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mRNA expression profiles of calmodulin and liver receptor homolog-1 genes in chickens

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ABSTRACT. Calmodulin (CALM), a calcium-binding protein, is expressed in the hypothalamic-pituitary-gonadal axis; it plays a pivotal role in the reproductive system by regulating gonadotropin-releasing hormone signaling. Downstream of hypothalamic-pituitary-gonadal signaling pathways, liver receptor homolog-1 (LRH-1) is involved in female gonadal hormone synthesis. In the chicken, although the two genes are known to be associated with reproductive traits, the interaction between gonadotropins and gonadal steroids remains unclear. We used quantitative real-time PCR to quantify the tissular (hypothalamus, pituitary, ovary, liver, kidney, oviduct, heart) and ontogenetic (12, 18, 32, and 45 weeks) mRNA expression profiles of *CALM* and *LRH-1* in Erlang Mountainous chickens to determine their roles in the endocrine control of fertility, and compared these profiles with expression in Roman chickens. We found that the relative expressions of *CALM* and *LRH-1* genes had the highest levels in the pituitary and ovary at 32 weeks. The expression level of *CALM* mRNA in the pituitary of Roman chickens was significantly higher than that in Erlang Mountainous chickens at 32 and 45 weeks, while the *LRH-1* transcript level in the ovaries of Roman chickens was significantly lower than that of Erlang Mountainous chickens at 32 and 45 weeks. In summary, the transcript

levels of *CALM* and *LRH-1* genes are associated with chicken reproductive traits; in addition, we found that the *CALM* gene is the key regulator in the hypothalamic-pituitary-gonadal signaling network.

Key words: Chicken; *CALM*; *LRH-1*; Quantitative real-time PCR

INTRODUCTION

Because the reproductive trait of chicken has high economic importance in the layer and breeding chicken industry, even a slight improvement in fertility ability have received considerable attention for breeders, geneticists, and farmers in recent years. Generally, female fertility requires normal ovarian follicular growth and ovulation, which depends on different hormones and growth factors in the follicular fluid and bi-directional communication between the oocyte and granulosa cells (Semiz and Evirgen, 2009). Several hormones and factors participate in the hypothalamic-pituitary-gonadal (HPG) signaling pathway, and they can be divided into 2 types by their biochemical characteristics, i.e., gonadotropins and gonadal steroids. Accordingly, candidate genes related to the gonadotropins and gonadal steroids can be defined, such as *GHR*, *GnRHR*, *IGF-1*, *ESR*, *PRL*, *PRLR*, and *FSHR* (Sharp et al., 1992; Contijoch et al., 1993; Linville et al., 2001; Sun et al., 2001; Gershon et al., 2007). Further studies are necessary to identify the key regulator of the HPG axis. In this study, we selected candidate genes on the basis of the knowledge obtained from other animal models and tested whether the relative expression levels of these genes are associated with reproductive performance in chicken.

Calmodulin (CALM) is the primary receptor protein of calcium (Ca^{2+}), and Ca^{2+} is a ubiquitous intracellular messenger responsible for controlling numerous cellular processes (Sun et al., 2001). CALM may play a critical role in specifically linking Ca^{2+} flux with extracellular signal-regulated protein kinase (ERK) activation within the gonadotropin-releasing hormone (GnRH) signaling pathway (Roberson et al., 2005). Further studies demonstrated that the frequency of the calcium pulse signal can differentially regulate α, LHβ, and FSHβ transcriptional activity and mRNA expression (Haisenleder et al., 2003a,b). Therefore, Ca^{2+}/CALM may play a pivotal role in the GnRH signal pathway. The expression profile of *CALM* can reflect the activity of GnRH in the anterior pituitary and its involvement in the production and secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

In ovary, the 2 main female hormones, 17 β-estradiol and progesterone, have been characterized as steroid hormones that are directly involved in follicular growth and ovulation (Roberson et al., 2005). Mice null for cytochrome P450 aromatase (Cyp19) exhibit arrested follicular growth (Fisher et al., 1998), and enhanced estradiol action can reverse the ovulatory block (Gershon et al., 2007). *LRH-1* belongs to the NR5A subfamily of nuclear receptors (Boerboom et al., 2000). In recent studies, it has became increasingly evident that *LRH-1* plays a critical role in regulating steroidogenesis, female gonadal hormone synthesis, and sex determination (Roberson et al., 2005; Mueller et al., 2006). Furthermore, *LRH-1* was reported to participate in ovarian follicular development by serving as a critical regulator of multiple mechanisms essential for the maturation of ovarian follicles and ovulation (Roberson et al., 2005).

Analysis of variation in gene expression would enable the study of the different reproductive performances (Cheung and Spielman, 2002). Using quantitative PCR (qPCR), we confirmed that the transcript abundance of the 2 candidate genes are associated with reproduction traits and that *CALM* is key regulator in the HPG signaling network.

MATERIAL AND METHODS

Birds

One hundred and twenty birds representing 2 breeds, namely, Erlang Mountainous chicken (EM, N = 80) and Roman hens (R, N = 40), were used in this study. The former is a Chinese indigenous chicken breed, while the latter is a commercial laying line. All birds were housed on the deep-litter bedding and were transferred to the growing pens at the age of 7 weeks. Birds had access to feed (commercial con-soybean diets meeting the National Research Council's [NRC] requirements) and water ad libitum. Chickens at 4 developmental stages (12, 18, 32, and 45 weeks) were randomly selected and raised under the same conditions on an experimental farm for poultry breeding at the Sichuan Agricultural University (Ya'an, China). Tissue samples (including those from the hypothalamus, pituitary, ovary, liver, kidney, oviduct, and heart) from EM (N = 20/stage) and R (N = 10/stage) chickens were collected at each time point. Fresh tissues were identified, excised, immediately frozen in liquid nitrogen, and stored at -80°C until mRNA was isolated.

RNA isolation and cDNA synthesis

Total RNA was isolated from the hypothalamus, pituitary, ovary, liver, and oviduct tissues by using the Trizol reagent (Invitrogen) according to manufacturer instructions. The quality of RNA was determined by the $A_{260/280}$ absorbance ratio (1.6-1.8) and the integrity of the 18S and 28S rRNA bands on 1% agarose gel. Isolated RNAs were treated with 8 µL DNase (TakaRa Biotechnology Co. Ltd., Dalian, China) for 20 min at 37°C and stored at -80°C. The cDNA was synthesized using the ImProm-II Reverse Transcription System (TakaRa Biotechnology Co. Ltd.) according to manufacturer instructions. The reaction was performed in a volume of 10 µL containing 5X PrimerScript buffer, 10 mM of each dNTP, 40 U/µL RNase inhibitor, and 2.5 µM oligo-dT primer. Reverse transcription was maintained at 30°C for 10 min, 45°C for 25 min, and 99°C for 5 min; it was stopped with incubation at 4°C for 5 min, and the samples were stored at -20°C.

Real-time qPCR (RT-PCR) assay for *CALM* and *LRH-1*

Total RNA extraction and reverse transcription were performed as described above. According to the mRNA sequence of chicken *CALM* and *LRH-1* recorded in GenBank (accession No. NM_205005.1 and NM_205078.1), gene-specific primers were designed by using Oligo 6.0 (Table 1) and the housekeeping gene *β-actin* was used as the reference. RT-PCR assays were carried out by using iQ5 real-time PCR thermal cycle instrument (Bio-Rad, USA). RT-PCR amplification was performed in 20 µL of the reaction mixture containing 2 µL cDNA sample, 10 µL SYBR Premix Ex TaqTM 2X (TakaRa Biotechnology Co. Ltd.), 0.8 µL of each primer (10 µm), and 6.4 µL ddH_2O. PCR was performed with a 30 s incubation at 95°C, followed by denaturation at 94°C for 5 s, annealing at 52.5°C for 30 s, and extension at 72°C for 50 s for 40 cycles. In order to check for non-specific amplification, after the completion of the PCR, melting curve analysis was also performed. The melting protocol consisted of 1 min at 95°C, 1 min at 55°C, and 30 s at 55°C, with 81 cycles in total.

Table 1. Primers for quantitative real-time PCR of *β-actin*, *CALM* and *LRH-1* genes.

Gene	Sequences of primer pairs (5'-3')	Amplicon size (bp)	Tm (°C)	Optimal Ta (°C)	GenBank ID
β-actin	F: TGATGGAGTTGAAGGTGGTCTC R: TCCCTGGAGAAGAGCTACGAG	113	60.0	60.0	NM_205518
CALM	F: GAAGTAGACGCTGATGGCAATG R: GTCAAACACACGGAACGCTTCT	120	62.5	53.5	NM_205005.1
LRH-1	F: GACTCAGGTGATCCAAGCTATGC R: GAGAGGTTACAAAGGGGCTTCTG	143	62.3	53.5	NM_205078.1

Tm = melting temperature. Ta = anneling temperature.

Data analysis

For each sample, the reactions were set up in triplicate to ensure the reproducibility of the results and were analyzed using the $2^{-\Delta\Delta Ct}$ method described previously (Livak and Schmittgen, 2001). Data were represented as means ± SD and were analyzed using the SAS 8.0 for Windows Software (SAS Institute Inc., Cary, NC, USA). The 2 breeds were compared for the expression levels of *CALM* and *LRH-1* in each tissue sample at each stage by using one-way ANOVA. P value of ≤0.05 was considered statistically significant.

RESULTS

Expression profiling of chicken *CALM* and *LRH-1*

The presence of non-specific PCR products and primer dimer artifacts was checked for by melting curve analysis. The amplification efficiency of *β-actin*, *CALM*, and *LRH-1* genes were 96.3, 91.9, and 95.1%, respectively, which were approximately within the expected theoretical values.

At 32 weeks, EM and R chicken were at the peak of egg production. Therefore, we selected the 32-week EM chicken populations for tissular studies of transcripts. Quantitative PCR analysis showed that *CALM* was expressed in all 7 chicken tissues, with the highest levels in the pituitary and hypothalamus, followed by the heart, liver, kidney, ovary, and the oviduct, in that order (Figure 1A). Then, we selected the pituitary, which had the highest expression level in the ontogenetic studies. The accumulation of the transcripts indicated that the abundances of the genes differed in the different stages. In the pituitary, *CALM* had the highest expression at 32 weeks and the lowest, at 45 weeks ($P \leq 0.05$; Figure 2A). In addition, as shown in Figure 1B, we found that at 32 weeks, the expression level of *LRH-1* was high in the ovary, liver, and heart, but low in the other tissues. Further, *LRH-1* expression in the ovary was the highest at 32 weeks and the lowest at 18 weeks ($P \leq 0.05$) (Figure 2B).

Comparison of gene expression levels between the R and EM chicken

To further determine the key regulator gene, we analyzed the expression levels of *CALM* and *LRH-1* in the 2 chicken populations. Figures 3A and B present the mRNA expression levels of *CALM* and *LRH-1* in EM and R birds at 32 and 45 weeks, respectively. The expression level of *CALM* mRNA in R chicken was higher than that in EM chicken at 32 and 45 weeks ($P \leq 0.05$), while the *LRH-1* transcripts of R was lower than that of EM chicken at 32 and 45 weeks ($P \leq 0.05$).

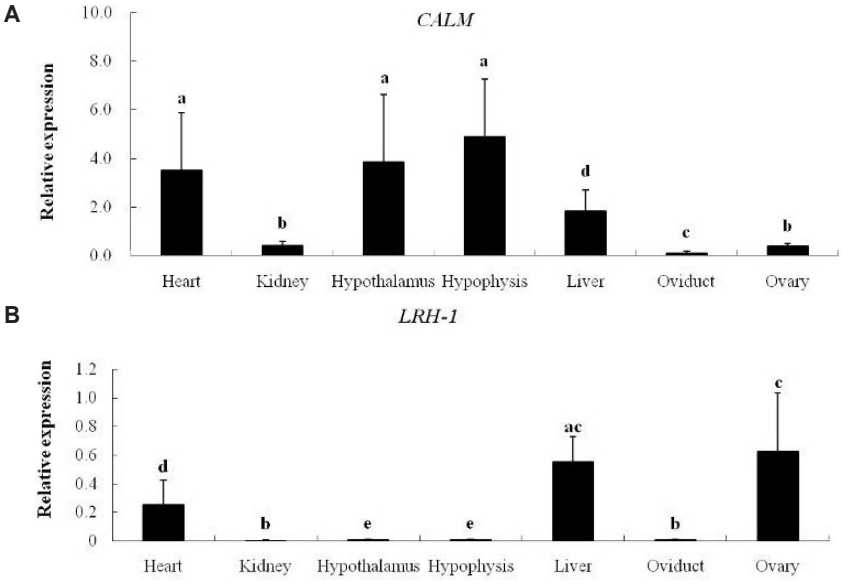

Figure 1. Relative mRNA expression level in different tissues from Erlang Mountainous chicken (N = 20) at growth point (32 weeks) when hens were in high egg-production peak. All expression data were normalized with β-actin mRNA in each tissue and are reported as means ± SD. Different letters above the columns indicate significant differences ($P \leq 0.05$). **A.** Relative mRNA of calmodulin (*CALM*) level in different tissues. **B.** Relative mRNA of liver receptor homolog-1 (*LRH-1*) level in different tissues.

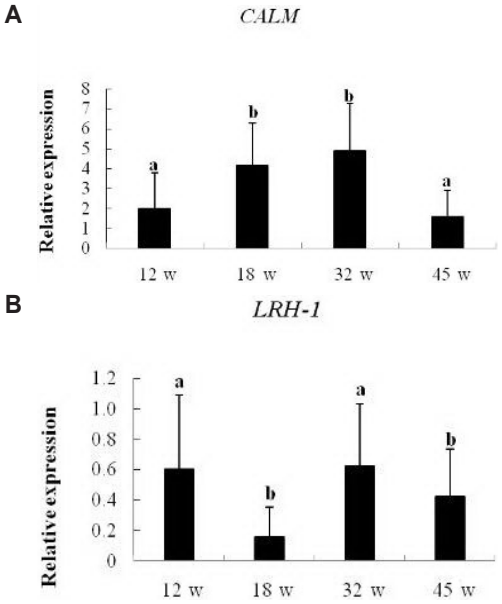

Figure 2. Relative mRNA expression levels at different stages from Erlang Mountainous chicken (N = 20) in predominant tissue. Different letters above the columns indicate significant differences ($P \leq 0.05$). **A.** Relative mRNA of calmodulin (*CALM*) level in pituitary at different stages. **B.** Relative mRNA of liver receptor homolog-1 (*LRH-1*) level in ovary at different stages. w = weeks.

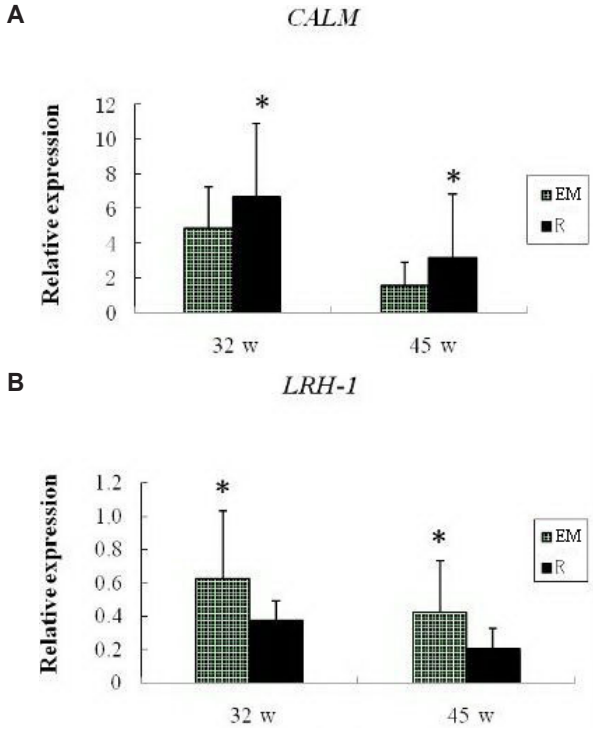

Figure 3. Relative expression levels between two different breeds at 32 and 45 weeks (w). Asterisks above the columns indicate significant differences ($P \leq 0.05$). **A.** Relative mRNA expression level of the calmodulin (*CALM*) gene. **B.** Relative mRNA expression level of the liver receptor homolog-1 (*LRH-1*) gene. EM = Erlang Mountainous chicken. R = Roman hens.

DISCUSSION

Because we limited our study to reproductive traits, it primarily involved a targeted assessment of candidate gene expression in hens. At 32 weeks, when the EM chickens are at the peak of egg production, we quantified the relative expressions in 7 tissue samples. *CALM* was expressed in all tissues, with the highest level in the pituitary and hypothalamus, which is consistent with Kahl's view (Kahl and Means, 2003). CALM is responsible for controlling numerous cellular processes, and studies indicate that CALM and its dependent kinase (CAMK) regulate the intracellular signaling cascades triggered by GnRH (Burger et al., 2008). Additionally, we found that the expression level of *LRH-1* was high in the ovary, liver, and heart, but very low in the other tissues. It provided evidence that *LRH-1* mRNA was expressed in steroidogenic organs (Fayard et al., 2004; Kudo and Sutou, 2006).

Consistent with previous reports, our findings showed that *CALM* and *LRH-1* are predominantly expressed in the pituitary and ovary. We monitored the relative expressions of *CALM* and *LRH-1* in the predominant tissues of EM chicken at 4 stages (12, 18, 32, and 45 weeks). The obtained results indicated that both *CALM* and *LRH-1* had the highest expression level at 32 weeks in the pituitary and ovary, respectively. In a previous study, a coordinated array of signals was attributed to the maturation of ovarian follicles, and for ovulation, go-

nadotropins, and gonadal steroids, such as LH and progesterone, were reported to be important factors (Brännström et al., 2010). During the peak of egg production, these hormones and their receptors were in activated states. Studies in rats showed that the mRNA expression levels of receptors of GnRH, FSH, and LH were elevated in the follicular stages of the estrous cycle (Patsoula et al., 2001; Crawford et al., 2009). Therefore, our results supported the views that *CALM* and *LRH-1* are associated with the synthesis of glycopeptides, gonadotropins, and steroid gonadal hormones in chicken; in other words, we confirmed that the 2 candidate genes are important regulators of HPG axis signaling. Notably, the mRNA expression levels of *CALM* and *LRH-1* were elevated at the 32 weeks group; however, since *CALM* regulates the components upstream of the HPG signaling pathway, it is difficult to determine whether the variations in mRNA expression of *LRH-1* were in response to the upregulation of *CALM*. In general, GnRH stimulates the synthesis and release of the pituitary gonadotropins LH and FSH, which in turn regulate the production of gonadal steroid hormones (Dunn et al., 2003). Thereafter, gonadal steroids act at the hypothalamus and/or pituitary levels to either positively or negatively influence LH and FSH synthesis and secretion; the control of hormone synthesis and secretion is complex, and the detailed molecular mechanism underlying them remains unclear. Therefore, we focused on comparing the expression profiles of the 2 genes in 2 breeds (EM and R) that have different genetic backgrounds.

The relative abundance of gene transcripts is significantly different between the 2 breeds: the expression level of *CALM* was 1.4-fold less and 2.1-fold less in EM chicken than in R chicken and the relative expression of *LRH-1* was 1.6-fold higher and 2.0-fold higher in EM chicken than in R chicken at 32 and 45 weeks, respectively. These findings revealed that the differences of the reproductive traits between the R and EM chicken were mainly attributed to the upregulation of *CALM* in the former. Compared to *LRH-1*, *CALM* is a more important regulator in the HPG signaling network.

Certainly, we cannot ignore the important role of steroid hormones. In the intricate interplay between the components of the HPG axis, GnRH serves as the initiator of the cascade and CALM regulates the activity upstream of the signaling network. The synthesis of steroid hormones may involve some compensation pathways in addition to LRH-1, which is a pivotal regulator. Because of the complexity in biological signal systems (Weng et al., 1999), an understanding of the entire signaling network of the HPG axis is difficult to understand (Brännström et al., 2010); further studies based on a systems biology might facilitate the elucidation of this problem (Zhu and Zhao, 2007).

In summary, we confirmed that *CALM* expression is related to the pituitary's activities, and *LRH-1*, to the ovary and liver. The upregulation of *CALM* mRNA expression may be mainly related to production characteristics. Further studies will be essential to explore the entire signaling network about the interplay of the components of the HPG axis by a system biology approach.

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