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Relative mRNA expression of *CRBP IV* gene and its association with layer economic traits in Rhode Island Red chicken

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ABSTRACT

Cellular retinol-binding protein (*CRBP*) *IV* gene plays an important role in absorption, transport, metabolism and homeostasis of retinol (Vitamin A) and its derivatives. Vitamin A plays vital role in ovarian follicular growth, oviduct and uterine environments, embryo and its development, and oocyte maturation. Present investigation was carried out in Rhode Island Red (RIR) chicken to assess relative mRNA expression of *CRBP IV* gene in kidney, liver and oviduct tissues, collected from 12 birds at 40 weeks of age from four different egg production-body weight groups, viz. high egg production-high body weight (HEP-HBW), high egg production-low body weight (HEP-LBW), low egg production-high body weight (LEP-HBW) and low egg production-low body weight (LEP-LBW), using qRT-PCR method. Data on body weight at 20 weeks of age (BW20) and layer traits, viz. age at sexual maturity (ASM), egg weight at 28 (EW28) and 40 (EW40) weeks and egg production up to 40 weeks of age (EP40) were analyzed by LS ANOVA taking sire as random and hatch as fixed effects. LS means of ASM, BW20, EW28, EW40 and EP40 were 135.19±1.15 days, 1347.13±15.28 g, 42.49±0.27 g, 48.19±0.42 g and 124.55±1.94 eggs, respectively. Expression data was analyzed by LS ANOVA using SAS. The basal mRNA expression of *CRBP IV* gene differed significantly among different egg production-body weight groups in kidney only. The 40-ΔC_t values of mRNA expression levels of *CRBP IV* gene in the four groups were 35.81±1.60, 29.49±1.60, 32.16±1.60 and 39.02±1.60, respectively. Significantly lower expression was observed in HEP-LBW group. Similarly, tissues differed significantly for the *CRBP IV* gene expression. The 40-ΔC_t values of mRNA expression levels of *CRBP IV* gene in kidney, liver and oviduct were 34.12±0.87, 31.67±0.87 and 37.97±0.87, respectively. Highest expression was observed in oviduct. The study generated significant information on basal mRNA expression of *CRBP IV* gene in poultry which may have great potential for use in selection for higher reproductive efficiencies.

Key words: Chicken, *CRBP IV* gene, Gene expression, *q*RT-PCR, Rhode Island Red, Tissues

Chicken is most widely domesticated species of poultry and plays a significant role in global food-security. Poultry farming in India has transformed from backyard farming to a vibrant organized industry during last four decades. Total poultry population in the country is 729.2 million (19th Livestock Census 2012), an increase of 12.39% over 2007 census. The annual egg production is approximately 88.1 billion pieces (BAHS 2017) bringing India at 2nd position in egg production.

Rhode Island Red (RIR), a dual-purpose breed, has gained more appreciation due to its good egg producing ability and its consumers preferred brown shelled eggs. It is getting more popularity in the rural areas also as it is well adapted to the local environmental conditions, possess more disease resistance and preferred by small flock owners. RIR is being maintained at this Institute since 1979. The selection program based on 40-weeks part-period egg production was initiated. The selected strain of RIR (RIR^S) has undergone 35 generations of selection and has attained annual egg production of 214 eggs (CARI Annual Report 2016–17). Long-term selection programme has slowed down the genetic progress over generations due to loss of phenotypic variation in the population. Faster genetic progress in layer breeder flocks is possible only by utilizing results from genomics which will have a significant impact on layer breeding (Albers and Van Sambeek 2002).

Cellular retinol-binding proteins are (CRBPs) are small molecular mass (~15 kDa) proteins that binds specifically to retinol and retinoic acid (Sundelin *et al.* 1985). There are four genes which control the expression of CRBP proteins, viz. *CRBP I*, *CRBP II*, *CRBP III* and *CRBP IV*. *CRBP IV* gene may have roles in absorption, transport, metabolism and homeostasis of retinol and its derivatives

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(Yin *et al.* 2014). Vitamin A (Retinol) plays a vital role in ovarian follicular growth, oviduct and uterine environments, embryo and its development, and oocyte maturation (Schweigert and Zucker 1988), making *CRBP IV* a golden candidate gene for enhancing reproductive traits in chicken and is recently been analyzed (Debnath *et al.* 2019). Therefore, the present study was carried out to estimate the relative mRNA expression of *CRBP IV* gene in various tissues and as well as in different egg production-body weight groups in RIR^S chicken.

MATERIALS AND METHODS

Experimental birds, tissue collection and storage: Twelve Rhode Island Red hens at 40 weeks of age were chosen on the basis of their production performance and body weights. Birds were grouped in to four groups, viz. high egg production-high body weight (HEP-HBW), high egg production-low body weight (HEP-LBW), low egg production-high body weight (LEP-HBW) and low egg production-low body weight (LEP-LBW). Three tissues, viz. liver, kidney and oviduct, weighing approximately 50–100 mg were aseptically collected in RNAlater® (Ambion, USA) and cryopreserved at –80°C until further use.

Isolation of total RNA: Total RNA from each tissue was isolated using TRIzol® reagent (Invitrogen, USA) as per Hongbao *et al.* (2008). The air-dried RNA pellet was re-suspended in 50 µl of nuclease-free water and stored at –80°C until further use. The purity and concentration of total RNA was determined spectrophotometrically taking absorbance at 260 and 280 nm by NanoDrop Spectrophotometer (Thermo Fisher Scientific Inc., USA). Samples showing absorbance ratio (260/280) between 1.8–2 were considered to have satisfactory purity and used further. The concentration of RNA was adjusted to 1000 ng/µl before proceeding to cDNA synthesis.

Synthesis of first strand cDNA: Required amount of total RNA from each sample was taken as template and first strand cDNA was prepared using Verso cDNA synthesis kit® (Thermo Fisher Scientific Inc., U.S.A.) in 20.0 µl reaction mixture as given in Table 1. The reaction tube was spun and then incubated at 42°C for 30 min; then at 95°C for 2 min and finally at 4°C forever. RT enhancer was added to remove contaminating DNA, eliminating the need for DNAse I treatment. Newly synthesized cDNA was checked by PCR for amplification of different genes using PCR amplification program consisting of heat inactivation at 95°C for 7 min followed by 40 cycles of denaturation at 95°C for 20 sec, annealing at 58°C for 20 sec, extension at 72°C for 20 sec, final extension at 72°C for 5 min and hold at 4°C.

Table 1. Reaction mixture for cDNA synthesis

Reagent	Volume	Final concentration
5× cDNA synthesis buffer	4 µl	1×
5 mM dNTP mix	2 µl	500 µM each
Random Hexamer (400 ng/µl)	1 µl	400 ng
RT enhancer	1 µl	
Verso enzyme mix	1 µl	
Template (RNA)	1 µl	1000 ng
Water, nuclease – free	10 µl	
Total volume	20 µl	

PCR products were checked by 1.4% horizontal submarine agarose gel electrophoresis and all cDNA samples were stored at –80°C until use. The concentration of cDNA was equalized to 75.0 ng/µl for subsequent usage in qRT-PCR.

Quantitative reverse transcriptase real time polymerase chain reaction (qRT-PCR): Primer pairs of *CRBP IV* gene and housekeeping or the reference gene (*GAPDH*) were selected from published literature and got synthesized from M/S Xcelris Genomics Labs Ltd., Ahmedabad, India. The nucleotide sequences of the primers, optimized annealing temperature, amplicon length and gene accession number/reference are given in Table 2.

Quantification of relative mRNA expression of *CRBP IV* gene was done by qRT-PCR method in CFX 96® - Real Time PCR detection system (Bio-Rad Laboratories Inc., USA) using DyNAmo ColorFlash SYBR Green qPCR Kit® (Thermo Fisher Scientific Inc., USA) in triplicate in 0.2 ml clear, thin walled nuclease-free 8-tube strips with optically clear flat lid (Axygen Scientific Inc., USA). The reaction mixture (20 µl) consisted of 10 µl of 2× SYBR Green master mix with blue dye, 0.5 µl each of 10 µM forward and reverse primer, 7.5 µl of nuclease free water and 1.5 µl of cDNA (37.5 ng). A negative control (NTC; no template control) was also set up each time. *GAPDH* gene was used as reference gene. Real-time PCR cycling conditions used were initial denaturation at 95°C for 7 min, then 40 cycles of denaturation at 95°C for 10 sec, annealing at 58°C for 20 sec and extension at 72°C for 20 sec; followed by detection of fluorescent signal by the real time detection system to generate amplification curve. The data were imported into MS-Excel file for further statistical analysis.

Determination of 40–ΔC_T: C_t values of the target gene, minimum two C_t values which showed high resemblance were chosen and averaged for subsequent analysis. ΔCt value for each sample was calculated after subtracting

Table 2. Primers used in qRT–PCR analysis

Target gene	Primer sequences (5'–3')	Ta (°C)	Amplicon size (bp)	References
CRBP IV	F: CATACCACAAGCACATTCAGAGA R: AGTTTGTCATTGTCCCAGGTAAC	58°C	397	Accession No. XM_417606.2
GAPDH	F: CCGTCCTCTCTGGCAAAGTCC R: AGCCCCAGCCTTCTCCATG	58°C	266	Bhanja *et al.* (2014)

average C_t value of housekeeping gene from average C_t value of target gene. The ΔC_t value was subtracted from 40 (total cycle number) so as to obtain 40-ΔC_t. Higher 40-ΔC_t value was considered as higher expression (MacKinnon *et al.* 2009).

Statistical analysis: Tissue-wise differential expression of target gene in four different egg production-body weight groups was analyzed by least squares analysis of variance (LS ANOVA) using JMP 9.0.0 statistical program package (SAS 2010), wherein the egg production-body weight group was taken as fixed effect in the model. Differential expression of target gene in four different egg production-body weight groups and three tissues were analyzed by least squares analysis of variance (LS ANOVA) wherein egg production-body weight groups and tissue were taken as fixed effects in the model.

RESULTS AND DISCUSSION

The basal relative mRNA expression of *CRBP* IV gene was determined in four egg production-body weight groups and in three tissues. For this purpose, first of all, the real time data was analyzed by LS ANOVA to study the influence of egg production-body weight groups on expression of mRNA of *CRBP IV* gene (as 40-ΔC_t), separately in kidney, liver and oviduct tissues (Table 3).

The analysis revealed that the basal expression of *CRBP IV* gene differed significantly among different egg production-body weight groups in kidney ($P \leq 0.05$) only. The least squares mean±standard error of 40-ΔC_t values in different groups is presented in Table 4.

The 40-ΔC_t values of mRNA expression levels of *CRBP IV* gene in the four groups was 35.81±1.60, 29.49±1.60, 32.16±1.60 and 39.02±1.60, respectively. Significantly lower expression was observed in HEP-LBW group. The remaining three groups did not differ statistically ($P>0.05$) for basal expression of *CRBP IV* gene. There was no report available on this aspect to compare or contrast.

Thereafter, the influences of egg production-body weight groups as well as tissues on relative mRNA expression levels (40-ΔC_t values) of *CRBP IV* gene were analyzed together by LS ANOVA and the results are given in Table 5. The expression of *CRBP IV* gene differed significantly ($P \leq 0.0001$) among the tissues. The factor-wise least squares mean±standard error of relative mRNA expression levels is presented in Table 6.

The 40-ΔC_t values of mRNA expression levels of *CRBP IV* gene in kidney, liver and oviduct were 34.12±0.87, 31.67±0.87 and 37.97±0.87, respectively. The highest expression was observed in oviduct, followed by kidney and liver although the latter two did not differ significantly. Contrarily, Debnath (2016) observed highest expression of *CRBP IV* gene in kidney (mean 40-ΔC_t=30.29±0.38) followed by oviduct (29.36±0.41) and liver (28.85±0.68) tissues. Significant tissue differences, viz. small intestine, heart, liver, kidney, oviduct, ovary, pituitary, and

Table 3. Least squares analysis of variance of relative mRNA expression levels (40–ΔC_t values) of *CRBP IV* gene in different egg production–body weight groups in three tissues in RIR chicken

Source of Variation	df	Mean Sum of Squares		
		Kidney	Liver	Oviduct
EP–BW	3	52.13*	8.37312	4.79040
Error/ Remainder	8	7.69	5.05595	3.05137

EP–BW, Egg production – body weight; *P≤0.05; df, degrees of freedom.

Table 4. Least squares mean±standard error of relative mRNA expression levels (40–ΔC_t values) of *CRBP IV* gene in different egg production–body weight groups in three tissues in RIR chicken

Group / Tissue	N	Kidney	Liver	Oviduct
HEP–HBW	3	35.81±1.60^{ab}	32.95±1.29	37.59±1.01
HEP–LBW	3	29.49±1.60^{b}	33.19±1.29	36.35±1.01
LEP–HBW	3	32.16±1.60^{ab}	30.81±1.29	39.15±1.01
LEP–LBW	3	39.02±1.60^{a}	29.75±1.29	38.77±1.01

HEP, High egg production; HBW, high body weight; LEP, low egg production; LBW, low body weight; N, number of observations. Means with different superscripts in a column differ significantly.

Table 5. Least squares analysis of variance of relative mRNA expression levels (40–ΔC_t values) of *CRBP IV* gene in different egg production–body weight groups and in three different tissues in RIR chicken

Source of variation	df	Mean sum of squares 40–ΔC_t values
EP–BW	3	15.35
Tissues	2	120.70****
Error/ Remainder	30	276.27

EP–BW, Egg production – body weight; ****P≤0.0001; df, degrees of freedom.

Table 6. Least squares mean±standard error of relative mRNA expression levels (40–ΔC_t values) of *CRBP IV* gene in different egg production–body weight groups and in three different tissues in RIR chicken

Effects	Group/ Tissue	N	40–ΔC_t values
EP–BW	HEP– HBW	3	35.45±1.01
	HEP– LBW	3	33.01±1.01
	LEP– HBW	3	34.04±1.01
	LEP– LBW	3	35.85±1.01
Tissues	Kidney	12	34.12±0.87^{b}
	Liver	12	31.67±0.87^{b}
	Oviduct	12	37.97±0.87^{a}

EP–BW, Egg production–body weight; HEP, high egg production; HBW, high body weight; LEP, low egg production; LBW, low body weight; N, number of observations. Means with different superscripts in a column differ significantly.

hypothalamus at different ages (12, 24, 32 and 45 weeks) in expression of *CRBP IV* mRNA in Erlang Mountainous chickens have also been reported by Yin *et al.* (2013) wherein the contrasting findings were reported as high expression was found in small intestine, pituitary, kidney and liver, whereas it was low in the heart and intermediate in oviduct. They reported that the *CRBP IV* mRNA levels changed with age in various tissues, as was also reported by Debnath (2016) in kidney ($P<0.05$) and oviduct ($P<0.1$). However, in present study, all the sampled birds were of the same age, hence could not be compared for this aspect.

Owing to the fact that scanty reports are available on *CRBP IV* gene analysis, present study revealed significant difference ($P\leq0.0001$) in basal mRNA expression of *CRBP IV* gene among different tissues and highest expression was observed in oviduct. However, the relative mRNA expression among the egg production-body weight groups, differed significantly ($P\leq0.05$) only in kidney. Further analysis of *CRBP IV* gene with regards to its expression at different ages and also in various other tissues would help in its further exploitation towards use in selection for higher reproductive efficiency in poultry.

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