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Expression Profile of Acetyl-CoA Carboxylase A (*ACACA*) Gene in Layer Chicken during Juvenile Stage

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ABSTRACT

Acetyl-CoA carboxylase A (*ACACA*) is a rate limiting enzyme and plays a crucial role in the production of precursors of fatty acid biosynthesis. The objective of this study was to analyse the expression levels of *ACACA* at different ages of juvenile stage in one control layer (CL) chicken strain. As the birds are slow growing, the expression of the gene exhibited an increasing trend. A negative correlation has been found between body weight and mRNA expression of the gene at 6 weeks of age indicating complete utilization of energy for growth. When compared to day 1 expression, gene expression was up-regulated by 1.5, 10.5 and 10.8 folds at day 14, 28 and 42 of the birds, respectively. It is concluded that the expression of *ACACA* gene was increased gradually as age progressed during juvenile stage in layer chicken.

Keywords: Fatty acid biosynthesis, Acetyl-CoA carboxylase A, *ACACA*, mRNA levels

Chicken contain about 13-14.5% fat of their total body weight (Latshaw and Bishop, 2001; Haro, 2005) and deposit at a rate of up to 6 g fat/kg body weight/day between 42 to 49 days of age (Havenstein *et al*., 2003). In chicken, fatty acids can come from the diet (up to 5%) or be synthesized from glucose (up to 95%) in lipogenic tissue such as liver and adipose tissue. In avian species, the liver is the principal site of *de novo* fatty acid synthesis, accounting for more than 95% and to a lesser (<5%) extent in adipose tissue (Leveille, 1969; Pearce, 1977; Griffin *et al*., 1992). In the process of fatty acid biosynthesis, Acetyl-CoA carboxylase (ACAC; EC 6.4.1.2) is the rate-limiting enzyme which catalyzes the irreversible carboxylation of acetyl-CoA to produce malonyl-CoA, which is an intermediate substrate that plays a pivotal role in the regulation of fatty acid metabolism. Malonyl-CoA is also required by chain elongation systems of the endoplasmic reticulum in many tissues for the synthesis of very long chain fatty acids (Nugteren, 1965). To facilitate the different roles, fatty acid synthesis and oxidation, two distinct isoforms have evolved (Lane *et al.,* 1974; Cronan *et al.,* 2002; Barber *et al*., 2005): acetyl-CoA carboxylase A (*ACACA or ACC-α or ACC1*) and acetyl-CoA carboxylase B *(ACACB or ACC-β or ACC2)*. *ACACA* (M_r ~265 kDa) is mainly localized in lipogenic tissues such as liver tissue, where the fatty acids are synthesized. The expression level of this particular gene at different age groups will form the base for understanding the gene action and subsequently can be used for manipulating the gene functionality with the aim to reduce fat deposition. Earlier studies revealed expression of this gene involved in hepatic lipogenesis process broiler chicken population (Legrand *et al.,* 1987a, b; Douaire *et al.,* 1992; Legrand and Hermier, 1992; Daval *et al.,* 2000). But, reports on genes involved in lipogenesis in layer chicken population were scanty. Keeping these facts in mind, the objective of the study was designed to analyze the expression levels of *ACACA* at different ages of juvenile stage in layer chicken population.

MATERIALS AND METHODS

Sample collection

The study was conducted on a control layer (CL) line which was a slow growing chicken (*Gallus gallus*) population maintained at the Directorate of Poultry Research (DPR), Hyderabad, India. This line was developed from White leghorn breed and mated randomly over last 12 generations. Five male and five female birds in total of 10 birds each were slaughtered at day 1, day 14, day 28 and day 42 days of juvenile period and liver samples were collected. In total, the experiment was conducted on forty birds of CL chicken line. All the birds were slaughtered following the approved slaughtered protocol of the Institutional Animal Ethics Committee (IAEC) of ICAR-DPR, Hyderabad. Liver samples were collected into 1.5 ml RNase free sterile polypropylene micro centrifuge tubes under aseptic conditions by using DEPC treated sterile instruments. Samples were chilled immediately on ice to minimize RNA degradation and kept at -80ºC for further RNA extraction.

RNA extraction

Total RNA was isolated from liver samples using Trizol (Amresco, USA) according to the manufacturer's protocol. Upon aqueous phase separation, RNA was precipitated in isopropanol and washed in 75% (v/v) ethanol. The RNA pellet was re-suspended in 50 µL of nuclease free water RNA quantity and quality were evaluated using the NanoDrop (Nanodrop technologies, Wilmington, DE, USA). The RNA integrity was checked by 2.2M formaldehyde denatured agarose gel electrophoresis ensuring that the 18s and 28s ribosomal RNA bands were intact. The RNA samples showing the OD_{260}/OD_{280} range 1.9 to 2.2 were considered as good quality and subjected to first strand complementary DNA (cDNA) synthesis.

First strand cDNA synthesis

cDNA was synthesized by using High-Capacity cDNA Reverse transcription Kit (Applied Biosystems, #4368814) in a final volume of 20 µL containing 10X Reverse Transcription (RT) buffer (2 µl), 10X RT random primers (2 µl), 100 mM of 25X dNTP mix (0.8 µl), RNase inhibitor (1 µl), MultiScribe™ Reverse Transcriptase (1 µl), Nuclease-free H_2O (3.2 µl) and 1 ng RNA (10 µl). Reverse transcription was carried out in thermocycler (Mastercycler, Eppendorf, Germany) following the manufacturer's instructions, which includes the incubation of reaction at 25˚C for 10 min followed by 37˚C for 2 hours and 85˚C for 5 min. Synthesized cDNA was stored at -20˚C till further use.

Real time quantitative PCR

The mRNA expression levels of target (*ACACA*) and reference genes (*GAPDH*) were quantified using thermal cycler Applied Biosystems® Step One Real Time PCR (Life Technologies) with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH)* was used as an internal control for normalizing different amounts of input RNA. All PCR reactions were prepared in duplicate with a final volume of 10 µL containing 5 µl of 2X SYBR Green master mix, 0.5 µl of each forward and reverse primer (20 ng), 1.0 µL of cDNA and 3 µL of nuclease free water. Thermal cycling conditions followed were initial denaturation at 95˚C for 10 min followed by 40 cycles of denaturation at 95˚C for 30 sec, primer annealing at 58˚C for 30 sec and extension at 72˚C for 30 sec. Melt curve analysis (95˚C for 15 sec and 60˚C for 1 min) was performed at the end of qPCR to check the specificity of amplification. The primers (Table 1) used for quantitative real time PCR were designed with the help of DNASTAR software (Lasergene Inc., USA) from two consecutive exons spanning an intron so that amplification will not be hampered from genomic DNA contamination and will be specific one.

Relative quantification

The quantification cycle (C_q) or threshold cycle (C_t) value of the target and the reference genes were determined for all qPCR reactions. The mRNA expression the gene was analyzed by comparative C_t method of relative quantification. The gene quantification was expressed as "n-fold up/down regulation of transcription" in relation to an internal control. The expression of target gene was calibrated by that of the reference gene (*GAPDH)*, at each time point and converted to the relative expression (fold of expression), as follows:

Table 1: Primer details for amplification of genes

Gene	Primer Sequences (5'-3')	Amplicon Size (bp)	Annealing temp (°C)	NCBI Accession number
ACACA	F: CAGATTTGTTGTCATGGTGAC R: ACAGCCTGCACTGGAATGC	162	58	NC_006106
GAPDH	F: CTGCCGTCCTCTCTGGC R: GACAGTGCCCTTGAAGTG	119	58	NM_204305

Fold of expression = $2^{-\Delta\Delta Ct}$, where

ΔC_t = Average C_t of target gene - Average C_t of reference gene (*GAPDH*)

$\Delta\Delta C_t$ = Average ΔC_t of target sample - Average ΔC_t of calibrator sample

Serial ten-fold dilutions of cDNA template were prepared to standardize real time PCR reactions for gene specific primers of target (*ACACA*) and reference (*GAPDH*) genes. At each dilution, mean Cq/Ct values were recorded separately and the relative standard curve was developed by taking cDNA on X-axis as independent variable (in log) and quantification cycle on Y-axis as dependent variable.

Statistical analysis

The raw fluorescence data of both target and reference genes were subjected to logarithmic transformation for further statistical analysis. Statistical analysis was carried out using SPSS (Version 19) software. The ANOVA test was performed to compare the mRNA expression levels of target gene at different ages. Univariate General linear model with Tukey's HSD and DMRT as post hoc test was performed to differentiate mRNA expression levels of target genes among age groups in layer population. The statistical model used for this analysis was:

$$Y_{ijk} = \mu + A_i + S_j + e_{ijk}$$

Where, Y_{ijkl} is expression of each individual at each gene group in the population, μ is overall mean, A_i is the fixed age of population, S_j is the fixed effect of sex, and e_{ijk} is random error distributed normally with mean zero and common variance σ2 (NID~0, σ^2_e).

RESULTS AND DISCUSSION

The standard curve pertaining to optimization of real time PCR for expression of *ACACA* and *GAPDH* genes were prepared in which the regression coefficient (slope) as -3.448 and -3.348. The coefficient of determination (R^2) for the expression analysis was observed as 0.992 and 0.9999 for *ACACA* and *GAPDH* genes, respectively. A significant regression coefficient was found for target and reference genes representing an optimum standardization of gene expression prior to carry out the actual experiments. Literature revealed preparation of standard curves for expression of *Activin II B, HPRT1* (in Control Broiler and Aseel), *Ovalbumin* and *β-actin* (in pigeon) genes (Guru Vishnu *et al.,* 2017 and Zhang *et al.,* 2014). They also reported higher magnitudes (0.926 – 0.9999) of R^2 value depicting better efficiency of gene expression analysis which corresponds to our result in *ACACA* and *GAPDH* in CL chicken.

As the age advances, the gene expression (40-ΔCt) showed an increasing trend with the highest and lowest magnitude of expression observed on day 42 (39.16) and day 1 (35.74), respectively (Fig. 1).

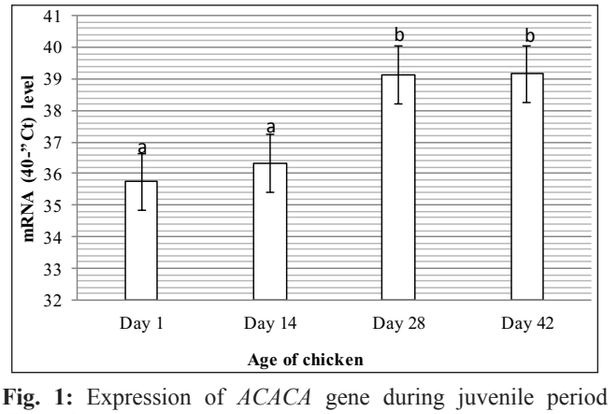

Fig. 1: Expression of *ACACA* gene during juvenile period (P<0.05)

Overall, a significant difference ($P<0.05$) in expression level ($40-\Delta C_t$) was observed among age groups (day 1, day 14, day 28 and day 42). Sex had no significant effect on the expression of target gene. However, the magnitude of gene expression in males was 1.8 fold higher than that of female birds. Guru Vishnu *et al.* (2017) reported non-significant fold change of expression of *Activin II B* gene in male birds over female birds. Fold change of expression in relation to different ages were mentioned in Table 2. When compared to day 1 expression, upregulation of gene expression was observed in control layer chicken population by 1.5, 10.5 and 10.8 folds at day 14, 28 and 42 of age, respectively (Table 2). When expression at day 42 was taken as calibrator, it was down regulated on day 14, 28 and day 42 by 10.77, 7.11 and 1.02 folds, respectively. At the day old age, the bird consumes less feed and also relatively requires less energy. But, as the age advances, the feed consumption was increased, so that the excess energy was stored as fat produced through fatty acid biosynthesis. As the age progressed, in chicken the size of the body as well as organs increased on account of cell growth, development and proliferation. In this process, lipids play a pivotal role as a structural component in cell membrane (Bieberich, 2012). In our study, we noticed the higher magnitude of expression of gene during 42 days of age as compared to day old, 14 and 28. However, the excess fat was stored and deposited in the inter-cellular space making the organ/body parts becoming fatty. Selecting broiler chickens for rapid growth rate has resulted in an increase in food intake and associated fat deposition in adipose tissues in muscle, abdomen, liver etc during post-hatch (Emmerson, 1997). Bai *et al.* (2015) also reported simultaneous increase in body weight and fat deposition in adipose tissue as age progressed. In human also the earlier study reported that occurrence of cancer enhanced de novo fatty acid biosynthesis in tumor cells which was proven by inoculating chemical inhibitors or by RNAi-mediated gene silencing (Jones *et al.,* 2017).

Table 2: Fold change of expression of *ACACA* gene among different age groups in layer chicken

	Day 14	**Day 28**	**Day 42**
Day 1	1.5	10.5	10.8
Day 14		7.1	6.9
Day 28			1.02

A significant negative correlation (-0.3) has been noticed in relation to body weight and expression at 42 days of age indicates the complete utilization of energy for the maintenance as the birds were slow growing and egg production has not been started at that age.

In conclusion, it may be stated that there was differential expression of *ACACA* gene in liver from day old to 42 days of age in layer chicken, which can form a base line for the research to reduce the fat accumulation in the body in order to improve the performance of layer birds during laying period.

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