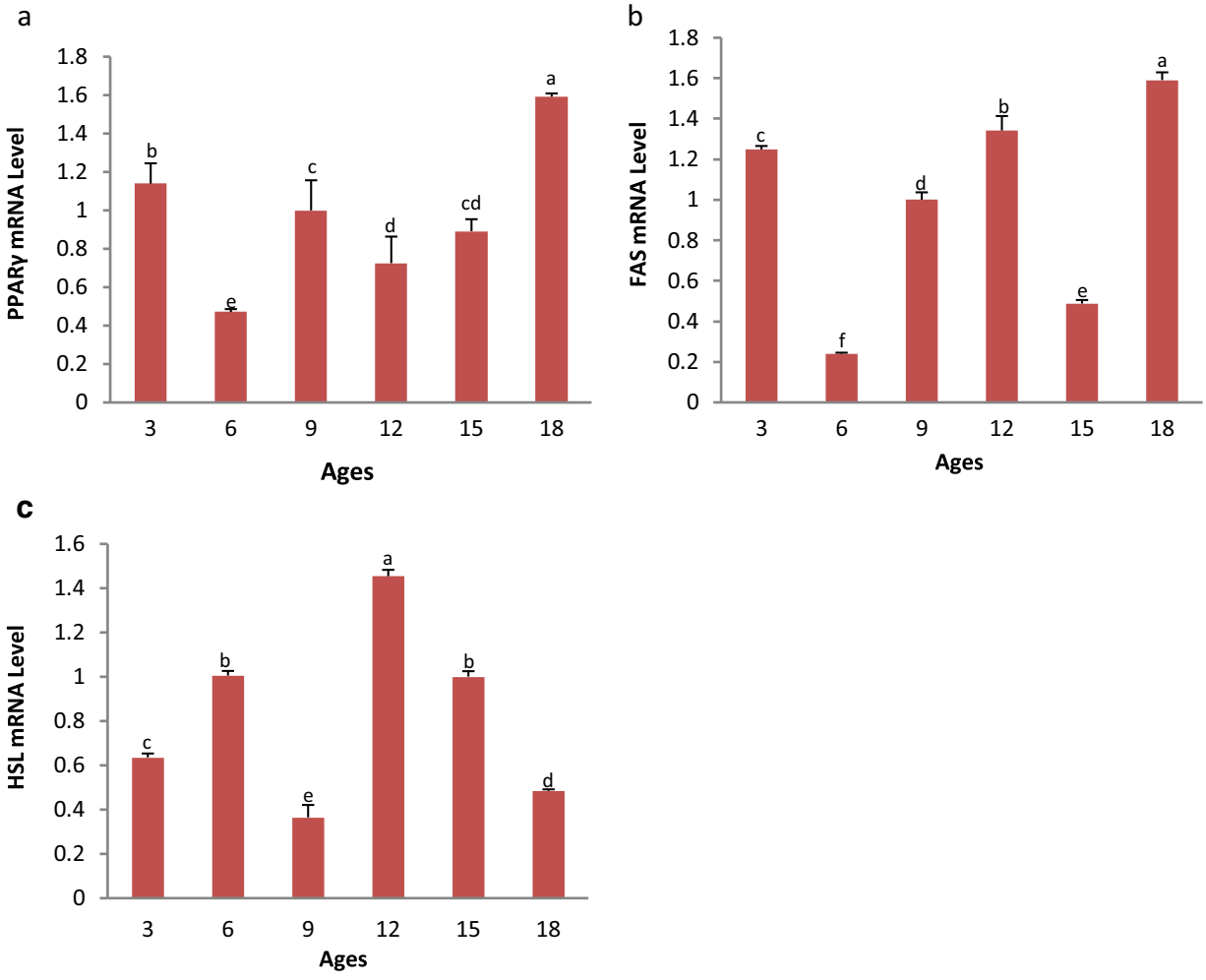


**Fig. 2.** Six ages: 3, 6, 9, 12, 15 and 18 months old. Data are ratios of PPARγ, FAS and HSL genes relative mRNA levels normalized to β-actin (housekeeping gene) mRNA levels. Each bar represents means ± SEM. Lowercases $P < 0.05$.

decreases production efficiency. Fat-tail weight cannot be measured on live animals, and the volume determined by water displacement is neither reliable nor feasible for large fat-tails. So we have developed an initial selection map for the fat tail trait in sheep. The length, width and girth of Tan sheep tails increased with the increase of their monthly age. It can be made easily on live animals, and could be used as a measure of tail volume in breeding programs. The results show that fat deposition content grows increasingly with age. Generally, tail length and tail width grow rapidly during the early stages of sheep growth; they grow slowly in the mid-season, and rebound in the late season. Tail girth grows slowly in the early period of sheep growth; it grows rapidly in the mid-to-late stages of sheep growth. This shows that there are large deposits of tail fat in later developmental stages as growth occurs increasingly in the middle period of sheep growth.

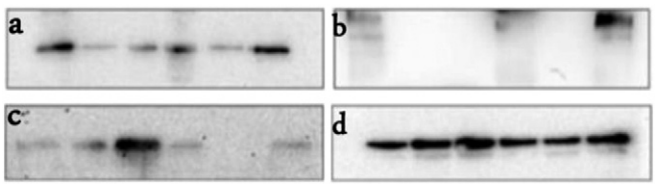


**Fig. 3.** (a–d) Representative western blots of relative expression of PPARγ, FAS, HSL and GAPDH in tail fat of different breeds, respectively. Each signal represents an individual animal. From left to right followed by 3, 6, 9, 12, 15 and 18 months old.

This pattern is consistent with body development in that the animal has a better ability to deposit fat. Studies have reported that tail length, tail width and tail depth of Altay sheep grows rapidly during 1–2 and 4–5 months of age [27]. These results show clearly that fat deposition rate is not maintaining substantial growth. This could be because the most energy is used for bones and other tissues during the development of sheep growth, but the energy for fat deposition decreases in the previous period of tail development.

PPARγ, FAS, HSL mRNA and protein expression levels are different from tail measurements during tail development. Tail measurements continue to grow, but PPARγ, FAS expression are mainly in a fluctuating growth trend, HSL expression is mainly in a fluctuating downward trend. The mRNA expression pattern of PPARγ and FAS exhibits a decrease first before it increases again and then the process is repeated. Conversely, the expression of HSL increases first before it decreases and then this process repeats at 3–18 months. Previous studies have demonstrated that the mRNA expression pattern of FAS and HSL increased first before it decreased and then this process repeated from 1 month to 13 months [24]. In this respect, our results are similar to those reported by others. FAS/HSL mRNA expression is greater than 1 at 3, 9 and 18 months, but it is less than 1 at 6, 12 and 15 months. These results show that anabolism of fat is higher than catabolism at 3, 9 and 18 months, but it is just the opposite at 6, 12 and 15 months. This illustrates that the rate of tail fat deposition is much faster at 3, 9 and 18 months; the rate of tail fat deposition is much slower at 6, 12 and 15 months. It is influenced by various

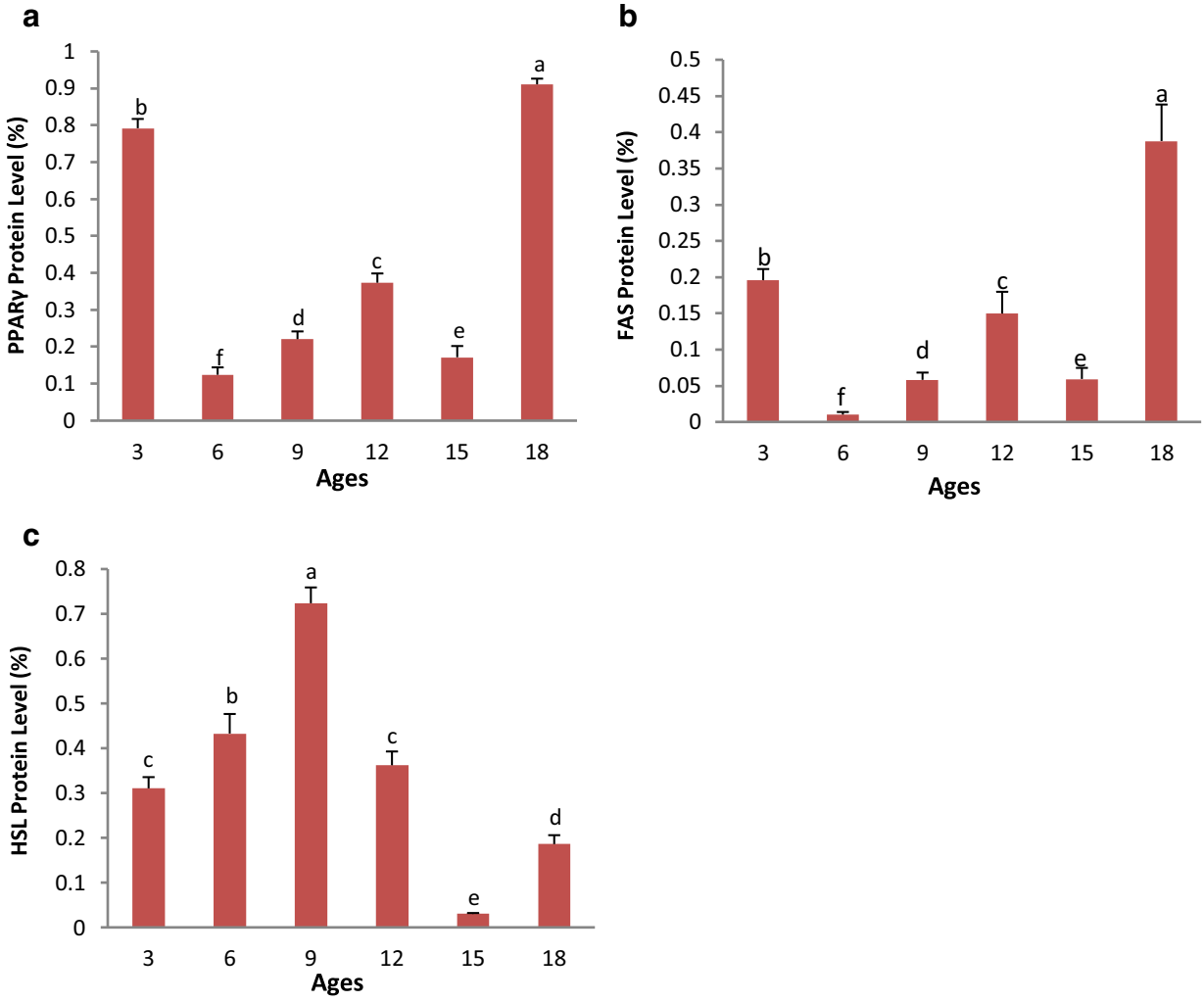


**Fig. 4.** Six ages: 3, 6, 9, 12, 15 and 18 months old. Data are ratios of PPARγ, FAS and HSL genes relative protein levels normalized to GAPDH (housekeeping gene) protein levels. Each bar represents means ± SEM. Lowercases $P < 0.05$.

factors. There is evidence that the FAS mRNA expression level of Rasa Aragonesa sheep decreased first before it increased again in 12–36 kg [28]. In addition, the FAS mRNA expression levels in kidney and subcutaneous fat increased with weight gain, while they increased first and then decreased in intramuscular fat [29]. The results show that the same gene is differentially expressed in different tissues. Yao's study has shown that HSL mRNA expression levels increased first before they decreased with weight gain. The enzyme activity decreased with weight gain. HSL mRNA expression levels were consistent with the changes of enzyme activity. Therefore, we speculate that gene expression is closely related to enzyme activity [30]. The protein expression pattern of PPARγ and FAS decreases first before it increases again and then this process repeats. Conversely, the protein expression pattern of HSL increases first before it decreases again. FAS/HSL protein expression was greater than 1 in 15 at 18 months, while it was less than 1 at 3, 6, 9 and 12 months. These results show that anabolism of fat is higher than catabolism at 15 and 18 months, but it is just the opposite at 3, 6, 9 and 12 months. This illustrates that the rate of tail fat deposition is much faster in sheep during late growth and development; the rate of tail fat deposition is much slower in sheep during early growth and development.

It is well known that gene expression includes the processes of gene encoding a protein from transcription, mRNA processing and maturity, RNA translation, and protein processing to the formation of an active (function) protein. Gene expression includes transcriptional regulation, RNA processing regulation, RNA transport regulation, translation regulation, mRNA stability regulation and post-translational regulation [31]. The patterns of mRNA expression are consistent with protein expression of PPARγ, FAS and HSL to a certain extent. The role of the mechanism, summed up, is mainly to promote adipocyte differentiation and improve adipocyte counts. Adipocyte volume is small, while adipocyte counts are limited for the growth of adipose tissue. The accumulation of lipids from mature adipocytes enables them to attain a large size. It is a major factor in adipose tissue growth. In general, we analyzed the expression patterns of the key genes in the fat deposition process, and reveal dynamic results during various developmental changes. Our findings have established a solid foundation for future studies on the molecular mechanisms of fat deposition in sheep.

These results are necessary for formulating a production plan and gaining the greatest economic benefits for farmers by choosing a proper slaughter time point. It has been suggested that stall-feeding Tan sheep should be slaughtered before 9 months of age. This approach would improve meat production rates and reduce fat weight [32]. Wang's et al. [33] study has shown that stall-feeding Tan sheep slaughtering at 12 months is superior to that in 3-year-old sheep. Our studies show that Tan sheep fat anabolism is greater than catabolism at 9 months in the mRNA levels and after 12 months in the protein levels. Tan sheep slaughter requires a combination of various factors, such as carcass weight, slaughter rate, meat tenderness and flavor. Therefore, we suggest that house breeding Tan sheep should be

slaughtered before 9 months of age to increase the price of feed and gain the maximum economic profits.

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## Conflict of interest

All authors declare the absence of any conflict of interests including any financial, personal, or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, their work.

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