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Gene Expression Profile of Estrogen Receptor Alpha and Beta in the Ovaries of Zi Geese (A*nser cygnoides*)

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The profile of ERalpha and ERbeta gene expression in the ovaries of Zi geese at 1 day and 1, 2, 3, 4, 5 and 8 months of age (n=8, respectively) was examined by quantitative real-time PCR (qRT-PCR). The results showed that the expression of ERalpha and ERbeta mRNA was greater at 1 to 5 and 8 months compared with that observed at 1 day. In particular, the level of expression of ERalpha and ERbeta at 8 months was greater, 2.47 ± 0.23 fold and 29.07 ± 1.25 fold, respectively, compared with that at 1 day ($P<0.05$). The expression of ERalpha mRNA was not significantly different at 1, 2, 3 and 4 months ($P>0.05$). The level of expression of ERalpha mRNA at 5 months was 1.86 ± 0.17 fold higher than at 1 day ($P<0.05$). The level of expression of ERbeta mRNA at 2, 3, 4, 5 and 8 months (1.96 ± 0.13, 2.58 ± 0.08, 2.08 ± 0.05, 3.25 ± 0.11 and 29.07 ± 1.25 fold, respectively, $P<0.05$) was significantly higher than at 1 day. In summary, the expression of ERalpha and ERbeta mRNA in the ovaries of geese was increased between newborn and the laying stage. These results suggest that ERalpha and ERbeta mediate the process of ovarian development and egg laying in geese. In addition, ERbeta may play a more important role in regulating the response of the ovary to estrogen during the developmental and egg-laying stages.

Key words: Estrogen receptor alpha, estrogen receptor beta, Zi goose, ovary, quantitative real-time PCR.

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Estrogens play a pivotal role in the development and maintenance of normal sexual and reproductive function (HELDRING *et al.* 2007; MURIACH *et al.* 2008). The biological actions of estrogens are manifested through two high-affinity estrogen receptors, estrogen receptor alpha (ERalpha) and estrogen receptor beta (ERbeta), which belong to a family of transcription factors, the nuclear receptor super family, and are expressed at different levels in target cells (GREENE *et al.* 1986; KUIPER *et al.* 1996; GRUBER *et al.* 2004; TENA-SEMPERE *et al.* 2004). With respect to the functions of estrogens, ERalpha and ERbeta have been chosen as candidate genes to study their relationship with reproductive traits in pigs (GOLIASOVA & WOLF 2004; MUNOZ *et al.* 2004; SPOTTER & DISTL 2006), cattle (SZREDER & ZWIERZCHOWSKI 2007) and sheep (BI *et al.* 2005). The expression of ERalpha and ERbeta in the ovary of pregnant swine has been examined, and the results suggest that estrogens act *via* both ERalpha and ERbeta in the regulation of ovarian function during pregnancy and are involved in the process of successful reproduction (KNAPCZYK *et al.* 2008).

In recent years, the role of ERalpha and ERbeta in the reproductive performance of poultry has been increasingly studied. The expression of ERalpha and ERbeta mRNA has been examined in the ovaries of chickens (KRUST *et al.* 1986) and quail (FOIDART *et al.* 1999; ICHIKAWA *et al.* 2003). The presence of ERalpha and ERbeta mRNA was detected in the ovarian stroma and in the white, yellowish, small yellow, granulosa and thecal layers of the walls of preovulatory follicles in the ovaries of laying hens (HRABIA *et al.* 2008). The change in expression of ERbeta mRNA in ovaries of prepubertal ducks has been elucidated (NI *et al.* 2007).

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Although remarkable progress has been made in understanding the impact of estrogens on ovarian function during ovarian development in poultry, *via* both ERalpha and ERbeta, studies of estrogen receptors in the ovaries of geese are rare. To our knowledge, the profile of ERalpha and ERbeta gene expression in the ovaries of developing and laying geese was not examined until the present study. Therefore, the purpose this study was to investigate the dynamic regulation of the expression of ERalpha and ERbeta mRNA in the ovaries of geese during the developmental and egg-laying stages.

Material and Methods

Experimental design and animals

Fifty-six female Zi geese (*Anser cygnoides*) were selected randomly from 100 geese in a local breeding farm and raised according to the standard program used at the farm. Geese were fed *ad libitum* with rice grain and were supplemented with green grass or water plants whenever possible during the experiment. Eight geese were killed at the age of 1 day and 1, 2, 3, 4, 5 and 8 months to obtain ovaries. The geese were sacrificed by electrical stunning followed by exsanguination. The ovaries were removed rapidly, frozen in liquid nitrogen, and then stored at -70°C until analysis.

Total RNA extraction and reverse transcription PCR

Following manufacturers' instructions, total RNA was prepared from the ovaries of Zi geese with Trizol reagent (Invitrogen Corporation, Carlsbad, California, USA), and then stored at -70°C until analysis. cDNA was synthesized using SuperScript III Reverse Transcriptase (Invitrogen). Reverse transcriptase negative control reactions were performed to ensure the absence of genomic DNA contamination. Gene-specific primers were designed by using Primer Premier 5.00 and synthesized commercially by Shanghai Sangon (Shanghai, China). The primers are listed in Table 1. The 50 μl reaction consisted of 1 μl of cDNA, 8 μl of 2.5 mM deoxynucleoside triphosphate (dNTP) Mix, 2 μl of 20 μM of PCR forward primer and PCR reverse primer, 5 μl of 10×LA PCR Buffer, 0.5 μl of 5U/μl LA Taq™ (Takara Bio Inc., Dalian, China), and 33.5 μl sterile MilliQ water. Thermal cycling was performed with an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 56°C for 30 s, and then 72°C for 30 s, and then a final extension at 72°C for 10 min.

Construction of ERalpha, ERbeta and GAPDH cDNA plasmid

The recombinant plasmids containing ERalpha, ERbeta and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA were termed pERalpha, pERbeta, and pGAPDH, respectively. The constructs were prepared from total RNA in the ovaries of Zi geese at 8 months of age, and the complementary double stranded cDNA fragments were subcloned into the pGEM-T Easy Vector System (Promega, Madison, Wisconsin, USA) as described (KANG *et al.* 2010).

qRT-PCR with SYBR Green I chemistry

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), 2 μl of 20 μM of PCR forward primer and PCR reverse primer, and 22 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 pERalpha, pERbeta or pGAPDH standards were also run. Relative quantitation of gene expression was performed (see KANG *et al.* 2010).

Table 1

List of primers used for quantitative RT-PCR

Target gene	Accession number	Primer sequence (5' - 3')	Amplicon size (bp)
ERalpha	EF502052	Forward: ACCCAAACAGACCATTCAACGAA	187
		Reverse: CGCCAGACTAAGCCAATCATCAG	
ERbeta	EF621308	Forward: AAGTGGGAATGATGAAATGTGGC	163
		Reverse: GGACTGACCGTGCTGAGGAGAAT	
GAPDH	DQ821717	Forward: GCTGATGCTCCCATGTTCGTGAT	86
		Reverse: GTGGTGCAAGAGGCATTGCTGAC	

Statistical analysis

Threshold and Ct (threshold cycle) values were determined automatically by the Line-Gene K Real-time PCR Detection software, using default parameters. The relative level of expression for ERalpha and ERbeta was calculated according to GAPDH (the normalizer) using the relative standard curve method. The relative level of expression was expressed as the mean of three or more means ±SD. The abundance of ERalpha and ERbeta in the ovaries of geese at 1 day of age was assigned a value of 1. All data were analyzed using SAS statistical software for Windows (SAS Institute Inc., Cary, NC, USA). The data were analyzed by ANOVA followed by Duncan's test. Differences were considered to be significant at $P<0.05$.

Results

The ratio 260 nm/280 nm of the RNA preparation was about 1.87. The integrity of 28S and 18S rRNA and the absence of genomic DNA were confirmed (proportionally 2:1, respectively) by agarose gel electrophoresis (data not shown).

The RT-PCR analysis showed the presence of ERalpha and ERbeta mRNAs in the examined ovaries during the developmental and laying stages. The products were 86, 187 and 163 bp for ERalpha, ERbeta and GAPDH mRNA (Fig. 1), respectively; the products corresponded to the approximate size for each as predicted, and their specificity was appropriate for qRT-PCR.

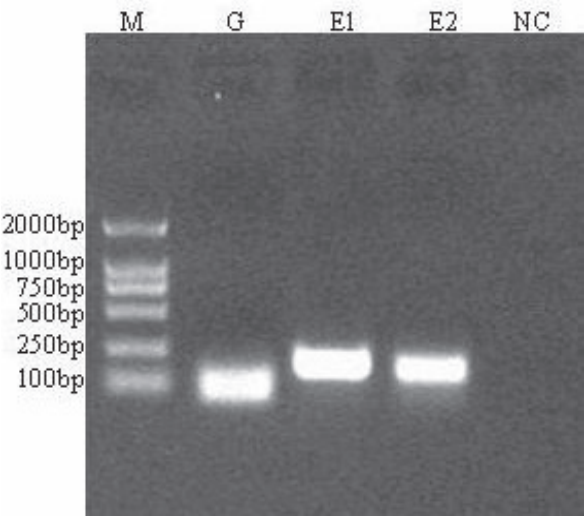

Fig. 1. Representative electrophoresis photograph of RT-PCR products for GAPDH (G), ERalpha (E1) and ERbeta (E2) in the ovaries of Zi geese. The amplicons of 86 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 187 bp estrogen receptor alpha (ERalpha) and 163 bp estrogen receptor beta (ERbeta) were separated on 1.5% agarose gels, stained with ethidium bromide, examined with ultraviolet light and visualized with a Gel-Pro Imager (Media Cybernetics, Maryland, USA). In addition, a negative control (NC) is shown that resulted in no bands after amplification. A 2000-bp molecular weight marker (M) was used.

The RT-PCR analysis showed the presence of ERalpha and ERbeta mRNAs in the examined ovaries during the developmental and laying stages. The products were 86, 187 and 163 bp for ERalpha, ERbeta and GAPDH mRNA (Fig. 1), respectively; the products corresponded to the approximate size for each as predicted, and their specificity was appropriate for qRT-PCR.

The PCR efficiencies (E) for the standard curves, when the plasmids pGAPDH, pERalpha and pERbeta were used as independent templates, are shown in Figure 2, in which the equations of

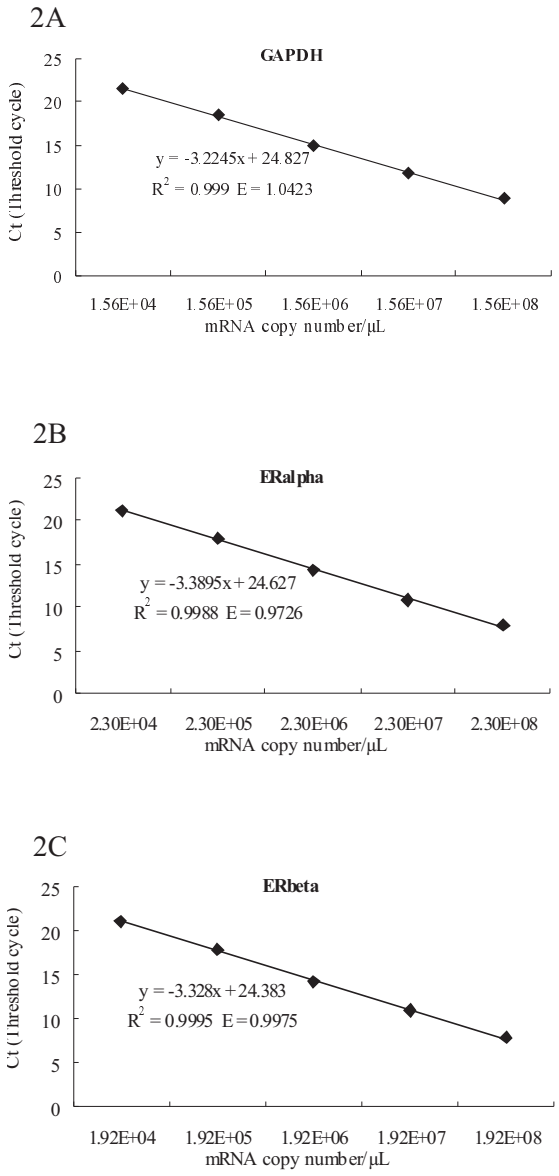

Fig. 2. Standard curves of quantitative real-time PCR (qRT-PCR) amplifications of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), estrogen receptor alpha (ERalpha) and estrogen receptor beta (ERbeta). The curve equations, efficiency values (E) and square of the Pearson correlation coefficient (R^2) were plotted.

the curves and the R^2 values are also presented. In all the standard curves of Ct versus DNA concentration, the R^2 values were more than 0.99, which indicated an excellent degree of colinearity between these parameters (PFAFFL 2001). In addition, the efficiency value (E) of amplification of ERalpha, ERbeta and GAPDH in the experiments was 0.9726, 0.9975 and 1.0423, respectively.

The qRT-PCR results showed that, in the ovaries of Zi geese, the expression of ERalpha and ERbeta mRNA was greater at 1 to 5 months and 8 months compared with 1 day (Figs 3 and 4). In particular, the expression of ERalpha and ERbeta mRNA at 8 months was 2.47 ± 0.23 fold and 29.07 ± 1.25 fold greater than at 1 day, respectively ($P<0.05$). The level of expression of ERalpha mRNA in the ovaries of the geese fluctuated and increased as the geese aged. The relative expression of ERalpha mRNA was not significantly different at 1, 2, 3 and 4 months of age (1.50 ± 0.11, 1.46 ± 0.12, 1.35 ± 0.10 and 1.39 ± 0.14 fold, respectively). The expression of ERalpha mRNA was significantly greater at 8 months compared with 5 months ($P<0.05$), and the relative levels of expression were 1.86 ± 0.17 and 2.47 ± 0.23, respectively (Fig. 3). The level of expression of ERbeta mRNA

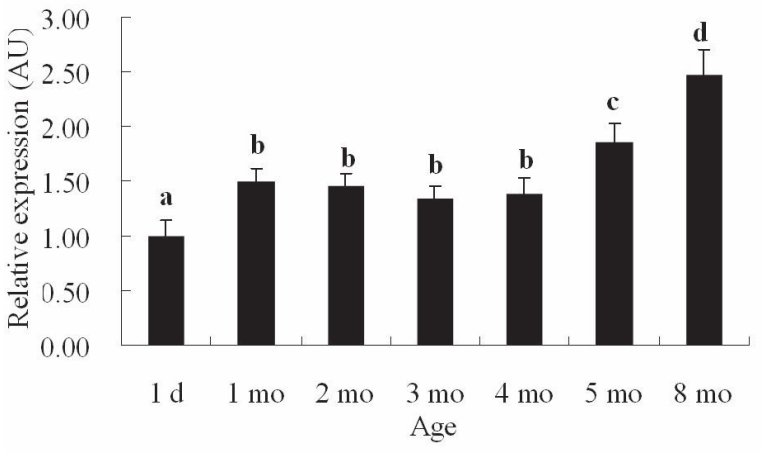

Fig. 3. Relative expression of estrogen receptor alpha (ERalpha) mRNA in the ovaries of Zi geese at the 1 day and 1, 2, 3, 4, 5 and 8 months of age (n=8, respectively). The expression levels of ERalpha were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The expression levels, calculated by the relative standard curve method, are presented in arbitrary units (AU). Values are means ± SD. The significance of differences in the levels of expression of ERalpha mRNA was determined by ANOVA. Means with the same letter are not significantly different (P 0.05) (The same below).

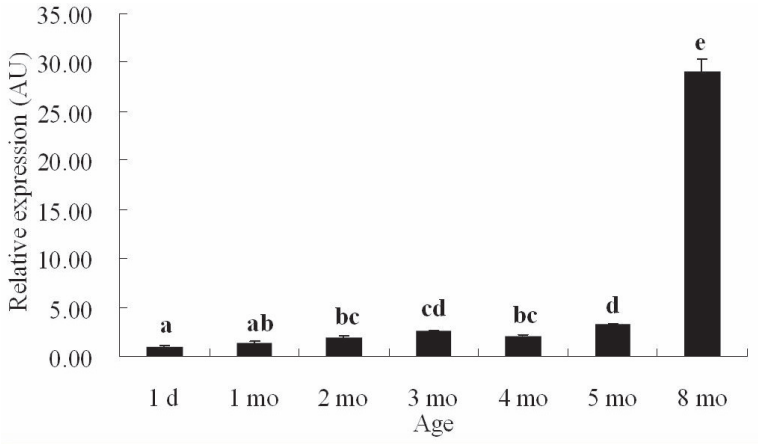

Fig. 4. Relative expression of estrogen receptor beta (ERbeta) mRNA in the ovaries of Zi geese at the 1 day and 1, 2, 3, 4, 5 and 8 months of age (n=8, respectively).

in the ovaries of geese was not significantly different between 1 day and 1 month of age (P>0.05). However, the level of expression of ERbeta in the ovaries of geese at 2 to 5 months and at 8 months (1.96 ± 0.13, 2.58 ± 0.08, 2.08 ± 0.05, 3.25 ± 0.11 and 29.07 ± 1.25 fold, respectively) was significantly greater than at 1 day of age (P<0.05). The relative expression of ERbeta mRNA in the ovaries of geese was not significantly different at 1, 2 and 4 months (P>0.05), and also between 3 and 5 months (P<0.05). The The expression of ERbeta mRNA was significantly greater at of sthat observeds.

Discussion

Estrogens play important role in the ovarian function of poultry. The primary mechanism of action of estrogen is *via* binding to and modulation of the activity of ERalpha and ERbeta, which are ligand-dependent nuclear transcription factors expressed at high levels in female tissues that are critical for reproduction (e.g. the ovary). However, the expression profile of these receptors in the ovaries of developing and laying geese was not examined until the present study. Quantitative PCR has become a standard method for the measurement of gene expression by evaluating the amount of mRNAproduced (ONG & IRVINE 2002). Therefore, in the present work, the level of expression of estrogen receptor mRNA in the ovaries of developing and laying Zi geese were investigated by qRT-PCR.

In the present study, the level of expression of ERalpha mRNA increased and fluctuated from the newborn to the egg-laying stage; furthermore differences in expression of ERalpha mRNA were not significant during the first 4 months of age. These results suggest that ERalpha remained stable and then increased during the developmental stage because it plays a pivotal role in the development of the ovaries of the female and in sexual maturity of geese (DRUMMOND *et al.* 1999; MUNOZ *et al.* 2007). Although the change in the level of expression of ERbeta was similar to that of ERalpha, the increment of the expression of ERbeta from 2 to 8 months was greater than that of ERalpha (KANG *et al.* 2009). Data from mice with knockout of the estrogen receptor gene have confirmed that ERbeta is essential for the early development of ovarian follicles (COUSE *et al.* 1997). The results of this experiment suggest that the physiological roles of estrogens in the ovary are mediated more by ERbeta than by ERalpha during early postnatal development (YING *et al.* 2000; NILSSON *et al.* 2001; KOWALSKI *et al.* 2002; NI *et al.* 2007).

Interestingly, the level of expression of ERalpha and ERbeta mRNA in the ovaries of geese at 5 and 8 months was much higher than in the ovaries of newborn geese, and the relative expression of both ERalpha and ERbeta at 8 months was the highest measured, especially for ERbeta. These findings indicate that both ERalpha and ERbeta play important roles in the maintenance of ovarian function and in the process of successful reproduction (DRUMMOND *et al.* 2002; KNAPCZYK *et al.* 2008). In particular, it is more noticeable during the egg-laying stage. ERbeta is critical for granulose cell differentiation and the ovulatory response to gonadotropins (COUSE *et al.* 2005). ERbeta is also the predominant form of estrogen receptor in the ovary (JEFFERSON *et al.* 2002; COUSE & KORACH 2004). Therefore, it is understandable that the level of ERbeta mRNA was much greater than that of ERalpha mRNA from 2 to 8 months of age.

In summary, the results of the current study establish that ERalpha and ERbeta mRNAs are expressed and exhibit changes in the ovaries of Zi geese during the developmental and egg-laying stages. These findings demonstrate that the expression of both ERalpha and ERbeta mRNA in the geese ovaries was fluctuant, and that it increased from the neonatal to laying stage. These results support the further possibility that both ERalpha and ERbeta mediate ovarian function and the process of egg laying, and that ERbeta may play a more important role in mediating the response of the ovary to estrogen during the developmental and egg-laying stages.

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