

Ontogenic Expression Pattern and Genetic Polymorphism of the Peroxisome Proliferator-Activated Receptor (*PPAR-γ*) Gene in Goose Populations

^{1,2}Hua-Li Wu, ¹Hui-Ying Wang, ¹Da-Qian He, ¹Yi Liu,
¹Shao-Ming Gong, ²Fan-Li Kong and ²Qing Zhu
¹Shanghai Academy of Agricultural Sciences,

Institute of Animal Husbandry and Veterinary Science, 201106 Shanghai, China

²Laboratory of Animal Genetics and Breeding, College of Animal Science and Technology,
Sichuan Agricultural University, Ya'an, 625014 Sichuan, China

Abstract: Peroxisome Proliferator-Activated Receptor γ (*PPAR-γ*) was a key regulator of proliferation and preadipocyte differentiation in mammals. The objective of this study was to investigate the effect of the *PPAR-γ* gene on slaughter traits of 170 individuals from Zhejiang White geese (ZW) and the ontogenetic expression pattern. PCR-SSCP technique was developed to analyze coding region of the *PPAR-γ* gene polymorphism. The results showed that two genotypes (AA and AG) which were the products of two alleles (A and G) were observed. Association analysis indicated that this SNP was significantly associated with percentage of goose leg muscle ($p < 0.05$) and the allele A was the predominant allele. Quantitative real-time PCR (qRT-PCR) assay measured the *PPAR-γ* mRNA expression in ZW and Landes goose five tissues of different ages (P1, P14, P21, P28, P35, P42, P49, P56 and P63). The results showed that the *PPAR-γ* mRNA had the highest expression level in goose abdominal fat and subcutaneous fat followed by lung and intestine. The *PPAR-γ* mRNA levels exhibited a rise-decline change in fat tissues except for ZW subcutaneous fat. The values in fat tissues were higher than those of other tissues at the same goose breed ($p < 0.01$). Besides, average values in ZW fat and liver tissues were higher than those of Landes goose totally. These results suggested that the *PPAR-γ* expressions were positively associated with goose fat development and had a breed-related tendency. The *PPAR-γ* gene polymorphism could be used in Marker Assisted Selection (MAS) as a genetic marker for goose slaughter traits.

Key words: Goose, *PPAR-γ* gene, SNP, carcass traits, expression

INTRODUCTION

PPAR-γ was one member of the PPAR family of nuclear receptors which was a ligand activated transcription and influenced human obesity, diabetes, atherosclerosis and hypertension (Brun and Spiegelman, 1997; Rosen and Spiegelman, 2001; Gelman *et al.*, 2007; Szeles *et al.*, 2007; Sanada *et al.*, 2011). It was highly abundant in adipose tissue (Yin *et al.*, 2008) with expression being induced early in preadipocyte differentiation (Saladin *et al.*, 1999; Grimaldi, 2001). Human *PPAR-γ* gene had four different isoforms, the *PPAR-γ1* isoform has a relative ubiquitous expression, the *PPAR-γ2* was restricted to adipose tissues and the *PPAR-γ3* expression is relatively to certain tissues such as macrophages, adipose tissue and colon and γ ORF4 may play a role in the tumorigenic process (Fajas *et al.*, 1998; Sabatino *et al.*, 2005).

There was one polymorphism in the duck *PPAR-γ* coding region which was linked with some growth and fatness traits such as carcass weight, sebum, percentage of sebum, abdominal fat weight, percentage of abdominal fat and IMF (Wu *et al.*, 2008). On the contrary, research showed that the *PPAR-γ* gene polymorphism was not related with chicken growth and body composition traits (Meng *et al.*, 2005). The current research indicates that the *PPAR-γ* is a key regulator of chicken preadipocyte differentiation (Wang *et al.*, 2008).

Modern strains of geese exhibit excessive body fat deposition which is one of the main problems encountered by goose industry today. Excess fat deposition has led commercial breeders to incorporate significant selection for reducing body fatness in breeding programs. Earlier research effort on duck has been applied to study factors associated with fat deposition and methods to reduce it (Shahin *et al.*, 2000; Wawro *et al.*, 2004).

Up to now, the current research about the goose *PPAR-γ* gene has seldom been reported. The objectives of the present study were to detect the *PPAR-γ* gene Single Nucleotide Polymorphism (SNP) and investigated its association with the Zhejiang White goose slaughter traits and to further determine the patterns of the *PPAR-γ* gene expression in ZW and Landes goose five tissues.

MATERIALS AND METHODS

Samples collection and preparation: Blood samples were collected from 170 individuals that belong to ZW populations. ZW was a Chinese indigenous breed in Zhejiang province with white feathers, medium size, early growth fast, coarse to feed and a favorable meat quality (Chen *et al.*, 2004). Geese were reared at the same management system in the Pinghu Breeding Company in Zhejiang, P.R. China. The 170 geese were slaughtered at the end of 70 days. These traits such as live weight, carcass weight, eviscerated weight, percentage of eviscerated weight, heart weight, liver weight, masticatory stomach weight, breast muscle weight, percentage of breast muscle, leg muscle weight, percentage of leg muscle, abdominal fat weight and percentage of abdominal fat were collected for statistical analysis. Genome DNA was obtained by phenol and chloroform (1:1) extraction and stored at -20°C.

Landes goose used world-wide for the production of fatty liver. ZW and Landes geese were selected from the Pinghu Breeding Company in Zhejiang province, eighty birds of each breed, hatched on the same day and reared under similar management conditions including a standard diet (commercial corn-soybean diets meeting the National Research Council's NRC requirements) and were transferred to the growing pens at the age of 9 weeks. The studied tissues included five tissues such as subcutaneous fat, abdominal fat, lung, intestine and kidney, researchers collected four individuals at two breed each stage, respectively. The collecting process has the nine postnatal periods (P1, P14, P21, P28, P35, P42, P49,

P56 and P63). Fresh tissues were removed, immediately frozen in liquid nitrogen and stored at -80°C for RNA isolating.

Primer design and PCR amplification: Seven primer pairs were designed according to the sequence of the goose *PPAR-γ* gene (GenBank Accession No: AF481798) (Table 1), the amplified products that contains the exons of the *PPAR-γ* gene. Polymerase Chain Reaction (PCR) amplification was carried out on Gene Amp PCR System 9700 (Bio-Rad) in a total volume of 10 μL reaction with 40 ng genomic DNA, 3 pmol of forward and reverse primer, 2×Taq PCR Master mix (including Mg²⁺, dNTP, Tag DNA Polymerase; Beijing TIAN WEI Biology Technique Corporation, Beijing, China), PCR program: initial denaturation for 10 min at 95°C, 30 cycles each 40 sec at 94°C, 40 sec at 58°C and 45 sec at 72°C and 10 min final extension at 72°C.

Single Stranded Conformation Polymorphism (SSCP) and sequencing: PCR products were resolved by SSCP analysis. Several factors were tested for each fragment in order to optimize the amount of PCR products, denaturing solution, gel concentration, glycerol, voltage, running time and temperature. Each PCR product was diluted in the denaturing solution (95% formamide deionized, 0.05% of bromophenol blue, 0.25% xylene cyanole and 10% glycerol) and was denatured at 99°C for 10 min and quickly cooled on ice. A total of 3 μL of mixture was subjected to 12% polyacrylamide gel (polyacrylamide: bisacrylamide = 39:1) in 1×TBE buffer and constant voltage (130 V) for 12~14 h. The gel was stained with silver nitrate. The bands on the gel were visualized by Gel Imaging System. The DNA samples showing different patterns on SSCP gel were further amplified and purified and then sequenced by Shanghai YingJun Biology Technique Corporation (Shanghai, China).

QRT-PCR assay for *PPAR-γ* mRNA expression in different tissues: Total RNA were isolated from

Table 1: Primers for screening the goose *PPAR-γ* gene polymorphisms

Primer name	Primer sequence (5'-3')	Product length (bp)	Annealing temperature (°C)
PPAR-γ-1F	ATGGTTGACACAGAAATGCCGT	217	60
PPAR-γ-1R	GGCAATCCTGGAGCTTGATAT		
PPAR-γ-2F	GTGCAATCAAAATGGAGCCTC	170	56
PPAR-γ-2R	CTTACAGCCTTACATGCATGC		
PPAR-γ-3F	GGCTTTTTTCGAAGAACCATC	138	60
PPAR-γ-3R	ATTGTGTGGCATTCCAACAG		
PPAR-γ-4F	CATCAGGTTTGGGCGAATG	199	58
PPAR-γ-4R	TGATTTGTCTGTCGTTTTCTGT		
PPAR-γ-5F	CCATTGTATTATGACATGAAT	205	55
PPAR-γ-5R	GATCATTGAGTCAAGATTACACA		
PPAR-γ-6F	AAGTAACTCTCCTGAAATACGGT	246	56
PPAR-γ-6R	CTCCACTTAGTATAATGACAGCTATA		
PPAR-γ-7F	GCCCAGGTTTGTTAAATGTGA	234	56
PPAR-γ-7R	CTTTATAGATTTCTTGTAGGAGTGG		

abdominal fat, subcutaneous fat, lung, intestine and kidney tissues using the Trizol reagent (Invitrogen) and treated with RNase-free DNase I to remove contaminated genomic DNA. The first strand cDNA was synthesized using the Reverse Transcription System (TaKaRa Biotechnology Co., Ltd.) according to the manufacturer's instructions. The 10 µL reaction contained 5×PrimerScript Buffer, 10 mM of each dNTPs, 40 U µL⁻¹ RNase Inhibitor, 2.5 µM oligo-dT Primer. The reverse transcription was maintained at 42°C for 30 min then 85°C for 5 sec. The cDNA product was stored at -20°C.

Expression of goose *β-actin* gene from GenBank (M26111.1) was used as an internal control. Two primer pairs (PPAR-γ-F, PPAR-γ-R; *β-actin*-F, *β-actin*-R) were to achieve the most efficient amplification (Table 1). Sequence authenticity was confirmed by direct sequencing of the amplified fragments. QRT-PCR analysis was performed on iQ5 Detection System (BIO-RAD Company) using SYBR Green II detection chemistry (TaKaRa Biotechnology Co., Ltd.).

All reactions were analyzed in triplicate. Under the same cycling conditions: 95°C for 160 sec; 40 cycles of 95°C for 5 sec; 60°C for 30 sec and a final extension step at 72°C for 15 sec. Fluorescent data for quantification were collected at 82°C. Melt curve analysis was performed over a range of 55–95°C in order to verify single product generation at the end of the assay. To exclude that the amplification-associated fluorescence was associated with residual genomic DNA or from the formation of primer dimers, controls without reverse transcriptase were analyzed.

The standard curves which were prepared from tenfold dilution series products were used to analyze the data. The Cycle threshold (Ct) was defined as the fractional number of cycles at which the reporter fluorescent emission reached a fixed threshold level in the exponential region of the amplification plot.

Statistical analysis: Statistical significance threshold was determined as $p < 0.05$, unless otherwise specified. The data of all carcass traits were verified for normal distribution by the Shapiro-Wilks test in SAS. t-tests were used to determine whether the individual variant was in accordance with Hardy-Weinberg equilibrium. The genetic parameters of allele gene and genotype frequency, effective Number of alleles (N_e), heterozygosity (h) and Polymorphism Information Content (PIC) were estimated.

Data were analyzed by the GLM procedure of SAS 8.0 (SAS Inst. Inc., Cary, NC, USA). The genetic effect of the diplotypes on carcass traits was analyzed by the following model:

$$Y_{ijk} = \mu + P_i + S_j + G_k(P_i) + bX + e_{ijk}$$

Where:

- Y_{ijk} = The dependant variable
- μ = The population mean
- P_i = Fixed effect of the population
- S_j = Fixed effect of the sex
- $G_k(P_i)$ = Nested effect of genotype within population
- e_{ijk} = Random error
- b = The regression coefficients of the linear regressions on X (BW)
- X = Included in the model as its effect on any trait was significant, the significance of least square means was tested with the Duncan test ($p < 0.05$)

The relative expression levels of PPAR-γ were calculated according to the equations as followed:

$$\text{Rel. Quantity} = \frac{(1 + \text{Eff})_{\text{GOI}}^{(\text{Ct}_{\text{Control}} - \text{Ct}_{\text{Sample}})}}{(1 + \text{Eff})_{\text{NORM}}^{(\text{Ct}_{\text{Control}} - \text{Ct}_{\text{Sample}})}}$$

Where:

- Rel. quantity = The relative quantity of PPAR-γ in a sample compared with that in the control
- GOI = The gene of interest
- NORM = The reference gene
- Eff = The efficiency of qRT-PCR

The PPAR-γ cDNA dilution curves were generated and used to calculate the individual qRT-PCR efficiencies ($E = 10^{(-1/\text{slope})}$). The amplification efficiencies of *PPAR-γ* and *β-actin* gene were 99.6 and 99.7%, respectively. Comparisons between groups were made by Kruskal-Wallis test and Mann-Whitney U-test. The $p < 0.05$ was considered statistically significant. The data were presented as means±SD.

RESULTS AND DISCUSSION

Single nucleotide polymorphism of goose *PPAR-γ* gene:

Researchers used seven pair of primers to amplify and screen SNPs in exons of the goose *PPAR-γ* gene. (Table 2). Primer (PPAR-γ-5F, PPAR-γ-5R) of amplification

Table 2: Primers for measuring the goose *PPAR-γ* gene expression

Primer name	Primer sequence (5'-3')	Product length (bp)	Annealing temperature (°C)
PPAR-γ-F	GCAGGAGCAGAACAAGAGGTTAG	158	60
PPAR-γ-R	GGACACCGTATTTAGGAGAGT		
<i>β-actin</i> -F	ACCACCGGTATTGTTATGGACT	398	60
<i>β-actin</i> -R	TTGAAGGTGGTCTCGTGGAT		

The forward and reverse primers are marked by F and R in the primer names

product showed polymorphism. One Single Nucleotide Polymorphisms (SNP) was detected by PCR-SSCP. Two genotypes (AA, AG) and two alleles (A and G) were observed in Zhejiang white goose populations (Fig. 1) and had resulted in one amino acid change which was sense mutations (Arg/His).

Genotype frequencies and association with ZW carcass traits: Allele gene and genotype frequency, p-values of Chi-square and Polymorphism Information Content (PIC) for Zhejiang White goose was presented in Table 3. A allele frequencies was higher than G allele in populations. Two genotypes were determined with average frequencies of AA (85.3%) and AG (14.7%) in 170 samples. The analysis of association between the *PPAR-γ* gene polymorphism with carcass traits was summarized in Table 4. We found one SNP of *PPAR-γ* gene and this nucleotide variation was sense mutations (Arg/His) and was association with the ZW percentage of leg muscle. The genotype has significant effect on ZW percentage of leg muscle. AG genotype has the higher percentage of leg muscle (12.38 ± 0.27 g) than the AA genotype (11.74 ± 0.11). In contrast, the other carcass traits did not significantly differ between AA and AG genotypes Table 5.

Expression of the *PPAR-γ* gene in different goose tissues: To discern the expression pattern of the *PPAR-γ* gene in different goose tissues, researchers performed real-time PCR analysis using the cDNA samples of ZW and Landes goose. Researchers measured the difference of the *PPAR-γ* mRNA levels in a variety of tissues.

Figure 1 shows the ontogenic expression pattern of the *PPAR-γ* mRNA in five tissues between the ZW and Landes goose. Researchers found that the *PPAR-γ* mRNA in subcutaneous fat was significantly different at P42 and P49 between two breeds ($p > 0.05$). In abdominal fat, the *PPAR-γ* mRNA expression levels all exhibited a rise-decline developmental change and had the highest expression at P21 Landes goose Table 6. In lung, the *PPAR-γ* mRNA expression was not significantly different at various weeks of age ($p > 0.05$) and had the highest expression at P14 ZW. In kidney, no significant difference was observed for the *PPAR-γ* mRNA expression levels at two goose various ages ($p > 0.05$). In intestine tissue, the

Table 3: Alleles and genotypes frequencies of the goose *PPAR-γ* gene

N	Alleles		Genotypes			p-values of Chi-square ^a	PIC ^b
	A	G	AA	GG	AG		
170	0.7643	0.2357	0.853	0.00	0.147	$p < 0.05$	0.03

The test of Hardy-Weinberg Equilibrium; ^a $p < 0.05$ suggested the significant deviation from Hardy-Weinberg Equilibrium; ^bPIC: Polymorphism Information Content

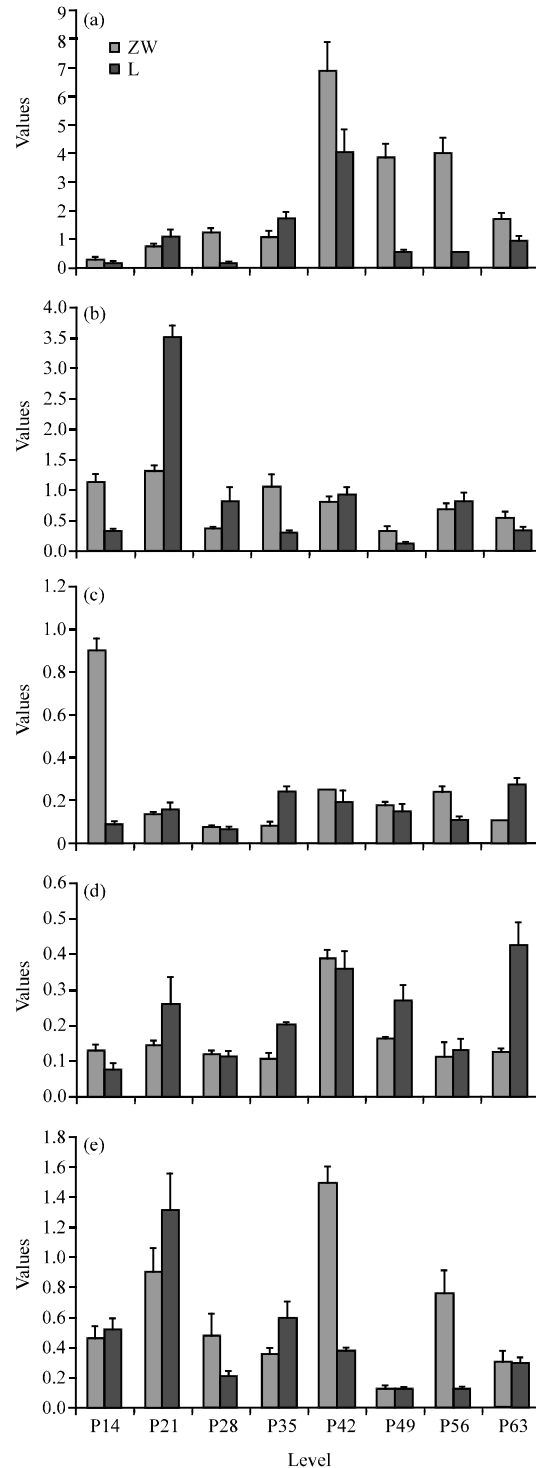


Fig. 1: The relative temporal expression of the *PPAR-γ* mRNA in different goose tissues at the different growth point; ZW = Zhejiang White goose; L = Landes goose. a) Subcutaneous fat; b) Abdominal fat; c) Lung; d) Kidney and e) Intestine

Table 4: Association of the single locus of the *PPAR-γ* gene SNP and Zhejiang White goose carcass traits (unit: g)

Site	Genotype	Traits												
		LBW	CW	EW	PEW	HW	LW	MSW	BMW	PBMW	LMW	PLMW	AFW	PAF
SNP	AA	3629.73±43.840	3174.47±29.95	2320.05±24.20	63.39±0.30	39.68±0.41	127.53±2.15	157.31±1.75	156.44±3.31	6.72±0.11	271.45±3.00	11.74±0.11	207.86±6.480	5.60±0.14
	AG	3623.89±108.32	3168.76±73.99	2291.15±59.95	63.11±0.75	39.7±1.010	124.86±5.31	165.35±4.34	152.08±8.19	6.57±0.27	280.05±7.40	12.38±0.27	204.00±16.02	5.50±0.36

The least square means within a row under the same SNP locus lacking a common lowercase superscript differ significantly ($p < 0.05$). The least square means within a row lacking a common uppercase superscript differ great significantly ($p < 0.01$). LBW = Live Body Weight (g), CW = Carcass Weight (g), EW = Eviscerated Weight (g), PEW = Percentage of Eviscerated Weight (g), HW = Heart weight (g), LW = Liver Weight (g), MSW = Masticatory Stomach Weight (g), BMW = Breast Muscle Weight (g), LMW = Leg Muscle Weight (g), AFW = Abdominal Fat Weight (g), PBMW, PLMW and PAF refer to the percentages of traits BMW, LMW and AFW relative to CW, respectively

Table 5: The difference of relative quality of the *PPAR-γ* mRNA at the different growth point in different tissues in Landes goose

Breed	Growth point	Subcutaneous fat	Abdominal fat	Lung	Kidney	Intestines
Landes goose	1	-	-	-	-	0.0935±0.023
	14	0.1434±0.038	0.3218±0.043	0.0836±0.011	0.0783±0.017	0.5102±0.079
	21	1.12±0.282 ^A	3.5075±0.185 ^B	0.1524±0.044	0.2578±0.079	1.3020±0.257
	28	0.1053±0.015	0.8102±0.247	0.0633±0.013	0.1131±0.020	0.2026±0.027
	35	1.6846±0.363	0.3089±0.045	0.2385±0.031	0.2023±0.008	0.5903±0.112
	42	3.9225±0.941 ^A	0.9300±0.154 ^B	0.1899±0.055	0.3547±0.052	0.3669±0.020
	49	0.5285±0.046	0.1320±0.016	0.1440±0.042	0.2685±0.051	0.1132±0.022
	56	0.5674±0.048	0.8377±0.168	0.1068±0.015	0.1280±0.038	0.1162±0.019
	63	0.9753±0.143	0.3388±0.061	0.2632±0.045	0.4238±0.067	0.2921±0.033

Table 6: The difference of relative quality of the *PPAR-γ* mRNA at the different growth point in different tissues in Zhejiang White goose

Breed	Growth point	Subcutaneous fat	Abdominal fat	Lung	Kidney	Intestine
Zhejiang white goose	1	-	-	-	-	1.0258±0.187
	14	0.3552±0.0312	1.1318±0.153	0.9024±0.070	0.1306±0.020	0.4592±0.085
	21	0.7807±0.0420	1.3070±0.124	0.1348±0.014	0.1425±0.015	0.8952±0.169
	28	1.2566±0.1330	0.3647±0.066	0.0732±0.014	0.1202±0.008	0.4670±0.149
	35	1.0835±0.2830	1.0512±0.233	0.0778±0.018	0.1053±0.017	0.3507±0.040
	42	6.597±1.01500 ^A	0.8226±0.135 ^B	0.2501±0.002 ^B	0.3863±0.028 ^B	1.4850±0.103 ^B
	49	3.8862±0.4360 ^A	0.3326±0.063 ^B	0.1737±0.025 ^B	0.1590±0.010 ^B	0.1175±0.035 ^B
	56	4.0230±0.5250 ^A	0.6958±0.092 ^B	0.2338±0.032 ^B	0.1134±0.039 ^B	0.7576±0.148 ^B
	63	1.7410±0.1920	0.5707±0.085	0.1023±0.003	0.1232±0.015	0.3018±0.068

Values in a line without a common uppercase mean that the relative quantities of the goose *PPAR-γ* mRNA in different tissues at the same growth points differ greatly significantly ($p < 0.01$)

higher expression level of the *PPAR-γ* mRNA was at P21 Landes goose and P42 ZW and presented a rise-decline developmental change.

As shown in Fig. 2, the *PPAR-γ* mRNA expression level had the highest value in adipose tissue than the other tissues during goose development. Despite the fact that the expression of the *PPAR* mRNA except for P21, P42, P49 and P56 had no significant differences among all tissues ($p > 0.05$), the expression levels of this gene reached the highest values in ZWSF, LSF and LAF. In addition, researchers found that the expression of the *PPAR-γ* mRNA at P42 SF was significantly different among other tissues ($p < 0.05$).

Lots of studies showed that the variants of the *PPAR-γ* genes influence the metabolism of glucide and lipid (Rangwala and Lazar, 2004; Christodoulides and Vidal-Puig, 2010). The Pro12Ala polymorphism of the *PPAR-γ* gene was found to increase the body mass index and directly related with the type 2 diabetes (Stumvoll and Haring, 2002; Tonjes *et al.*, 2006). The patients who had the Pro15Gln polymorphism of the *PPAR-γ* gene were extremely obesity (Gurnell *et al.*, 2003). The same mutation of the *PPAR-γ* gene could induce the coronary heart disease, severe insulin resistance and hypertension (Clark, 2002; Szeles *et al.*, 2007). In this study, the *PPAR-γ* gene was selected as a candidate gene to investigated associations of gene polymorphisms with ZW carcass traits. Researchers found one SNP (A651G) of *PPAR-γ*

gene and this nucleotide variation was sense mutations (Arg/His) and was association with the ZW percentage of leg muscle. Earlier studies showed that the *PPAR-γ* gene in skeletal muscles coordinated the utilization of energy rather than regulated glucose homeostasis or insulin sensitive (Koutnikova *et al.*, 2003). The *PPAR-γ* activation play role in C2C12 skeletal muscle cell differentiation, whether up-regulation or down-regulation of the *PPAR-γ* expression in skeletal muscle cells had effect on the utilization of fatty acids by skeletal muscle (Verma *et al.*, 2004). The specific absence of the *PPAR-γ* in fat robustly increases bone mass by favouring osteogenic over adipogenic differentiation of Mesenchymal Stromal Precursor (MSP) cells. In addition, absence of the *PPAR-γ* in adipocytes limits their capacity to secrete antiosteogenic signaling factors such as leptin, further enhancing the bone phenotype (Cock *et al.*, 2004).

Poultry meat quality is very important to keep up with consumer demand and its final expression is the result of interaction among genetic, nutritional, age and environmental factors. Fatness plays an important role in meat quality. The candidate gene approach is a very powerful method to investigate associations of gene polymorphisms with economically important traits in farm animals. Many studies showed that goose *PPAR-γ* gene has been cloned and expressed higher at adipose tissues (Meng and Wang, 2004). There was three SNP in the chicken *PPAR-γ* 5' region and significantly different with

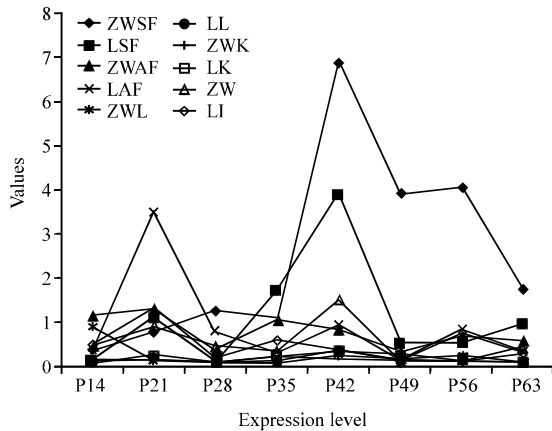


Fig. 2: The relative temporal expression of the PPAR- γ mRNA during goose development. ZWSF = Zhejiang White goose Subcutaneous Fat; LSF = Landes goose breast muscle; ZWAF = Zhejiang White goose Abdominal Fat; LAF = Landes goose Abdominal Fat; ZWL = Zhejiang White goose Lung; LL = Landes goose Lung; ZWK = Zhejiang White goose Kidney; LK = Landes goose Kidney; ZWI = Zhejiang White goose Intestine; LI = Landes goose Intestine

carcass traits such as weight of abdominal fat, weight of liver, weight of thigh bone and length of keel and shin (Han *et al.*, 2009). The PPAR- γ gene is expressed in white and brown adipose tissues, placenta, large intestine and macrophages (Picard and Auwerx, 2002). In the study, the PPAR- γ gene was expressed to a high extent in adipose tissue and kidney, lung and intestine expressed lower amounts of the PPAR- γ . Abdominal fat the PPAR- γ mRNA were relatively decreased after overfeeding however, the expression in liver, spleen, lung, small intestine and abdominal fat were increased (Luo *et al.*, 2010).

The combination of traditional genetics and breeding methods and modern molecular biology methods may be preferred for genetic improvement of goose in the future. In addition, the expression patterns of the PPAR- γ gene in Zhejiang White goose are different from Landes goose. It might suggest that different functions of the PPAR- γ gene are responsive to different breed.

CONCLUSION

Therefore, the research indicated that the PPAR- γ gene may be a major candidate gene that impact goose carcass traits. However, an explanation for the results was also probably due to the low animal numbers. Researchers will conduct further tests with higher number of samples in the future.

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