Differential expression profiling of estrogen receptor in the ovaries of two egg duck (*Anas platyrhynchos*) breeds

Y. Wu[1,2*], H.W. Xiao[1*], Z.H. Liang[1], A.L. Pan[1], J. Shen[1], J.S. Pi[1], Y.J. Pu[1], J.P. Du[1], Z.H. Chen[1]

[1]Institute of Animal Husbandry and Veterinary, Hubei Academy of Agricultural Science, Wuhan, P.R. China

[2]Hubei Key Laboratory of Animal Embryo and Molecular Breeding, Wuhan, P.R. China

ABSTRACT: In the present study, quantitative real-time PCR was employed to investigate expression profiling and expression difference of *ESR1* and *ESR2* in ovaries of Shaoxing duck and Jingjiang duck during three laying stages. Results showed the expression levels of *ESR1* and *ESR2* in ovaries were increased from the age of the first egg to the age of 500 days in both duck breeds. The expression of *ESR1* in Shaoxing duck was lower than that in Jingjiang duck for the age of the first egg and of 180 days, and for the age of 500 days it was higher in Shaoxing duck than in Jingjiang duck. The *ESR2* showed converse expression profiling in the two duck breeds. The results suggest that *ESR1* and ESR2 mediate the process of egg laying in ducks, and that *ESR2* may play a more important role for the ovary during egg-laying stages and may be closely related to the laying performance of the ducks.

Keywords: egg duck; ovary; laying stage; gene expression; *ESR* gene

INTRODUCTION

The quest for improved egg production is an important focus of poultry breeding and management (Kang et al., 2009). Estrogens play a pivotal role in the development and maintenance of normal sexual and reproductive functions (Heldring et al., 2007; Muriach et al., 2008). The biological action of estrogens is manifested through two high-affinity estrogen receptors, estrogen receptor alpha and estrogen receptor beta, which belong to the family of the 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; Tean-Sempere et al., 2004). The research results in pregnant swine reported by Knapczyk (2008) suggest that in the regulation of ovarian function during pregnancy estrogen acts both via *ESR1* and *ESR2* and these are involved in the process of successful reproduction. Studies of the role of *ESR1* and *ESR2* in the reproductive performance of poultry have become still more intensive in recent years. Expression of estrogen receptors was examined in the ovaries of chicken (Krust et al., 1986) and quail (Foidart et al., 1999; Ichikawa et al., 2003). Presence of *ESR1* and *ESR2* mRNA was detected in the ovarian stroma and in the white, yellowish, small yellow, granulosa, and theca layers of the walls of the preovulatory follicles in the laying hens' ovaries (Hrabia et al., 2008). The change in the expression of *ESR2* mRNA in ovaries of prepubertal ducks was elucidated (Ni et al., 2007). Although a remarkable progress has been made in understanding the impact of estrogens on the ovarian function during the ovarian development in poultry, via both *ESR1* and *ESR2,* studies of estrogen receptors in the ovaries of different duck species are rare. The objective of the

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*Y. Wu and H.W. Xiao contributed equally to this work

present study was to investigate the expression profiling and the expression difference of *ESR1* and *ESR2* mRNA in the ovaries of Shaoxing duck and Jingjiang duck during three egg laying stages.

MATERIAL AND METHODS

Animals and tissue collection. Shaoxing duck and Jingjiang duck are two local Chinese breeds. Shaoxing duck is characterized by a small body size (about 1255 g), early maturity (ca. at day 102), and high productivity (more than 300 eggs per year) (Zhao et al., 2005). Jingjiang duck is a local variety of the Hubei Province which is characterized by small body size (about 1226 g), early maturity (ca. at day 100), and low productivity (about 200 eggs per year) (Ding et al., 2004). The two species are bred throughout the year. In this study, 100 Jingjiang and 100 Shaoxing female ducklings were raised from hatching up to the age of 500 days. All ducks were housed individually in wire cages in a room with controlled temperature (25 ± 3°C) and fed a commercial diet before the commencement of the experiments. At the onset of laying, the illumination scheme for all ducks was 16 h. All animal procedures and handling were conducted in compliance with the Guild for Care and Use of Laboratory Animals. At each of the three laying stages – (1) at the age of the first egg, (2) at day 180 (period of the best laying performance), and (3) at day 500 (at this stage the laying performance rapidly lowers) – six Jingjiang ducks and six Shaoxing ducks were anaesthetized by immersion in a saturated solution of 3-aminobenzoic acid ethyl ester (Sigma-Aldrich, St. Louis, USA). Then the ducks were immediately dissected to collect ovaries including small oocytes (diameter less than 5 mm), but excluding oviduct. Dissected ovaries were immediately frozen in liquid nitrogen. Once in the laboratory, samples were conserved at –80°C until RNA isolation was performed.

RNA isolation and cDNA synthesis. Total RNA was isolated using Gibco BRL TRIzol Reagent kit according to the instructions of the manufacturer (Life Technologies, Barcelona, Spain). The total RNA samples of ovaries from ducks of different age stages (age of the first egg, of 180 days, and of 500 days) were pooled separately for the two duck breeds. And cDNA was obtained from 1 μg of total RNA from the two different pools by reverse transcriptase PCR (SuperScript™ First-Strand Synthesis System; Invitrogen, Leek, the Netherlands) using random hexamers as primers and following manufacturer's recommendations.

Semi-quantitative RT-PCR. Real-time quantitative PCRs were run for 1 μg total RNA of each tissue in all ducks, by fluorescent monoplex PCR using 2×SYBRGreen Master Mix (Taq DNA polymerase, dNTP, and reaction buffer) (TaKaRa Bio Inc., Otsu, Japan). PCR mixture contained 1 μl of cDNA (1 μg/μl), 7.5 μl of 2×SYBRGreen Master Mix, 0.5 μl of each primer (10μM), and water in a final volume of 15 μl. Real-time quantitative PCR was conducted using a Chromo4 Real-Time Detection System (MJ Research Inc., Waltham, USA). Amplifications were carried out at a final volume of 20 μl, containing 1.0 μl of DNA sample, 10 μl of SYBRGreen Real time PCR Master Mix (TaKaRa), 1.0 μl of each primer, and 7.0 μl of ddH_2O. The reaction carried out without template was used as blanks. PCR amplification was performed in triplicate wells, using the following conditions: 5 min at 94°C, followed by 45 cycles consisting of 20 s at 94°C, 20 s at 50, 56, and 55°C (for *β-actin*, *ESR1*, and *ESR2*, respectively), and 30 s at 72°C.

All PCR primers (Table 1) for real-time quantitative RT-PCR were chosen within the target sequences of *β-actin*, *ESR1*, and *ESR2* for duck to generate specific products around 200 bp. The target sequences of *β-actin* (EF667345), *ESR1* (EF502052.1), and *ESR2* (NM_204794) for designed degenerate primers were retrieved from the GenBank (http://www.ncbi.nlm.nih.gov/; NCBI, US National Library of Medicine, USA). The cDNA concentrations were measured at OD_{260} and the

Table 1. Primer sequences used for analyses of the expression pattern of duck *ESR1* and *ESR2* genes

Primer name	Product size (bp)	Primer sequence (5'-3')	Annealing temperature (°C)
β-actin	150	F-5'-ACGGTGCTGTCTGGTGGTA-3' R-5'-TGTCTGACATGGGAGAGCAG-3'	50
ESR1	189	F-5'-TAGAGGGCATGGTGGAAATC-3' R-5'-TGTCCAGAACACGGTGGATA-3'	56
ESR2	238	F-5'-GGTTCCGAGAGCTGAAACTG-3' R-5'-TGTCTGACATGGGAGAGCAG-3'	55

corresponding copy numbers were based on the formula that 1 μg of 1000 bp DNA is equivalent to 9.1×10^{11} molecules. Serial 10-fold dilutions of the resulting plasmid clones, e.g., ranging from 109 down to 104 input cDNA copies, were used as a standard curve in each PCR run.

Western blot analysis. The ESR1 proteins from each tissue sample were extracted and run on 8–16% gradient SDS polyacrylamide gels. The separated proteins were transferred onto a nitrocellulose membrane. Antibodies specific to ESR1 and ESR2 were used at 1 : 1000 dilutions. All incubations were performed in Tris-HCl buffer, pH 7.5, with 0.1% Tween 20 and 5% dry milk. Dry milk was not added for the final washing. Immunodetection was performed by using chemiluminescence (TianGen Biotech, Beijing, China). Signal intensity was determined by densitometry.

Statistical analyses. After the completion of real-time quantitative PCR amplification, data were obtained using Opticon Monitor software (Version 2.03, 2005). The exact copy number of constitutive transcripted mRNA in each tissue was derived from each threshold value according to the standard curve. The data which presented as the fold change in tissues for *ESR1* and *ESR2* gene expression were normalized to *β-actin* using that of the age of the first egg as a calibrator of one. Normalized Ct values from *ESR1* and *ESR2* were compared using two-tailed paired Student's *t*-test (after the data passing the Kolmogorov-Smirnov test of normality).

RESULTS AND DISCUSSION

Estrogens play an important role in the ovarian function of poultry. The effect of estrogen on ovarian function is mediated by the receptors, ESR1 and ESR2. However, the expression profile of these receptors in the ovaries of developing and laying ducks has not been examined until this study. Quantitative PCR has become a standard method for the measurement of gene expression by evaluating the amount of mRNA produced (Ong et al., 2002). Therefore, in the present work, the level of expression of *ESR1* and *ESR2* mRNA in the ovaries of ducks at the age of the first egg, of 180 days, and of 500 days was investigated by real-time quantitative PCR.

The mRNA expression of *ESR1* in Jingjiang and Shaoxing ducks at three laying stages was examined by real-time PCR. As show the results given in Figure 1A, the *ESR1* gene occurred throughout all the examined ovaries of Jingjiang and Shaoxing duck. The relative expression level at 180 days was higher than that at the age of the first egg, and that at 500 days was higher than at 180 days both in Jingjiang and Shaoxing duck. For both duck species, there were significant differences in the relative expression levels of *ESR1* mRNA in ovaries between the age of the first egg (or of 180 days) and of 500 days, but there was no significant difference in the results for the age of the first egg and for 180 days. The results for the mRNA expression of *ESR2* in the ovaries of Jingjiang and Shaoxing duck at three laying stages are shown in

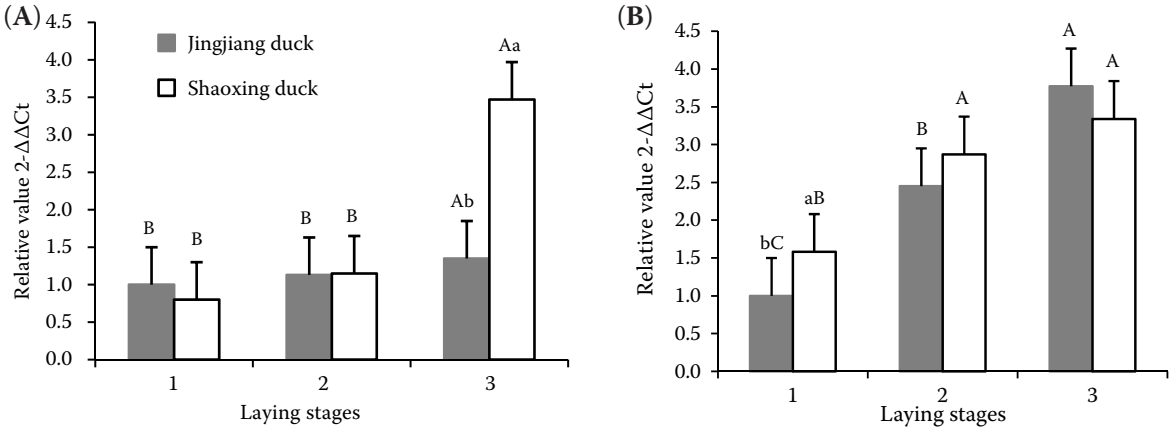

Figure 1. Development changes of the relative expression levels for *ESR1* (**A**) and *ESR2* (**B**) mRNA in ovaries of Jingjiang and Shaoxing ducks

1 = stage of the first egg, 2 = stage of 180 days, 3 = stage of 500 days

[a,b]($P < 0.05$) significant difference in Jingjiang duck and Shaoxing duck

[A,B]($P < 0.05$) significant difference at the individual stages of age

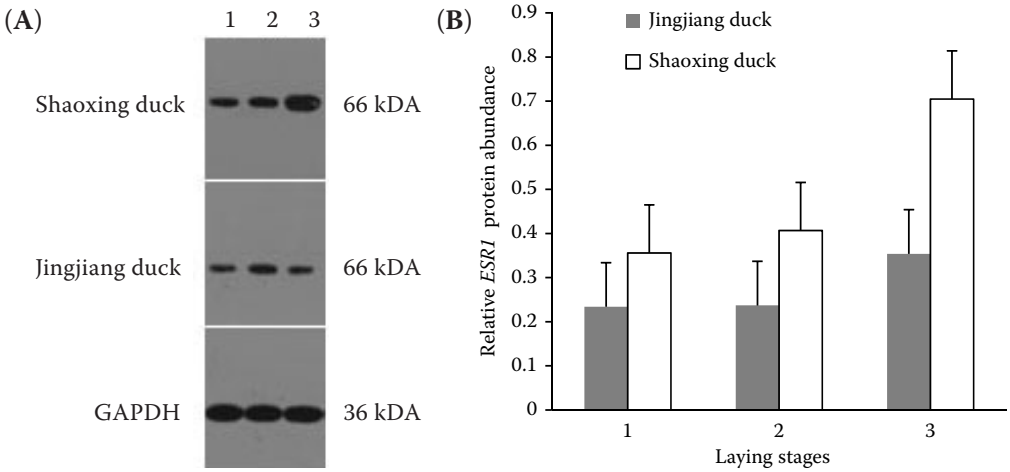

Figure 2. The Western-blot results for *ESR1* in different duck ovaries

1 = stage of the first egg, 2 = stage of 180 days, 3 = stage of 500 days

Figure 1B. Like the *ESR1* mRNA, the *ESR2* gene was detected throughout all the examined ovaries of Jingjiang as well as Shaoxing duck and the relative expression levels of *ESR2* mRNA in ovaries were increasing from the age of the first egg to 500 days both in Jingjiang and Shaoxing duck. For both duck species, there were significant differences in the relative expression level of *ESR2* mRNA in ovaries between the three laying stages (age of the first egg, of 180 days, and of 500 days).

The relative expression levels of *ESR1* mRNA in ovaries of Jingjiang duck and Shaoxing duck differed at the three laying stages. There was no significant difference in the relative expression levels between the stage of the first egg and that of 180 days in all duck. But there was a considerable difference between the two duck species for the stage of 500 days. At different laying stages, the relative expression levels of *ESR2* mRNA in ovaries of Jingjiang duck and Shaoxing duck were different. There was insignificant difference in the relative expression levels between the stage of 500 days for Jingjiang duck and Shaoxing duck. But at the stages of the first egg and of 180 days, the relative expression levels of *ESR2* mRNA in Shaoxing duck were significantly to very significantly higher than those in Jingjiang duck.

Protein lysates from each ovary sample were examined by the Western blot analysis to seek for the presence of ESR1 and ESR2 proteins in the ovaries. The predicted ESR1 and ESR2 duck proteins were 66 kDa and 54 kDa, respectively. The results are given in Figures 2 and 3. For ESR1 and ESR2 proteins, although they were detected in all

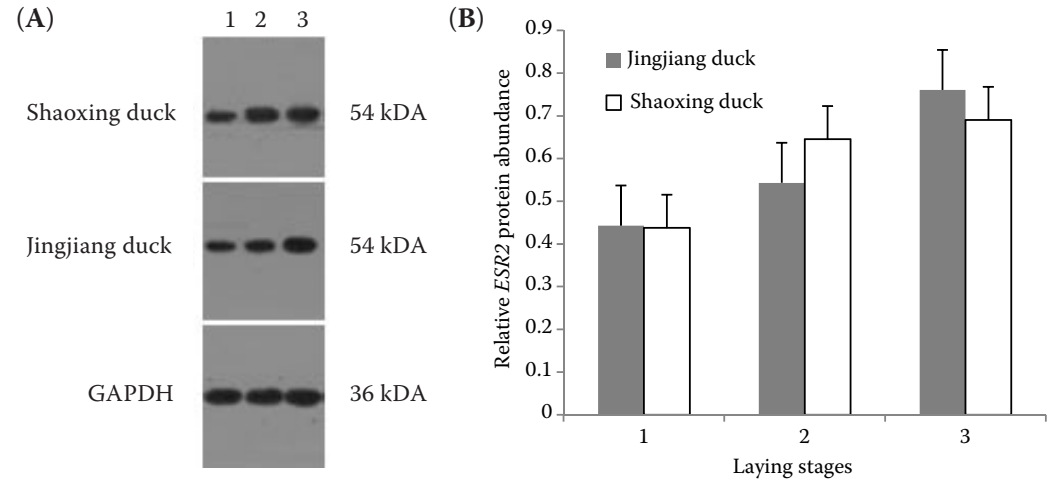

Figure 3. The Western-blot results for *ESR2* in different duck ovaries

1 = stage of the first egg, 2 = stage of 180 days, 3 = stage of 500 days

ovaries of both duck species and showed similar expression pattern with *ESR1* and *ESR2* mRNA expression, the expression abundance at different laying stages was not significantly different ($P > 0.05$) for the two species. It was probably caused by the fact that parts of mRNA of *ESR1* and *ESR2* were not translated to protein at different stages of the ovarian development.

In the present study, the levels of expression of *ESR1* mRNA were significantly different for Jingjiang ducks and Shaoxing ducks between the age of 500 days and the age of the first egg (180 days) ($P < 0.05$). These results showed similar pattern to that in geese (Kang et al., 2011) and suggest that *ESR1* remained stable from newborn to the egg-laying stage and then increased during the different ovarial stages and in sexual maturity of egg ducks because it plays a pivotal role in the ovary (Drummond et al., 1999; Munoz et al., 2007). Previous studies revealed that both *ESR1* and *ESR2* play the role in the maintenance of ovarian function and in the process of successful reproduction (Knapczyk et al., 2008). *ESR2* is a predominant form of estrogen receptor in the ovary (Jefferson et al., 2002; Couse and Korach, 2004) and is localized primarily in the granulose cells of small and growing follicles (Byers et al., 1997). It is essential for the efficiency of normal ovulation (Drummond et al., 2002). In contrast, *ESR1* occurs mainly in the mammalian uterus (Byers et al., 1997; Couse et al., 1997; Drummond et al., 1999) and it is essential for female sexual differentiation, fertility, and lactation (Drummond et al., 2002). Previous studies showed that the physiological roles of estrogens in the ovary are mediated more by *ESR2* than by *ESR1* during early postnatal development (Ying et al., 2000; Nilsson et al., 2001; Kowalski et al., 2002; Ni et al., 2007). Therefore, this probably explains why the increase of *ESR2* expression from the age of the first egg to 500 days was higher than that of *ESR1*.

The Shaoxing duck is a high-yielding Chinese local breed. The Jingjiang duck is also a local breed, but its yield is lower. In the present study, we observed that the expression level of *ESR2* mRNA and protein in Shaoxing duck at the stages of age of the first egg and of 180 days was higher than in Jingjiang duck; at the stage of 500 days the expression level of *ESR2* mRNA and protein in Shaoxing duck was lower than in Jingjiang duck. The expression pattern of *ESR1* mRNA and protein was converse. The results imply that *ESR2* is closely related to the laying performance of ducks and that it may play a more important role for the ovary during the egg-laying stages.

CONCLUSION

In conclusion, the results of this study establish that ESR1 and *ESR2* mRNAs (protein) are expressed and reflect changes in the ovaries of Jingjiang ducks and Shaoxing ducks during the three laying stages (age of the first egg, of 180 days, and of 500 days). These findings demonstrate how the expression of both *ESR1* and *ESR2* mRNA (protein) in the ducks' ovaries was changing, and that its level increased from the age of the first egg till day 500. These results support the possibility that *ESR1* and *ESR2* maintain the ovarian function and the process of successful reproduction, and that *ESR2* plays a more important role for the ovary during the egg-laying stages and that it is closely related to the ducks' laying performance.

REFERENCES

Byers M., Kuiper G.G., Gustafsson J.A., Park-Sarge O.K. (1997): Estrogen receptor-beta mRNA expression in rat ovary: down-regulation by gonadotropins. Molecular Endocrinology, 11, 172–182.

Couse J.F., Korach K.S. (2004): Estrogen receptor-alpha mediates the detrimental effects of neonatal diethylstilbestrol (DES) exposure in the murine reproductive tract. Toxicology, 205, 55–63.

Couse J.F., Lindzey J., Grandien K., Gustafsson J.A., Korach K.S. (1997): Tissue distribution and quantitative analysis of estrogen receptor-α (ERα) and estrogen receptor-β (ERβ) messenger ribonucleic acid in the wild-type and ERα-knockout mouse. Endocrinology, 138, 4613–4621.

Ding S.H., Chen H.S., Liu J.P., Zhao S.Q. (2004): Breeds of Livestock and Poultry in Hubei Province. 1st Ed. Hubei Science and Technology Press, Wuhan, China. (in Chinese)

Drummond A.E., Baillie A.J., Findlay J.K. (1999): Ovarian estrogen receptor alpha and beta mRNA expression: impact of development and estrogen. Molecular and Cellular Endocrinology, 149, 153–161.

Drummond A.E., Britt K.L., Dyson M., Jones M.E., Kerr J.B., O'Donnell L., Simpson E.R., Findlay J.K. (2002): Ovarian steroid receptors and their role in ovarian function. Molecular and Cellular Endocrinology, 191, 27–33.

Foidart A., Lakaye B., Grisar T., Ball G.F., Balthazart J. (1999): Estrogen receptor-beta in quail: cloning, tissue expression and neuroanatomical distribution. Journal of Neurobiology, 40, 327–342.

Greene G.L., Gilna P., Waterfield M., Baker A., Hort Y., Shine J. (1986): Sequence and expression of human estrogen receptor complementary DNA. Science, 231, 1150–1154.

Gruber C.J., Gruber D.M., Gruber I.M., Wieser F., Huber J.C. (2004): Anatomy of the estrogen response element. Trends in Endocrinology and Metabolism, 15, 73–78.

Heldring N., Pike A., Andersson S., Matthews J., Cheng G., Hartman J., Tujague M., Strom A., Treuter E., Warner M., Gustafsson J.A. (2007): Estrogen receptors: how do they signal and what are their targets. Physiological Reviews, 87, 905–931.

Hrabia A., Wilk M., Rzasa J. (2008): Expression of alpha and beta estrogen receptors in the chicken ovary. Folia Biologica-Krakow, 56, 187–191.

Ichikawa K., Yamamoto I., Tsukada A., Saito N., Shimada K. (2003): cDNA cloning and mRNA expression of estrogen receptor a in Japanese quail. Journal of Poultry Science, 40, 121–129.

Jefferson W.N., Couse J.F., Padilla-Banks E., Korach K.S., Newbold R.R. (2002): Neonatal exposure to genistein induces estrogen receptor (ER)alpha expression and multioocyte follicles in the maturing mouse ovary: evidence for ERbeta-mediated and nonestrogenic actions. Biology of Reproduction, 67, 1285–1296.

Kang B., Guo J.R., Yang H.M., Zhou R.J., Liu J.X., Li S.Z., Dong C.Y. (2009): Differential expression profiling of ovarian genes in prelaying and laying geese. Poultry Science, 88, 1975–1983.

Kang B., Jiang D.M., Liu B., Zhou R.J., Zhen L., Yang H.M. (2011): Gene expression profile of estrogen receptor alpha and beta in the ovaries of Zi geese (*Anser cygnoides*). Folia Biologica, 59, 135–140.

Knapczyk K., Duda M., Durlej M., Galas J., Koziorowski M., Slomczynska M. (2008): Expression of estrogen receptor alpha (*ERalpha*) and estrogen receptor beta (*ERbeta*) in the ovarian follicles and *corpora lutea* of pregnant swine. Domestic Animal Endocrinology, 35, 170–179.

Kowalski A.A., Graddy L.G., Vale-Cruz D.S., Choi I., Katzenellenbogen B.S., Simmen F.A., Simmen R.C. (2002): Molecular cloning of porcine estrogen receptor-beta complementary DNAs and developmental expression in periimplantation embryos. Biology of Reproduction, 66, 760–769.

Krust A., Green S., Argos P., Kumar V., Walter P., Bornert J., Chambon M. (1986): The chicken oestrogen receptor sequence: homology with verb A and the human oestrogen and glucocorticoid receptors. EMBO Journal, 5, 891–897.

Kuiper G.G., Enmark E., Pelto-Huikko Nilsson S., Gustafsson J.A. (1996): Cloning of a novel receptor expressed in rat prostate and ovary. Proceedings of the National Academy of Sciences of the United States of America, 93, 5925–5930.

Munoz G., Ovilo C., Estelle J., Silio L., Fernandez A., Rodriguez C. (2007): Association with litter size of new polymorphisms on ESR1 and ESR2 genes in a Chinese–European pig line. Genetics Selection Evolution, 39, 195–206.

Muriach B., Carrillo M., Zanuy S., Cerda-Reverter J.M. (2008): Distribution of estrogen receptor 2 mRNAs (Esr2a and Esr2b) in the brain and pituitary of the sea bass (*Dicentrarchus labrax*). Brain Research, 1210, 126–141.

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*). Animal Reproduction Science, 100, 318–328.

Nilsson S., Makela S., Treuter E., Tujague M., Thomsen J., Andersson G., Enmark E., Petterssonk K., Warner M., Gustafsson J.A. (2001): Mechanisms of estrogen action. Physiological Reviews, 81, 1535–1565.

Ong Y.L., Irvine A. (2002): Quantitative real-time PCR: a critique of method and practical considerations. Hematology, 7, 59–67.

Tean-Sempere M., Navarro V.M., Mayen A., Bellido C., Sanchez-Criado J.E. (2004): Regulation of estrogen receptor (*ER*) isoform messenger RNA expression by different *ER* ligands in female rat pituitary. Biology of Reproduction, 70, 671–678.

Ying C., Hsu W.L., Hong W.F., Cheng W.T., Yang Y. (2000): Estrogen receptor is expressed in pig embryos during preimplantation development. Molecular Reproduction and Development, 55, 83–88.

Zhao R.Q., Zhou Y.C., Ni Y.D., Lu L.Z., Tao Z.R., Chen W.H., Chen J. (2005): Effect of daidzein on egg-laying performance in Shaoxing duck breeders during different stages of the egg production cycle. British Poultry Science, 46, 175–181.

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Corresponding Author

Jin-ping Du, Hubei Academy of Agricultural Science, Institute of Animal Husbandry and Veterinary, 430064 Wuhan, P.R. China
Phone: +862 787 380 190, fax: +862 787 380 190, e-mail: youngwuyan@163.com