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Shivaprasad Ch
Livestock Farm Complex, College of Veterinary Science, Korutla, Jagtial, Telangana, India

Vinoo R
Department of Animal Genetics and Breeding, N.T.R College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Chatterjee RN
ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, Telangana, India

Muralidhar M
Department of Animal Genetics and Breeding, College of Veterinary Science, Garividi, Andhra Pradesh, India

Narendranath D
Department of Poultry Science, N. T. R College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Aswanikumar K
Department of Veterinary Biochemistry, N.T.R College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Bhattacharya TK
ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, Telangana, India

Sushma G
Department of Animal Genetics and Breeding, N.T.R College of Veterinary Science, Gannavaram, Andhra Pradesh, India

Kanakachari M
ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, Telangana, India

Prasad AR
ICAR-Directorate of Poultry Research, Rajendranagar, Hyderabad, Telangana, India

Corresponding Author:
Shivaprasad Ch
Livestock Farm Complex, College of Veterinary Science, Korutla, Jagtial, Telangana, India

Expression profile of acetyl CoA carboxylase beta (ACACB) gene in white leghorn line during the pre and post-hatch period

Shivaprasad Ch, Vinoo R, Chatterjee RN, Muralidhar M, Narendranath D, Aswanikumar K, Bhattacharya TK, Sushma G, Kanakachari M and Prasad AR

Abstract
The present study initiated with an objective of expression profile of Acetyl CoA carboxylase beta (ACACB) gene during pre and post-hatch period in White leghorn IWK line. ACACB gene plays a critical role in fatty acid metabolism thereby potentially regulating meat and egg quality. Temporal expression pattern of the ACACB gene in Quantitative real-time PCR (QPCR) indicated that the gene expresses in all stages in the skeletal muscle of chicken during pre-hatch (embryonic day (ED) 5th to ED 18th) and post-hatch (18th, 22nd and 40th week) period. During pre-hatch period highest expression at ED10 (37.86±0.37) and lowest at ED13 (32.15±0.21) was found. However, a significant (p<0.05) differences of gene expression was observed in different embryonic days. Whereas expression levels of ACACB gene during post-hatch period (p<0.005) within group was not significant. The magnitude of ACACB gene expression was highest on 40th week (32.21±0.44) and lowest on 18th week (29.27±1.16) was observed. The present study suggests that the ACACB gene plays a crucial role in pre and post-hatch period in IWK line.

Keywords: ACACB gene, expression, pre and post-hatch, QPCR, chicken

Introduction
During the past six decades, the application of selection and breeding methods based on population genetics and statistics allowed the development of chicken with high productive efficiency. These systems were based on simplified models of genic action that assume a large number of genes with small individual effects in the expression of the phenotype. By using molecular techniques through a candidate gene approach help to solve some of the limitations of the current methods. The Acetyl-CoA Carboxylase Beta (ACACB or *ACC-β* or *ACC2*) plays a key role in β-oxidation pathway. Two major isoforms of *ACC*, *ACACA* (Acetyl-CoA carboxylase A) and ACACB, which is an intermediate substrate that plays a pivotal role in the regulation of fatty acid metabolism and it is the rate-limiting step in fatty acid synthesis. The ACACB generated malonyl-CoA functions as inhibitor of the carnitine/palmitoyl-transferase 1 (CPT1) activity and the transfer of the fatty acyl group through the carnitine/palmitoyl shuttle system to inside the mitochondria for β-oxidation. (McGarry *et al*., 1978; Ruderman *et al*., 1999; Wakil and Abu-Elheiga. 2009) [15, 22, 28]. The net result is reduced fatty acid oxidation and increased fatty acid and triglyceride synthesis, at the expense of glucose utilization. ACACB was first discovered in rat heart (Thampy, 1989) [26], localized in chromosome 15 (NCBI). ACACB (M_r~280 Kda) is found primarily in non lipogenic tissues such as skeletal and heart muscle and to a lesser extent in liver (Abu-Elheiga *et al*., 1995, 1997; Lopaschuk *et al*., 1994; Ha *et al*., 1996) [1-2, 14, 11]. Ji *et al.* (2012) [12] studied avian adipose tissue transcriptome and metabolism in chicken under two different diets, one under fasting and the second under insulin deprivation in commercial chicken and observed that several genes were up regulated in fasting, including the fatty acid oxidation gene ACACB suggesting its role in fat metabolism.
The prevalence of accumulation of fat affects body composition and quality of meat and egg. Although high mass of white adipose tissue reduces the nutritional and economic value of a chicken, because it wastes feed by its conversion to adipose rather than lean tissue. Excess fat may contribute to the development of detrimental traits arising from genetic selection for rapid growth, such as reduced fertility and immunocompetence, both of which are seen in obese

humans (Siegel and Wolford 2003) [24]. High-fat content in poultry can be a risk to human health (Milićević *et al.*, 2014) [17]. Obesity is a serious public health problem around the world that increases the risk of some common diseases, such as type 2 diabetes, cardiovascular disease, metabolic syndrome, hypertension, and a few types of cancer (Kopelman, 2000; Alvehus *et al.*, 2010) [13, 5]. However, the excessive fat in modern poultry strains has been one of the major problems facing the poultry industry (Zhou *et al.*, 2006) [31]. For example, Choct *et al.* (2000) [9] found that modern broiler strains contain 15% to 20% fat and >85% of this fat is not physiologically required for body function. Laying hens also exhibit excessive fat accumulation, which negatively affects their reproductive performance (Xing *et al.*, 2009) [29]. So the chicken industry requires new strategies to reduce the fatness in chicken meat and egg, which would economically benefit producers and likely improve health and welfare of chicken and human being. The expression level of this particular gene at different age groups will form the base for understanding its action and subsequently used for manipulating the gene functionality to reduce fat deposition. Therefore, the present study was conducted to know the expression levels of the ACACB gene during the pre and post-hatch period in the white leghorn IWK line.

Materials and Methods

Experimental birds

The present study was conducted on IWK lines of the White Leghorn breed. IWK line is being subjected to selection for higher egg production up to 64 weeks of age as well as for higher egg weight at 28 weeks of age. Which are maintained at ICAR-Directorate of Poultry Research (ICAR-DPR), Hyderabad.

Sample collection

Breast muscle tissues were collected to study the trend of ACACB gene expression. Whole embryos were collected through embryonic days (ED) ED 5 to ED 8 (n=4 per day) and breast muscle tissues were collected in the course of ED 9 to ED18 (n=4 per day). While during post-hatch before on set of egg production (i.e.18th WK) 4 birds, after onset of egg production (22nd WK) 4 birds and during 40th week, 4 birds were slaughtered to collect breast muscle tissues under aseptic conditions following the approved slaughtered protocol of ethical committee of ICAR-Directorate of Poultry Research, Hyderabad. Breast muscles were collected in 1.5 ml DNAse, RNase free sterile polypropylene tubes under aseptic conditions using 0.1% Diethyl Pyrocarbonate (DEPC) treated sterile instruments. Samples were immediately chilled on ice to minimize RNA degradation and transferred to lab on ice in ice pack box and kept in -80 °C until further use.

RNA extraction

Total RNA was isolated from embryo and muscle tissues using Trizol (Amresco), according to the manufacturer's protocol. Resulted RNA pellet was re-suspended in 50µl nuclease-free water and the concentration and quality were determined using respective Genova plus Nano Drop and 1.2% formaldehyde agarose gel. The RNA sample showing the OD_{260}:OD_{280} values in between 1.8 to 2.2 were considered as good quality and were used further.

First strand cDNA synthesis

Each sample of RNA was treated with DNaseI (Fermentas) and converted in to cDNA using Verso cDNA synthesis kit (Thermo Scientific, #00775881). This reverse transcription was carried out in thermocycler (Himedia) using the components 5X cDNA Synthesis Buffer (4 µl), Anchored Oligo dT (0.25 µl), Random Hexamer (0.75 µl), dNTP Mix (2 µl), RT Enhancer (1 µl), Verso Enzyme Mix (1 µl), RNA template (RNA) (2 µg) and Nuclease-free H_2O (make up the volume to 20 µl) under the condition 42 °C @ 30 minutes and 95 °C @ 2 minutes. The resulted cDNA was confirmed by agarose gel electrophoresis (Fig.1) and stored at -20 °C until further use. The ACACB and GAPDH genes were amplified by using cDNA as a template in RT-PCR with gene specific primers and were then confirmed by 1% agarose gel electrophoresis. The GAPDH gene was used as internal housing keeping gene for quantification of ACACB expression in real-time PCR (Bhattacharya *et al.*, 2011) [7].

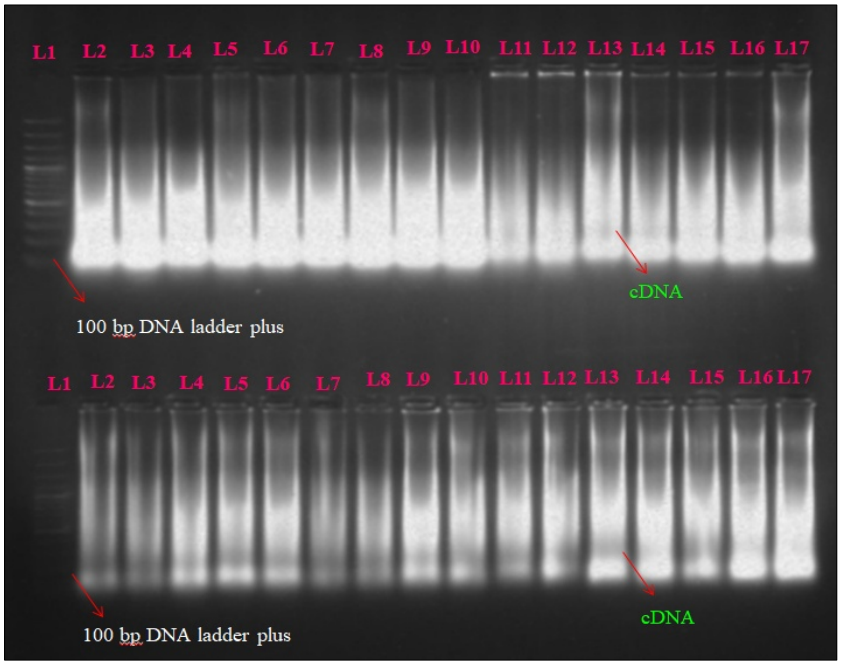

Fig 1: Agarose gel electrophoresis of cDNA amplified product. Lane 1: 100 BP ladder plus; Lane 2-17 cDNA.

Real-time quantitative PCR (qPCR)

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalizing different amounts of input RNA. Intron-spanning primers used for qPCR expression were designed using the IDT Primer Quest software (Table 1). The mRNA expression levels of the target (ACACB) and reference (GAPDH) genes were quantified by using thermal cycler Applied Biosystems® Step One Real-time PCR (Life Technologies) with Bright Green 2x qPCR Master Mix-ROX (abm). All the PCR reactions were performed in three biological replicates with a final volume of 10µl containing 5µl of Bright Green 2x qPCR Master Mix-ROX, 0.5µl of each forward and reverse primer (10 µM), 1µl of cDNA and 3µl of nuclease-free water. Thermal cycling conditions followed were initial denaturation at 95 °C for 10 minutes followed by 40 cycles of PCR stage (95 °C for 15 seconds and 58 °C for 1 minute) and melting stage (95 °C for 15 seconds, 60 °C for 1 minute and 95°C for 15 seconds) was performed at the end of the qPCR to check the specificity of amplification.

Table 1: Primer details for amplification of genes

Gene Symbol	Primer Sequences (5'-3')	Amplicon Size (bp)	Annealing temp (°C)
ACACB	F: GCTCCTGCTGCCCATATATTA R: GTCCGTGATGACACCTTTCT	94	58
GAPDH	F:ATGGGAAGCTTACTGGAATGG R:TCATCATACTTGGCTGGTTTCT	97	58

Relative quantification

Comparative Ct method was used for estimating the expression levels of gene (Bhattacharya *et al.*, 2013) [8]. Threshold cycle (Ct) values of ACACB and GAPDH genes were obtained after performing qPCR. These Ct values were used for estimating the "n-fold up/down-regulation of transcription" and ACACB gene expression levels relative to the internal control by using the following formula;

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

Where

ΔCt = Average Ct of the target gene (ACACB) - Average Ct of the reference gene (GAPDH)

ΔΔCt = Average ΔCt of the target sample – Average ΔCt of the calibrator sample.

Results and Discussion

The acetyl-coenzyme A carboxylase beta (ACACB) gene known to be associated with nephropathy (Tang *et al*., 2010) [25], obesity, diabetes (Riancho *et al*., 2011) [21], end stage renal diseases (Zain *et al.*, 2017) [30] etc. The enzyme plays a crucial role in fatty acid oxidation by catalysing the synthesis of malonyl-CoA, which is a substrate for fatty acid synthesis and a regular fatty acid oxidation (Abu-Elheiga *et al*., 2001) [3]. Mice lacking ACACB gene are reported to be protected against obesity and diabetes (Abu-Elheiga *et al*., 2003) [4].

In order to understand the temporal expression profile of ACACB gene in chicken, particularly during pre and post-hatch period in breast muscle. During early embryonic stage, i.e. up to ED8, whole embryo was used. Merkin *et al.* (2012) [16] while studying evolutionary dynamics of gene regulation in mammalian and bird tissues, indicated that ACACB gene expressed at low to medium level in colon, heart, kidney, lung, skeletal muscle tissue, spleen and testis while its expression is below cut off level in brain and liver. Similarly, a study by Barborasa-Morais *et al.* (2012) [6] also showed that ACACB gene is low to medium in kidney, skeletal muscle and heart and below cut-off in brain and liver. Hence, in chicken, skeletal muscle is targeted for its expression analysis. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as the reference gene in the present study. It showed a considerable amount of stability across different muscle tissues. Serial ten-fold dilutions of cDNA template were made to conduct real time PCR with gene specific primers of target (ACACB) and reference (GAPDH) genes. At each dilution, mean Ct values were recorded separately and the relative standard curve was made by taking cDNA on X-axis as independent variable (in log) and quantification cycle on Y-axis as dependent variable. This standard curve determined the regression coefficient (slope) as -2.941, -4.661 and and coefficient of determination (R^2) as 0.9543 and 0.8374 for ACACB and GAPDH genes, respectively (Fig.2). A significant regression coefficient was found for target and reference genes representing an optimum standardization of gene expression prior to carry out the actual experiment.

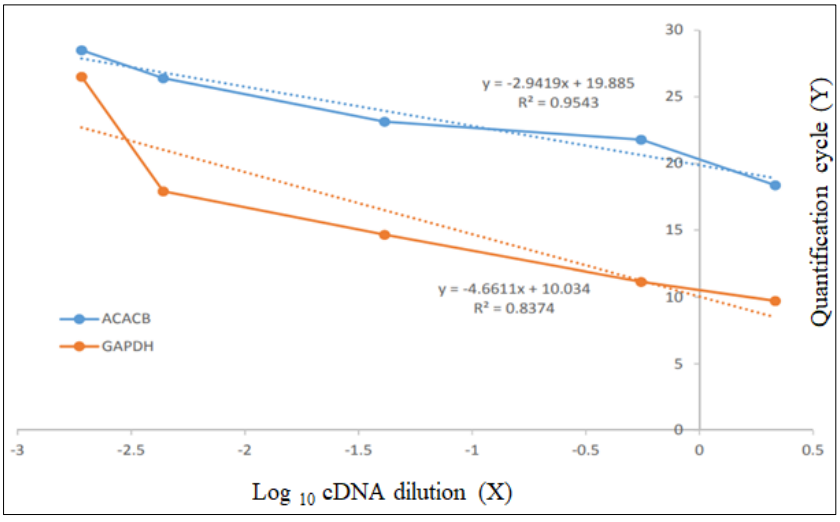

Fig 2: Relative standard curves of reference and target genes in qPCR

ACACB and GAPDH genes were amplified from cDNA using conventional PCR. PCR conditions were standardised for different concentrations of primers and annealing temperatures. Using CDNA as template with gene specific primers, ACACB (fig.3) gene and the reference (GAPDH*;* fig.4) genes were amplified with QPCR. The common comparative threshold method of analysis was used to study the relative gene expression. The expression profile in terms of adjusted ΔCt value (40-ΔCt) *i.e.,* ΔCt value of target gene subtracted from the 40, was computed for the line during pre-hatch and post-hatch period as the number amplification cycle in QPCR was 40. High 40-ΔCt value indicates high expression and low 40-ΔCt indicates low expression of gene. The expression at ED5 (pre-hatch period) and 18th week (post-hatch period) were used as a calibrator to determine the fold change in expression of ACACB gene during different embryonic days (ED) and post-hatch up to 40th week in IWK chicken line.

Expression of ACACB gene during pre-hatch period

The amplification plot of ACACB gene and amplification plot and melt curve of reference gene of IWK line was presented in Fig. 5 and Fig.6, respectively. The Expression and fold change in expression of ACACB gene during pre-hatch period was depicted in Fig.7.

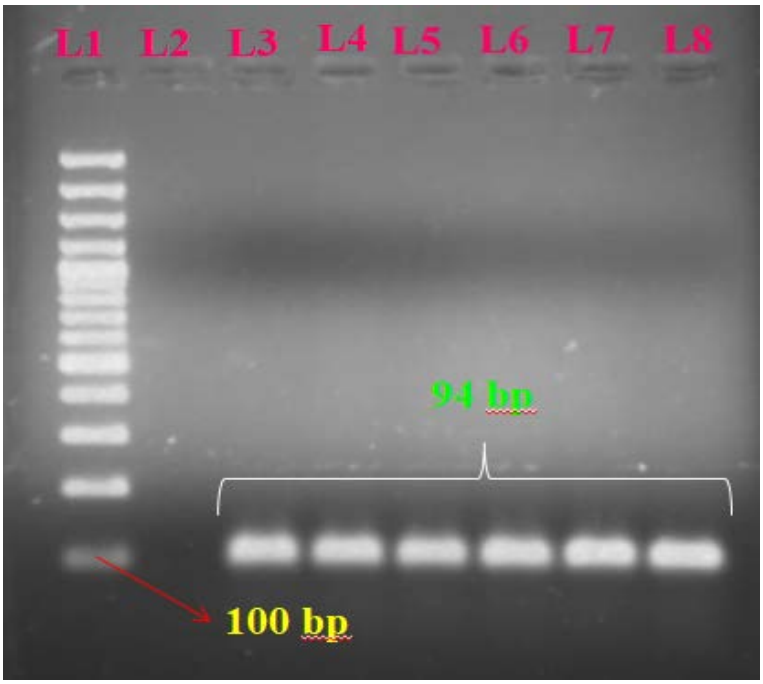

Fig 3: AGAROSE gel electrophoresis of QPCR amplified product of ACACB gene (94 BP); Lane 1: 100 BP ladder Plus; Lane 2: Negative control; Lane 3-8: ACACB fragment (94 BP).

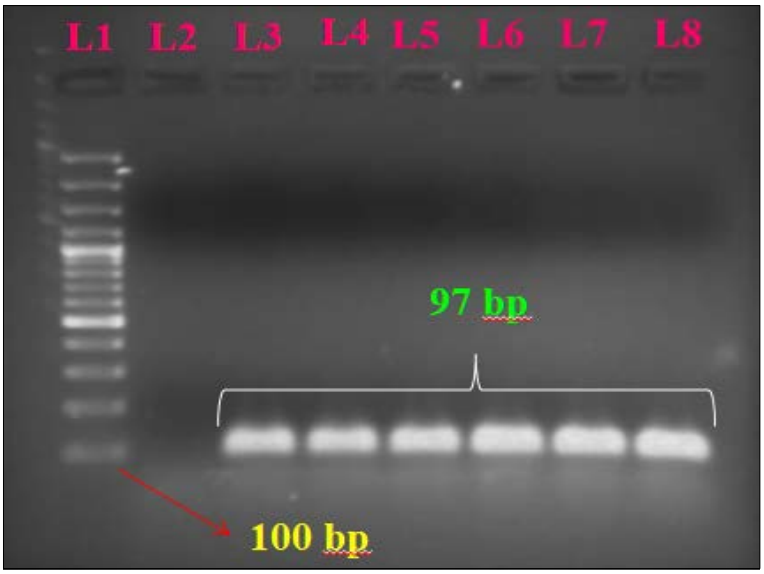

Fig 4: AGAROSE gel electrophoresis of QPCR amplified product of GAPDH gene (97 BP); Lane 1: 100 BP ladder plus; Lane 2: Negative control Lane 3-8: GAPDH fragment (97 BP).

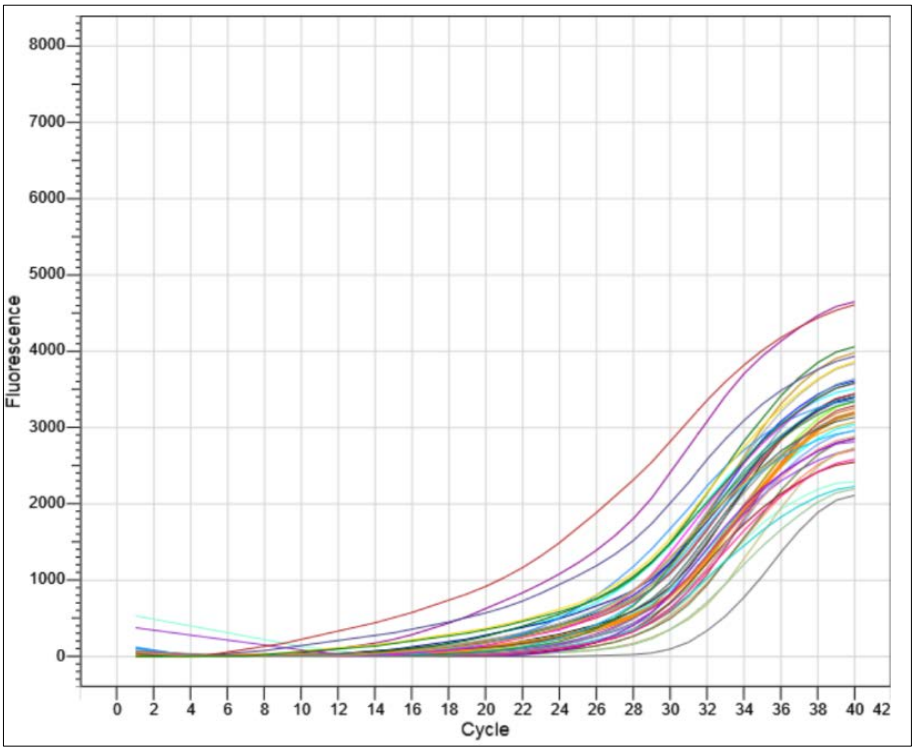

Fig 5: Amplification plot of ACACB gene during pre-hatch period

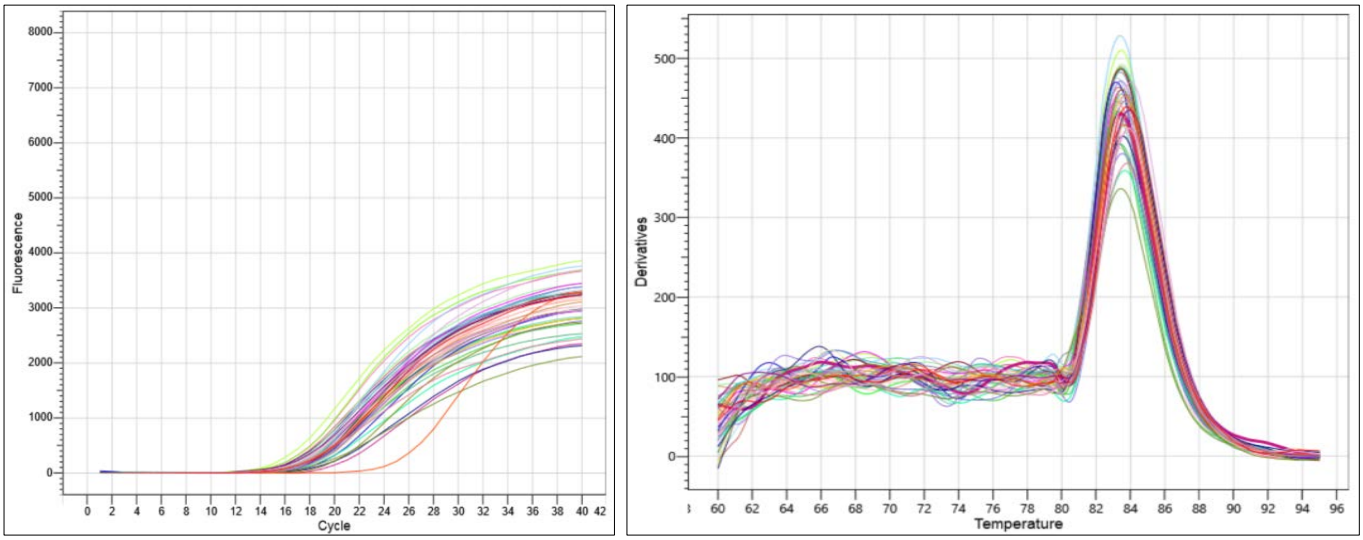

Fig 6: Amplification plot and melt curve of GAPDH gene during pre-hatch period

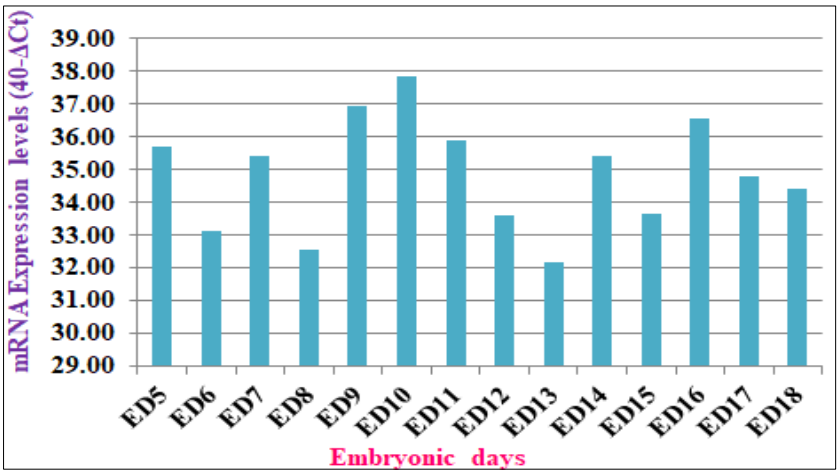

Fig 7: Expression levels of ACACB gene during pre-hatch period

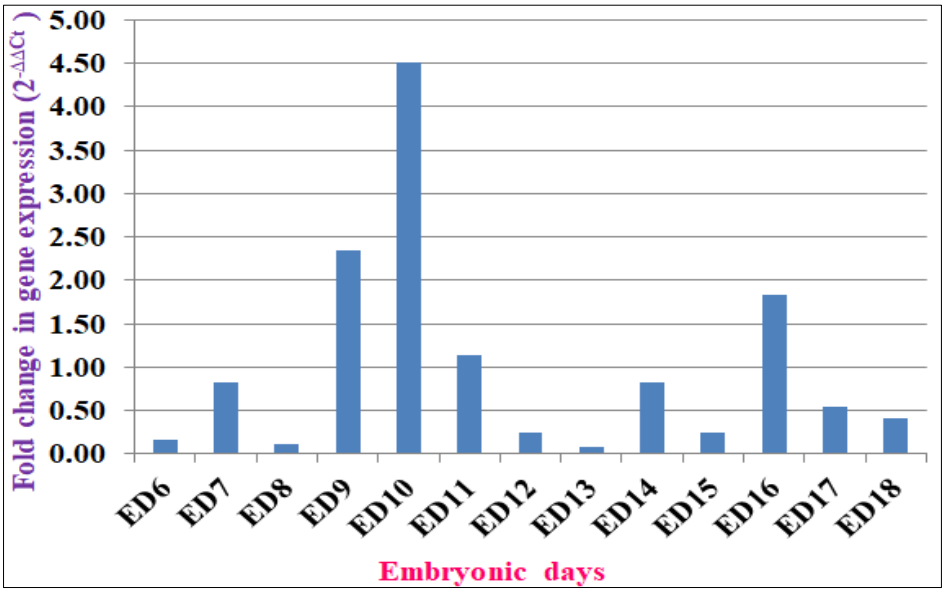

Fig 8: Fold change in expression of ACACB gene during pre-hatch period.

And Fig.8, respectively. The gene was expressed in all the days studied during pre-hatch and post-hatch period. Highest expression at ED10 (37.86±0.37) and lowest at ED13 (32.15±0.21) was found with zigzag expression was observed from ED5 to ED18. However, a significant (p<0.05) differences of gene expression was observed in different embryonic days (Table 2). When expression at ED5 was taken as calibrator, fold change in gene expression was initially down regulated from ED6 to ED8 i.e. 0.17, 0.82 and 0.11 then up regulated up to ED11 i.e. 2.34, 4.51, and 1.14 followed by down regulated up to ED18 except ED16 i.e. 0.24, 0.09, 0.83, 0.24, 1.83, 0.54 and 0.41 folds.

Table 2: Expression levels of ACACB gene during pre-hatch period.

Pre-hatch period	IWK line
ED5	35.69 ± 0.65^{abcd}
ED6	33.13 ± 1.75^{abc}
ED7	35.41 ± 1.39^{abcd}
ED8	32.56 ± 1.13^{ab}
ED9	36.92 ± 1.26^{cd}
ED10	37.86 ± 0.37^{d}
ED11	35.88 ± 2.75^{abcd}
ED12	33.61 ± 1.18^{abc}
ED13	32.15 ± 0.21^{a}
ED14	35.41 ± 0.66^{abcd}
ED15	33.66 ± 0.37^{abc}
ED16	36.56 ± 1.39^{bcd}
ED17	34.80 ± 0.71^{abcd}
ED18	34.39 ± 0.94^{abcd}

Means with different superscripts (a-d) within each column differ significantly (P≤0.05) (Duncan's MRT)

Expression of ACACB gene during post-hatch period

The amplification plot and melt curve of respective target and reference genes were presented in Fig.9 and 10, respectively. The expression and fold change in gene expression of ACACB gene during post-hatch period was depicted in Fig.11 and Fig.12. Expression levels of ACACB gene during post-hatch period (p<0.005) within groups was not significant. The magnitude of ACACB gene expression was highest on 40th week (32.21±0.44), whereas it was the lowest on 18th week (29.27±1.16). ACACB gene expression on 18th week was used as calibrators to calculate fold change in gene expression at different weeks. When compared to 18th week, the magnitude of ACACB gene expression was up regulated by 1.25 fold on 22nd week and 7.71 fold on 40th we

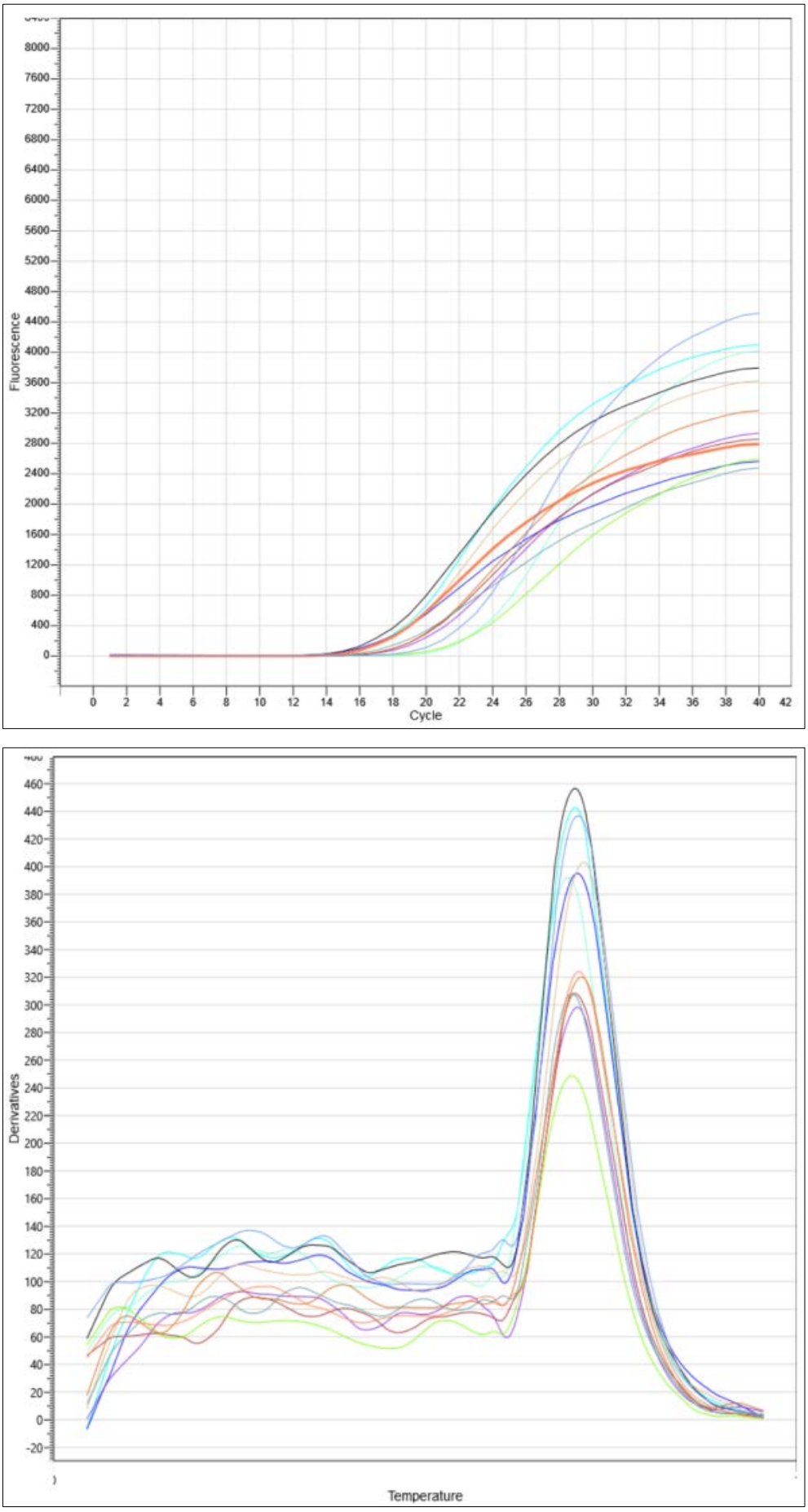

Fig 9: Amplification plot and melt curve of ACACB gene during post-hatch period.

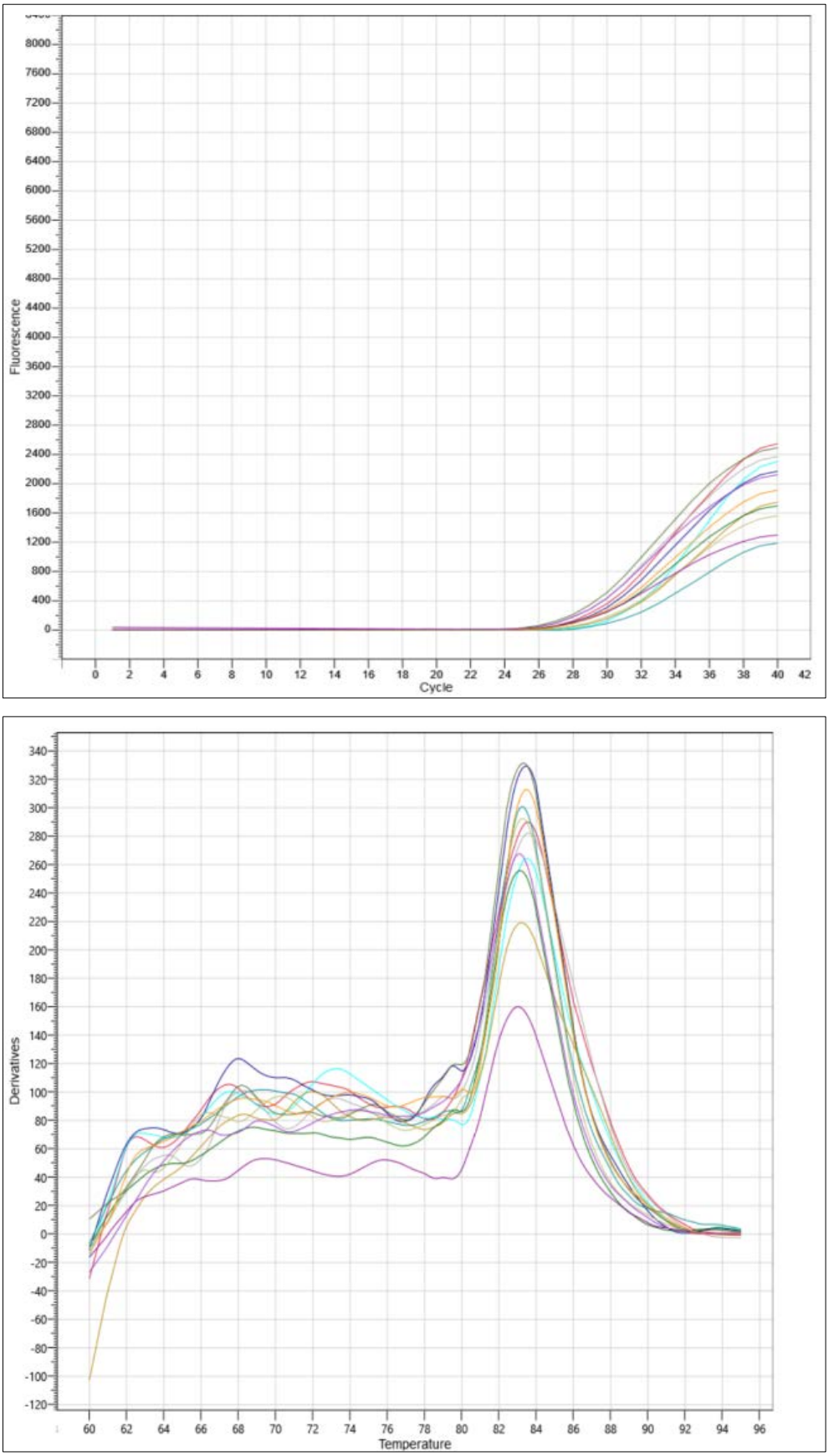

Fig 10: Amplification plot and melt curve of GAPDH gene during post-hatch period.

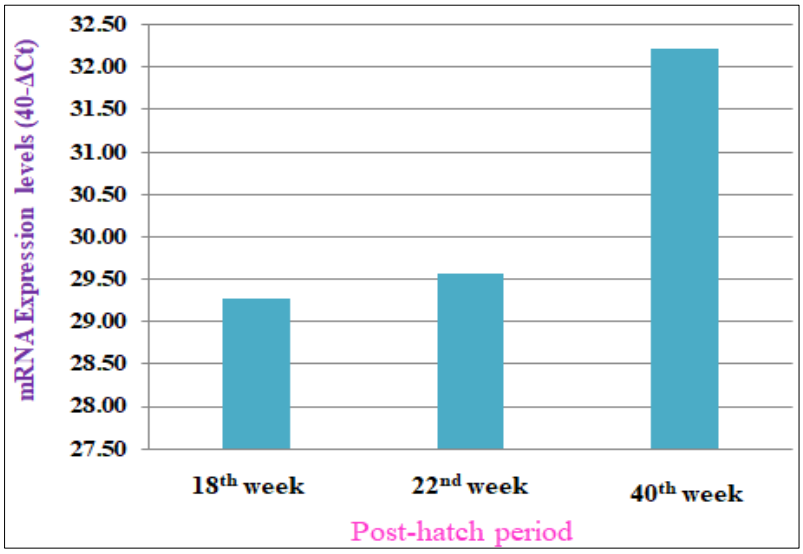

Fig 11: Expression levels of ACACB gene during post-hatch period.

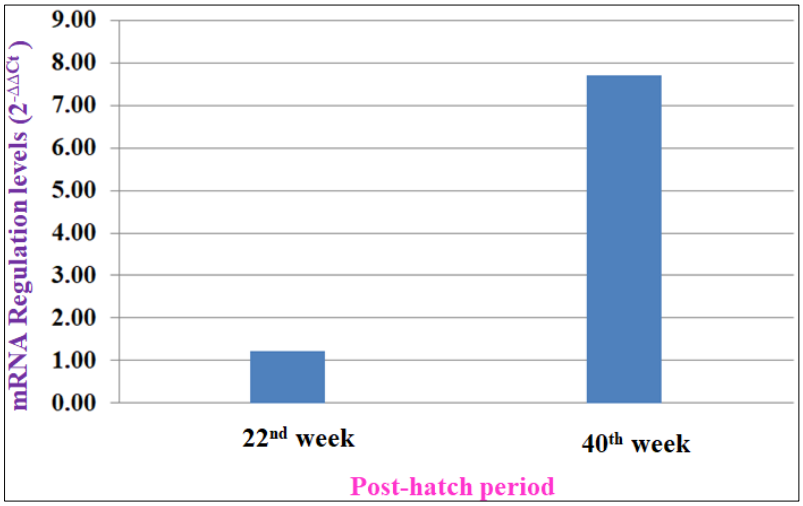

Fig 12: Fold change in expression of ACACB gene during post-hatch period.

There was no reports are available on expression of the ACACB gene during the pre and post-hatch period in chicken. However, some other genes are studied and reported during pre and post-hatch period in chicken. In domestic chicken, the higher embryonic growth was reported during late embryonic stage (Cogburn *et al*., 1989) [10]. In both control broiler and Aseel, the *ACTRIIB* gene expressed at ED7, ED11, and ED16 (Vishnu *et al*., 2017) [27]. Similar results were also found in the post-hatch period in layer chicken line, where the highest and lowest expression level of the *SCD* gene was found on the 2nd (40.75) and 6th week (27.75), respectively (Sagar *et al*., 2019) [23]. In the control layer, the expression of *ACACA* gene was up-regulated during 2nd, 4th and 6th weeks of age, when compared to the day one post-hatch period (Prasad *et al*., 2018b) [19]. In two indigenous breeds i.e. Ghagus and Aseel, the *myoglobin (Mb)* gene expression was studied in different organs like muscle, bursa, heart, spleen, and gizzard at day old and 4th week, respectively (Prasad *et al*., 2019) [20]. In both Aseel and control broiler, the *ACTRIIB* gene expression was studied and showed up-regulated on day one, and downward up to 4th week, and gradually increased at 6th week, respectively (Vishnu *et al*., 2017) [27]. In Ghagus, the expression of *TNNC1* gene was studied in different organs such as muscle, bursa, heart, spleen, and gizzard, respectively (Prasad *et al*., 2018a) [18].

Conclusion

Suggest that the gene is expressed in skeletal muscle of chicken with variation in intensity of expression. This variation could be to meet specific functions during those stages. Exploring protein profile in chicken skeletal muscle may provide further insights into functional role of ACACB in chicken skeletal muscle. Further studies with spatial expression pattern of the gene and experiments aiming at precise functional analysis would provide additional insights into the role of ACACB gene in chicken in providing healthy eggs and meat.

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