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Expression of Follicle-stimulating Hormone Receptor (FSHR) mRNA in the Ovary of Zi Geese During Developmental and Egg Laying Stages

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In order to evaluate the expression profile of follicle-stimulating hormone receptor (FSHR) mRNA in the ovary of Zi geese during developmental and egg laying stages, the expression levels of FSHR mRNA in the ovary of Zi geese at the ages of 1 day, 1, 2, 3, 4, 5 and 8 months (n=8, respectively) were examined by quantitative real-time PCR (qRT-PCR). The results showed that FSHR mRNA expression was greater at the age of 1 to 5 and 8 months compared to expression at day 1 ($P<0.05$). Particularly, the expression of FSHR mRNA at 4, 5 and 8 months was much greater, 1.86±0.14, 3.50±0.19 and 5.11±0.27 fold, respectively, compared to expression at day 1 ($P<0.01$). The level of FSHR mRNA expression at 1, 2 and 3 months was 1.35±0.12, 1.31±0.05 and 1.28±0.09 fold greater, respectively, compared to day 1 ($P<0.05$). The results indicate that the expression of FSHR mRNA remains at a stable level during the early developmental stage, and increases initially from 4 months until the egg laying stage. In addition, these results support the possibility that FSHR plays a pivotal role in mediating the response of the goose ovary to follicle-stimulating hormone during the developmental and egg laying stages, and especially during the latter.

Key words: Follicle-stimulating hormone receptor, Zi goose, ovary, quantitative real-time PCR.

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Follicle-stimulating hormone (FSH), a member of the glycoprotein hormone family, plays a key role in the regulation of reproductive function and ultimately the production of gametes and fertility (HERMANN & HECKERT 2007; MINJ *et al*. 2008). The hormonal activity is mediated through specific cell-surface receptors whose expression ultimately determines the site of action of these hormones. Many studies have demonstrated that follicle-stimulating hormone receptor (FSHR) is expressed only in a subpopulation of gonadal somatic cells, Sertoli cells of the testis and granulosa cells of the ovary (CAMP *et al*. 1991; HOUDE *et al*. 1994; SITES *et al*. 1994). FSHR belongs to the large family of G protein coupled receptors (KOBAYASHI & ANDERSEN 2008; MCFARLAND *et al*. 1989) and is characterized by a large extracellular N-terminal domain involved in high affinity binding of hormone (MEDURI *et al*. 2008; SIMONI *et al*. 1997).

Advances in molecular biology, particularly functional genomics, have led to several studies indicating the role of FSHR in the regulation of ovarian function. The FSHR gene has been studied in poultry including chicken (LIU & ZHANG 2008; WAKABAYASHI *et al*. 1997; YOU *et al*. 1996), duck (ZHOU *et al*. 2003) and quail (AKAZOME *et al*. 1996). Considering the importance of FSHR in the regulation of ovarian function through mediating FSH functional action, an understanding of the regulation of FSHR mRNA expression is a key determinant of endocrine control for developmental and reproductive processes. During the last several decades, the expression profiles of FSHR mRNA were adequately demonstrated in sexual differentiation, follicular development and follicle selection in poultry. Chicken embryonic FSHR gene expression has been quantified from day 10 of incubation to day 14 after hatching using northern

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hybridization (MAO *et al*. 2000). Furthermore, FSHR mRNA was identified in the undifferentiated gonads of chicken embryos from day 4 to day 6 of incubation and has been shown to increase on day 6 in female gonads (AKAZOME *et al*. 2002).The FSHR expression profile in granulosa and thecal layers of developing preovulatory follicles has been adequately studied in chickens (YAMAMURA *et al*. 2001; YOU *et al*. 1996; ZHANG *et al*. 1997). It was observed that the relative expression of FSHR mRNA within granulosa tissue was highest in follicles 6-8 mm in diameter and recently selected follicles 9-12 mm in diameter, and that levels subsequently decreased in large preovulatory follicles (WOODS & JOHNSON 2005). FSH and its receptor are the factors responsible for selection of follicles into the hierarchy (HERNANDEZ & BAHR 2003).

To date, however, and to our knowledge, no studies have addressed the dynamic regulation of FSHR mRNA expression in the ovaries of geese during the developmental and egg laying stages. Therefore the present experiments were conducted to determine the expression levels of FSHR mRNA in the ovary of the goose during these stages, providing a better understanding of the functional action of FSHR with regard to the processes of goose ovary development and egg laying.

Material and Methods

Geese and tissue collection

Fifty-six female Zi geese were selected from 100 geese in a local breeding farm and raised according to the standard program used at the farm. Eight geese were killed at the age of 1 day, 1, 2, 3, 4, 5 and 8 months, respectively, for samples of ovaries. Geese were sacrificed by electrical stunning followed by exsanguination. Ovary samples were rapidly removed, wrapped in foil, frozen in liquid nitrogen, and then stored at -70°C until analysis.

Total RNA isolation

Total RNA was prepared from Zi geese ovaries at 1 day, 1 to 5 and 8 months according to the Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA) manufacturer's instructions. Each of the samples of total RNA (n=8, respectively) were pooled, and then stored at -70 °C until analysis. The yield and quality of total RNA were determined spectrophotometrically using 260 nm absorbance and 260 nm/280 nm absorbance ratio, respectively.

Reverse transcription

Total RNA (1.5 μg) from Zi geese ovary tissues (at 1 day, 1 to 5 and 8 months, n=8), 500 ng/μl of oligo-dT primer (Promega, Madison, WI, USA), 10 mM deoxynucleoside triphosphate (dNTP) Mix (Takara Bio Inc., Dalian, China) and sterile MilliQ water (to a total volume of 12 μl) were heated to 65°C for 5 min in order to disrupt possible secondary structures and then quickly chilled on ice. Thereafter, 5 × First-Strand Buffer was combined with 0.1 M dithiothreitol (DTT) and 40 units/μl of RNaseOUT™ Recombinant Ribonuclease Inhibitor (all from Invitrogen, Beijing, China). The mixture was mixed gently and incubated at 37°C for 2 min. Then a total of 200 units of M-MLV reverse transcriptase was added and incubated at 25°C for 10 min. Reverse transcription was performed at 37°C for 50 min, and the reaction mixture was finally heated to 70°C for 15 min. The first strand cDNA products were then stored at -20°C for later use as template for qRT-PCR.

Construction of FSHR and GAPDH cDNA plasmids

The recombinant plasmids containing FSHR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were termed pFSHR and pGAPDH, which corresponds to the genes FSHR and GAPDH, respectively. Gene-specific primers were designed according to the cDNA sequences using Primer Premier 5.00 (PREMIER Biosoft International, Palo Alto, California, USA) and synthesized com-

Table 1

List of primers used for quantitative RT–PCR

Target gene	Accession number	Primer sequence (5'- 3')	Amplicon size (bp)
FSHR	NM_205079	Forward: TCCTGTGCTAACCCTTTCCTCTA	207
		Reverse: AACCAGTGAATAAATAGTCCCATC	
GAPDH	DQ821717	Forward: GCTGATGCTCCCATGTTCGTGAT	86
		Reverse: GTGGTGCAAGAGGCATTGCTGAC	

FSHR, follicle-stimulating hormone receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

mercially by Takara (Takara Bio Inc., Dalian, China). The primers are listed in Table 1. These constructs were prepared from total RNA in the Zi geese ovary at 8 months, and the complementary double strand cDNA fragments subcloned into the pGEM-T Easy Vector System (Promega, Madison, WI, USA). The exact sequences of the cloned amplicons were analyzed by Takara (Takara Bio Inc., Dalian, China). The plasmid concentrations were spectrophotometrically measured using the GeneQuant pro RNA/DNA Calculator (GE Healthcare, Piscataway-NJ, USA), and the corresponding copy number was calculated using the following equation:

$$\text{Molecules of DNA} = \frac{\text{Mass (in gramms)} \times \text{Avogadro's number}}{(\text{Average mol. wt. of a base} \times \text{template length})}$$

Serial dilutions from the resulting clones were used to produce standard curves, each containing a known amount of input copy number.

qRT-PCR with SYBR Green I chemistry, standard curves and quantitation

The qRT-PCR was performed on the first strand cDNA using the Line-Gene K Real-time PCR Detection System and software (Bioer Technology, Hangzhou, China) with SYBR® Premix Ex Taq™ (Takara Bio Inc., Dalian, China). Briefly, the 50 μl reaction consisted of 1 μl of cDNA, 25 μl of SYBR® Premix Ex Taq™ (2 × Concentration), 0.5 μl of 20 μM of PCR Forward Primer and PCR Reverse Primer, and 23 μl of nuclease-free water. Thermal cycling was performed with an initial denaturation step of 10 s at 94°C, followed by 45 cycles of 5 s at 94°C, and 56°C for 30 s, and then a final extension at 72°C for 20 s. For generation of the standard curves, the pFSHR or pGAPDH standards were also run. Specificity of each reaction was ascertained after completion of the amplification protocol. The PCR products were separated on a 1.5% agarose gel, stained with 0.5 μg/ml of ethidium bromide, and visualized with a Gel-Pro Imager (Media Cybernetics, Inc., Maryland, USA). Relative quantitation of gene expression was performed in three replicates for each sample and normalized to GAPDH. The quality of standard curves was judged by the slope of the standard curve and the square of the Pearson correlation coefficient (R^2). The slope of the line was used to estimate the efficiency of the target amplification using the equation $E = (10^{-1/\text{slope}}) - 1$.

Statistical analysis

Threshold and Ct (threshold cycle) values were automatically determined by the Line-Gene K Real-time PCR Detection software, using default parameters. In the present study, all data were analyzed using SAS statistical software for Windows (SAS Institute Inc., Cary, NC, USA). Relative expression levels for FSHR were calculated relative to GAPDH (normalizer) using the relative standard curve method. The Relative expression level data were expressed as means of three or more measurements ±S.D. The abundance of FSHR in the goose ovary at day 1 was assigned a value of 1. The Student's *t*-test was employed for the comparison of stage-dependent differences in FSHR mRNA. Differences were considered significant at $P<0.05$ and extremely significant at $P<0.01$.

Results

The expression of FSHR mRNA was examined in the ovaries on day 1, and 1, 2, 3, 4, 5 and 8 months. The products were 86 and 207 bp for GAPDH and FSHR mRNA, respectively, and corresponded to the approximate size for each as predicted, and the specificity of these RT-PCR products was available for the qRT-PCR (Fig. 1).

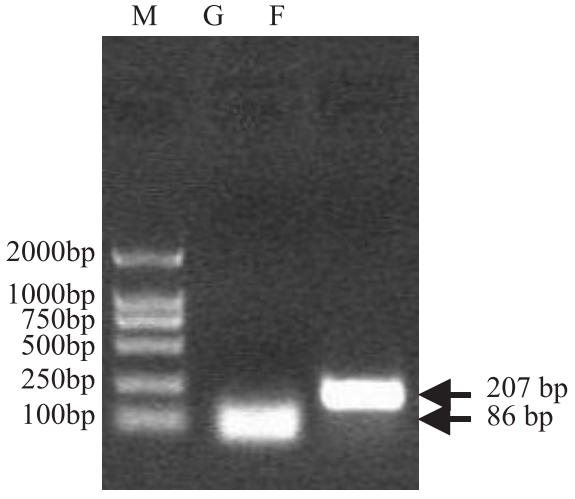

Fig. 1. Representative electrophoresis photo of RT-PCR products for GAPDH (G) and FSHR (F) mRNA in the Zi geese ovary. The amplicons of 86 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 207 bp follicle-stimulating hormone receptor (FSHR) were separated on 1.5 % agarose gel, stained with ethidium bromide and examined with ultraviolet light and visualized with a Gel-Pro Imager (Media Cybernetics, Maryland, USA). A 2000-bp molecular weight marker (M) was used.

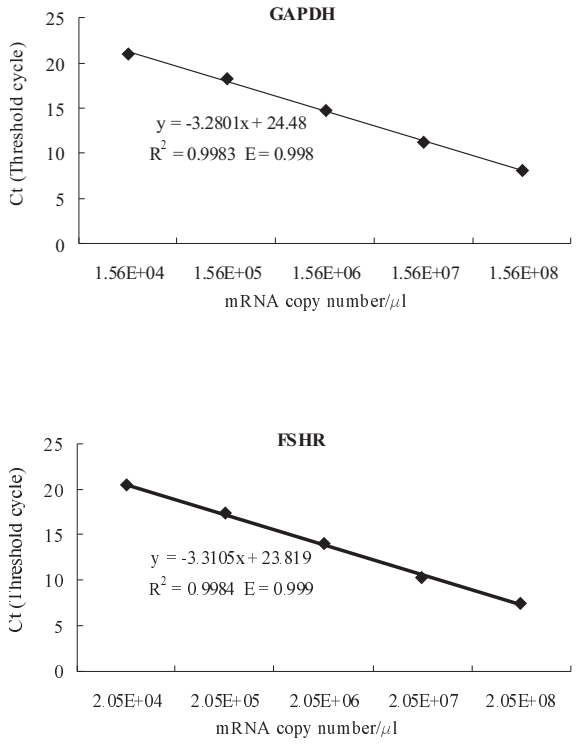

Fig. 2. Standard curves of quantitative real-time PCR (qRT-PCR) amplifications of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and follicle-stimulating hormone receptor (FSHR). Serial dilutions of cDNA plasmids (pGAPDH, pFSHR) were performed for quantification experiments. The curve equations, efficiency values (E) and square of Pearson correlation coefficient (R^2) were plotted.

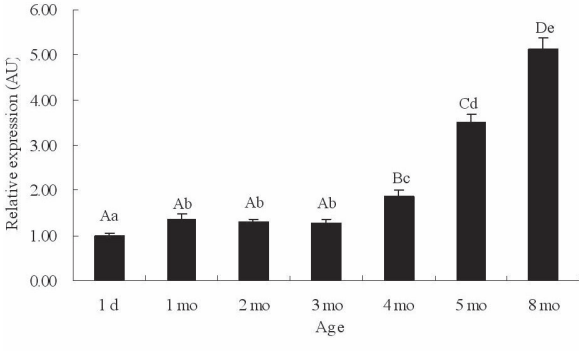

Fig. 3. Relative expression of follicle-stimulating hormone receptor (FSHR) mRNA in the ovary of Zi geese at the ages of 1 day, 1, 2, 3, 4, 5 and 8 months (n=8, respectively). The geese were killed and ovaries were dissected for RNA extraction. The abundance of FSHR transcripts were determined by quantitative reverse transcription-PCR (qRT-PCR) analysis by using gene-specific primers and SYBR Green chemistry (Takara Biotechnology Company, Dalian, China). The expression of FSHR was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene expression levels, calculated by the relative standard curve method, are presented in arbitrary units (AU).Values are means ± S.D. The significance of difference for the amount of FSHR mRNA expression was determined by the Student's *t*-test. Means with the different uppercase letters are extremely different (P<0.01). Means with the different lowercase letters are significantly different (P<0.05).

The PCR efficiencies (E) for standard curves when the plasmids pGAPDH and pFSHR were used as independent templates, are shown in Figure 2, where the curve equations and the R^2 values are also presented.

During the developmental and egg laying stages, the qRT-PCR results showed that FSHR mRNA expression in the Zi geese ovary was greater at the age of 1 to 5 and 8 months compared to day 1 (P<0.05), respectively. Especially, the expression of FSHR mRNA at 4, 5 and 8 months was much greater, 1.86 ± 0.14, 3.50 ± 0.19 and 5.11 ± 0.27 fold, as compared to day 1 (P<0.01), respectively. The level of FSHR mRNA expression at 1, 2 and 3 months was 1.35 ± 0.12, 1.31 ± 0.05 and 1.28 ± 0.09 fold higher than expression at day 1 (P<0.05), respectively. However, the difference of FSHR mRNA expression was not significant among 1, 2 and 3 months (P<0.05) (Fig. 3).

Discussion

The dynamic regulation of FSHR mRNA expression in the ovary of geese during the developmental and egg laying stages still remains to be clarified. Quantitative PCR has become a standard method for measuring gene expression by evaluating the amount of mRNA (ONG & IRVINE 2002). Therefore, in the present work the expression levels of FSHR mRNA in Zi geese ovaries was examined by qRT-PCR. To achieve this purpose, total RNA from geese ovaries of different ages was reversed transcribed, and the synthesized cDNA were submitted to qRT-PCR. The prepared total RNA were of viable quality, as assessed by the integrity of the 28S and 18S rRNA subunits, absence of genomic DNA, and the 260 nm/280 nm ratio (data not shown).

Standard curves were constructed for the relative expression of FSHR mRNA in the Zi geese ovaries. In all standard curves of Ct versus DNA concentration, the R^2 values were more than 0.99, which indicated outstanding linearity between these parameters (PFAFFL 2001). In addition, the efficiency values (E) of FSHR and GAPDH amplification in the experiments were 0.999 and 0.998, respectively. The efficiency values were close to 1.0, indicating the excellent quality of the real-time PCR amplifications (STAHLBERG *et al.* 2003).

In the present study, the expression level of FSHR mRNA in the geese ovary during the developmental and egg laying stages was described for the first time. The level of FSHR mRNA expression in the ovary increased significantly from postnatal to egg laying stage, with the exception of ovaries at the 2 and 3 months. FSH plays a pivotal

role in vertebrate reproduction by regulating cell differentiation, proliferation and steroidogenesis in gonadal tissues (RICHARDS 1994). To exert their hormonal actions on the target cells they bind to their G protein-coupled receptors FSHR. The results from the present study further support the possibility that FSH mediated by FSHR is important for the development of the ovary. The relative expression of FSHR mRNA was not significant during 1, 2 and 3 months in this experiment. The ovary retains silent status prior to the onset of puberty in most mammalian and non-mammalian species (NI *et al*. 2007). Therefore, it is understandable that the FSHR mRNA level is stable during the initiation of the developmental stage. Remarkably, the amount of FSHR mRNA in the ovary of geese at 4, 5 and 8 months was much higher than expression at day 1, and the FSHR level at 8 months was the highest. Zi geese become sexually mature at approximately 5 months of age, and reach peak egg laying at 8 months in Northeast China. Therefore, these findings indicate that the developing ovary is initially highly responsive to FSH at 4 months and remains so until the egg laying stage in geese. In summary, it is possible that the functional actions of FSH involve not only the differentiation of the gonads but also follicular development and selection, and then ultimately egg laying performance in geese.

Taken together, the present study is the first to demonstrate the expression profile of FSHR mRNA in the goose ovary during the developmental and egg laying stages. The expression of FSHR mRNA remains at a stable level during the early developmental stage, and increases initially from 4 months until the egg laying stage. These results support the possibility that FSHR plays a pivotal role in mediating the response of the goose ovary to follicle-stimulating hormone during developmental and egg laying stages, and especially during the latter.

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References

AKAZOME Y., ABE T., MORI T. 2002. Differentiation of chicken gonad as an endocrine organ: expression of LH receptor, FSH receptor, cytochrome P450c17 and aromatase genes. Reproduction **123**: 721-728.

AKAZOME Y., SHIMIZU F., PARK M. K., MORI T., KAWASHIMA S. 1996. Molecular characteristics of the N-terminal region of the quail follitropin receptor. In Vivo **10**: 345-349.

CAMP T. A., RAHAL J. O., MAYO K. E. 1991. Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNAs in the rat ovary. Mol. Endocrinol. **5**: 1405-1417.

HERMANN B. P., HECKERT L. L. 2007. Transcriptional regulation of the FSH receptor: new perspectives. Mol. Cell. Endocrinol. **260-262**: 100-108.

HERNANDEZ A. G., BAHR J. M. 2003. Role of FSH and epidermal growth factor (EGF) in the initiation of steroidogenesis in granulosa cells associated with follicular selection in chicken ovaries. Reproduction **125**: 683-691.

HOUDE A., LAMBERT A., SAUMANDE J., SILVERSIDES D. W., LUSSIER J. G. 1994. Structure of the bovine follicle-stimulating hormone receptor complementary DNA and expression in bovine tissues. Mol. Reprod. Dev. **39**: 127-135.

KOBAYASHI T., ANDERSEN O. 2008. The gonadotropin receptors FSH-R and LH-R of Atlantic halibut (Hippoglossus hippoglossus), 1: isolation of multiple transcripts encoding full-length and truncated variants of FSH-R. Gen. Comp. Endocrinol. **156**: 584-594.

LIU H. Y., ZHANG C. Q. 2008. Effects of daidzein on messenger ribonucleic Acid expression of gonadotropin receptors in chicken ovarian follicles. Poult. Sci. **87**: 541-545.

MAO X., ZHANG C., SAITO N., SHIMADA K. 2000. Expression of messenger ribonucleic acids of luteinizing hormone and follicle-stimulating hormone receptors in the embryonic and posthatch gonads of the chicken. J. Poult. Sci. **37**: 212-220.

MCFARLAND K. C., SPRENGEL R., PHILLIPS H. S., KOHLER M., ROSEMBLIT N., NIKOLICS K., SEGALOFF D. L., SEEBURG P. H. 1989. Lutropin-choriogonadotropin receptor: an unusual member of the G protein-coupled receptor family. Science **245**: 494-499.

MEDURI G., BACHELOT A., COCCA M. P., VASSEUR C., RODIEN P., KUTTENN F., TOURAINE P., MISRAHI M. 2008. Molecular pathology of the FSH receptor: new insights into FSH physiology. Mol. Cell. Endocrinol. **282**: 130-142.

MINJ A., MONDAL S., TIWARI A. K., SHARMA B., VARSHNEY V. P. 2008. Molecular characterization of follicle stimulating hormone receptor (FSHR) gene in the Indian river buffalo (*Bubalus bubalis*). Gen. Comp. Endocrinol. **158**: 147-153.

NI Y., ZHOU Y., LU L., GROSSMANN R., ZHAO R. 2007. Developmental changes of FSH-R, LH-R, ER-beta and GnRH-I expression in the ovary of prepubertal ducks (*Anas platyrhynchos*). Anim. Reprod. Sci. **100**: 318-328.

ONG Y. L., IRVINE A. 2002. Quantitative real-time PCR: a critique of method and practical considerations. Hematology **7**: 59-67.

PFAFFL M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. **29**: e45.

RICHARDS J. S. 1994. Hormonal control of gene expression in the ovary. Endocr. Rev. **15**: 725-751.

SIMONI M., GROMOLL J., NIESCHLAG E. 1997. The follicle--stimulating hormone receptor: biochemistry, molecular biology, physiology, and pathophysiology. Endocr. Rev. **18**: 739-773.

SITES C. K., PATTERSON K., JAMISON C. S., DEGEN S. J., LABARBERA A. R. 1994. Follicle-stimulating hormone (FSH) increases FSH receptor messenger ribonucleic acid while decreasing FSH binding in cultured porcine granulosa cells. Endocrinology **134**: 411-417.

STAHLBERG A., AMAN P., RIDELL B., MOSTAD P., KUBISTA M. 2003. Quantitative real-time PCR method for detection of B-lymphocyte monoclonality by comparison of kappa and lambda immunoglobulin light chain expression. Clin. Chem. **49**: 51-59.

WAKABAYASHI N., SUZUKI A., HOSHINO H., NISHIMORI K., MIZUNO S. 1997. The cDNA cloning and transient expression of a chicken gene encoding a follicle-stimulating hormone receptor. Gene **197**: 121-127.

WOODS D. C., JOHNSON A. L. 2005. Regulation of follicle-stimulating hormone-receptor messenger RNA in hen granulosa cells relative to follicle selection. Biol. Reprod. **72**: 643-650.

YAMAMURA N., TAKEISHI M., GOTO H., TAGAMI M., MIZUTANI T., MIYAMOTO K., DOI O., KAMIYOSHI M. 2001. Expression of messenger RNA for gonadotropin receptor in the granulosa layer during the ovulatory cycle of hens. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. **129**: 327-337.

YOU S., BRIDGHAM J. T., FOSTER D. N., JOHNSON A. L. 1996. Characterization of the chicken follicle-stimulating hormone receptor (cFSH-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. Biol. Reprod. **55**: 1055-1062.

ZHANG C., SHIMADA K., SAITO N., KANSAKU N. 1997. Expression of messenger ribonucleic acids of luteinizing hormone and follicle-stimulating hormone receptors in granulosa and theca layers of chicken preovulatory follicles. Gen. Comp. Endocrinol. **105**: 402-409.

ZHOU Y. C., FU Q. G., ZHAO R. Q., NI Y. D., CHEN J. 2003. Expression of mRNAs for GHR, IGF-IR, FSHR and LHR in granulosa and theca layers of ovarian follicles of Shaoxing ducks. Yi Chuan Xue Bao **30**: 840-846.