

The expression of pituitary FSH β and LH β mRNA and gonadal FSH and LH receptor mRNA in the chicken embryo

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Received: 15 May 2009; accepted: 29 October 2009

SUMMARY

In avian species, synthesis of sex steroids by embryonic gonads is regulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In order to elucidate the role of the two gonadotropins in gonadal axis development during the second half of chicken embryogenesis, pituitary expression of LH β subunit (LH β) and FSH β subunit (FSH β) mRNAs as well as gonadal expression of LH and FSH receptor (LHR and FSHR) mRNAs were determined on days 11 (E11) and 17 (E17) of embryonic development and after hatching (D1). In the pituitary of the female embryo, the gene expression of FSH β was the lowest on E11 and increased on E17. In the male pituitary, the expression of FSH β did not differ among the studied days. The FSH β mRNA expression on E11 was higher in the male than in the female pituitary gland. The expression of LH β mRNA in the female pituitary increased on D1 in comparison to E11. In the male pituitary gland,

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the expression of LH β gene was relatively constant. The expression of mRNA encoding FSHR in the ovary increased on E17, while in testes it did not differ among the studied days. There were no significant alterations in LHR gene expression in the ovary or in the testes in the examined period however, the gene expression on E17 was higher in the ovary than in the testes. We observed positive correlations between the pituitary FSH β mRNA expression and ovarian expression of FSHR mRNA ($r=0.63$; $p<0.01$) as well as between LH β mRNA and LHR mRNA in the testes ($r=0.65$; $p<0.01$). The reported alterations in gene expression of FSH β , LH β and their receptors between sexes and among the stages of embryonic development indicate time- and sex-dependent action of gonadotropins in gonads of chicken embryos. *Reproductive Biology* 2009 9 3: 253-269.

Key words: FSH β , LH β , FSH receptor, LH receptor, ovary, testis, hypophysis, chicken embryo

INTRODUCTION

In chicken embryos, gonadal sex is bipotential and morphologically indistinguishable up to six days of egg incubation. By days 8-10 of incubation, the gonads differentiate enough to be morphologically recognized. Gonads of genetic females (ZW heterozygotes) develop as asymmetric ovaries, while gonads of males (ZZ homozygotes) develop as symmetric testes [25, 29]. Differentiation of the bipotential gonad is regulated by specific sex-determining genes as well as by sex steroids [27, 29, 30]. In birds, estrogens play an essential role in sex-dependent differentiation of the ovarian tissue and the blockage of estrogen synthesis causes a phenotypical sex reversal in the genetic female [1, 5, 8, 29].

In both female and male chicken gonads, secretion of sex steroids is regulated by two pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), composed of common α [31], but distinct β subunits [23]. The cDNA sequences for the avian LH β [20, 42] and FSH β [28] have been determined. The gene encoding LH β is expressed in chicken since day 4 of embryonic development and LH β immunopositive

cells start to appear in the ventral part of the caudal lobe in the anterior pituitary gland on day 8 of egg incubation [16, 24]. Expression of mRNA for FSH β is expressed from day 7, and immunopositive cells start to appear in the ventral part of the caudal lobe in the anterior pituitary gland from day 8 of incubation. The distribution of FSH positive cells spreads from the ventral part to the dorsal part in the caudal lobe around day 10 and the cells subsequently expand to the cephalic lobe from day 12 to day 20 of embryogenesis [16, 24]. Previous studies demonstrated that LH plays a regulatory role in embryonic steroidogenesis [10, 34]. Treatment of female and male chicken embryos with equine LH on day 7.5 [38] or with human chorionic gonadotropin (hCG) on day 8 of incubation [33] increased plasma concentrations of estradiol (E₂) and testosterone (T), respectively. The function of FSH in steroidogenesis in chicken embryos has already been investigated [9, 21], however its effect on gonadal function is not fully elucidated.

The biological action of LH and FSH in gonadal tissue is mediated *via* membrane receptors for LH (LHR) and FSH (FSHR), respectively. Since chicken LHR [14, 19] and FSHR [37, 43] cDNAs were sequenced, the expression of chicken gonadotropin receptor genes has been studied by several scientific groups in an attempt to elucidate: (i) the role of gonadotropins in differentiation of the bipotential gonad, and (ii) the establishment of the hypothalamo-pituitary-gonadal axis (HPG) in chicken embryos [2, 3, 15]. Akazome et al. [3] demonstrated gonadal expression of LHR and FSHR mRNAs on day 4 of egg incubation in both sexes. The amounts of gonadal mRNA encoding LHR and FSHR in females exceeded those of males both on day 6, when sexual differentiation of chicken gonads was initiated, as well as on day 12 when gonadal differentiation was completed.

Despite numerous studies concerning the expression of gonadotropin mRNA in the differentiated pituitary gland and their receptors in chicken embryonic gonads, there are no data regarding their simultaneous measurement and analysis during the last stages of incubation. Thus, the aim of the present study was to investigate the relationship between the mRNA expression of gonadotropins (LH β , FSH β) and their receptors (LHR and FSHR) during embryogenesis.

MATERIALS AND METHODS

Animals and collection of tissue

The experiment was approved by the Local Animal Ethics Committee in Krakow. Fertilized eggs (n=180) of Hy-Line Brown strain were incubated at standard conditions for 21 days (1-18 days of incubation: t=37.8°C, relative humidity [RH]=55%, 19-21 days of incubation; t=37.2°C, RH=70%; incubator Masalles 65 DIGIT). The eggs were candled on day 5 of the incubation to eliminate unfertilized eggs and dead embryos. On day 11 (E11) and day 17 (E17) of embryonic development and just after hatching (D1) embryos were decapitated and sexed, and the pituitary gland, the left ovary in females and testes in males were isolated. Tissues were snap frozen and kept in liquid nitrogen until total RNA isolation.

Total RNA extraction and reverse transcription

The total RNA of each tissue was isolated using TRI-Reagent (Molecular Research Center, USA; [7]). The tissues were homogenized (UltraTurrax T25; IKA-Labortechnik, Germany), and phase separation was made with bromochloropropane. RNA was precipitated from the aqueous phase by mixing with isopropanol, washed with 75% ethanol and dissolved in pure RNAase free water (Promega, USA). The concentration of RNA was measured spectrophotometrically (Biophotometer; Eppendorf, Germany). Each sample was tested for RNA degradation by separation on agarose gel and spectrophotometrical analysis of absorbance 260/280 nm ratio. To avoid genomic DNA contamination, RNA samples were treated with DNase (RQ1 RNase-Free DNase, Promega, USA). Reverse transcription (RT) reactions were performed in 20 µl volume. The reaction mixture contained 5 µg total RNA, 200U M-MuLV reverse transcriptase (Fermentas, Lithuania), 0.5 µg oligo(dT)₁₈ as a primer, 1 mM of each dNTP, 20U Ribonuclease Inhibitor (Fermentas, Lithuania) and 4 µl of 5×reaction buffer containing 250 mM Tris-HCl, 250 mM KCl, 20 mM MgCl₂, 50 mM DTT

(Fermentas, Lithuania). The resulting first strand of cDNA (1 μ l) was used for PCR reactions.

Expression of the FSH β , LH β , FSHR and LHR genes

The expression of FSH β and LH β in the hypophysis and FSHR and LHR in the testes and left ovaries was investigated by RT-PCR. The primers sequences used in PCR reactions, their localization in genome and product size are shown in Table 1. All PCR amplifications were carried out in a total volume of 25 μ l PCR reaction mixture containing 1 μ l of cDNA, 0.2 mM of each dNTP, 0.2 μ M of each primer, 1.5 mM MgCl₂ and 0.625U of TaqDNA Polimerase (Fermentas, Lithuania). The amplification of a housekeeping gene (18S rRNA) was acquired in a separate sample. All PCR reactions were performed in the Thermocycler Gradient (Eppendorf, Germany). The temperature profiles used for PCR were programmed as follows: initial template denaturation at 95°C for 5 min, denaturation at 95°C for 30s, annealing at gene specific temperature (tab. 1) for 30s, followed by a final extension at 72°C for 30s. Thirty cycles of the appropriate profile were run, and the final extension step for each gene amplification was increased to 7 min at 72°C. Amplification products were separated by electrophoresis on 1.5% agarose gel containing ethidium bromide. The PCR products were semi-quantitatively estimated from the density of the gel band using the Scion Image for Windows Software (Scion Corporation, Maryland, USA). The relative density of each gene product was compared with 18S rRNA products, and expressed as the mean of six samples.

Statistical analysis

The data were analyzed by two-way ANOVA followed by Duncan's multiple range test. Values were expressed as the mean \pm SEM and considered significantly different at $p < 0.05$ and highly significant at $p < 0.01$. To estimate the relationship between the expression of gonadotropins mRNA in the pituitary gland and the appropriate receptor in female or male go-

Table 1. Oligonucleotide sequences of primers, nucleotide positions, GeneBank accession number, product size, and annealing temperature of PCR reaction.

Gene	Position (bp)	GenBank accession number	Sequences: (5'-3' forward, reverse)	Primers sequence according to:	Product size (bp)	Annealing temperature
FSH β	23-44	NM204257	CAGCACTCTACTAGAATACAGG	[16]	441	56°C
	446-463		CAAGCTGCCATATCAAGT			
LH β	217-238	L35519	GTATGGCTGTGACCCACCACGG	[16]	233	64°C
	431-449		CTGCACGGTGCAGTCGGAG			
FSHR	363-382	D87871	AGAAGGCCAACAAACCTCGTG	[44]	521	54°C
	863-883		ACAGCAATGGCTAGGATAGGT			
LHR	676-695	ABO09283	CTCAGGCGGATACACAACGA	[44]	193	60°C
	849-868		TCAGAACAGCTTCCAGCAGG			
18S rRNA	160-179	AF173612	CGCGTGCATTTTATCAGACCA	[35]	167	60°C
	308-326		ACCCGTGTCCACCATGGTA			

nads correlation analysis was performed. These statistical analyses were completed using SigmaStat 2.03 (SPSS Science Software, USA).

RESULTS

FSH β and LH β mRNAs were detected in male and female embryonic pituitary glands on all examined days (fig. 1A). Similarly, expression of FSHR and LHR mRNAs was demonstrated in the testes and left ovary during this time (fig. 1B). In the female pituitary gland, the expression of FSH β mRNA increased ($p < 0.01$) sharply by 436% on E17 compared to that on E11, and subsequently decreased ($p > 0.05$) by 42% on D1. In males, the pituitary expression of FSH β mRNA on E11 was 5.6-fold greater ($p < 0.01$) than that of females. The high abundance of the transcript was maintained until hatching and did not change among the examined stages of embryogenesis (fig. 2A). The expression of LH β mRNA in female pituitary gland did not differ between E11 and E17. However, in comparison with E11 it significantly increased ($p < 0.05$) by 41% on D1. There were no significant differences in the expression of LH β mRNA between sexes in each of the embryonic stages (fig. 2B).

The expression of FSHR mRNA in the left ovary on E17 was greater by 138% in comparison with E11 ($p < 0.05$). In testes, the expression of FSHR did not differ ($p > 0.05$) among the examined stages of embryogenesis. However, FSHR expression was 71% greater on D1 than on E11 (fig. 2C). The expression of LHR in the ovary and in the testes did not change among stages. LHR expression on E17 was greater by 52% ($p < 0.05$) in the ovary than in the testes (fig. 2D).

The statistical analysis revealed that the pituitary expression of LH β mRNA on E11 and D1 in the female embryo was higher ($p < 0.05$) in comparison with the FSH β expression (fig. 2B vs. fig. 2A). Moreover, the ovarian expression of LHR mRNA on E11 was higher ($p < 0.05$) than the expression of FSHR mRNA (fig. 2D vs. fig. 2C). Positive correlations (tab. 2) were found between the expression of FSH β mRNA in the pituitary glands of the female embryos and FSHR mRNA in the ovaries ($p < 0.01$) as well as between the expression of LH β mRNA in the male pituitary and LHR mRNA in testes ($p < 0.01$).

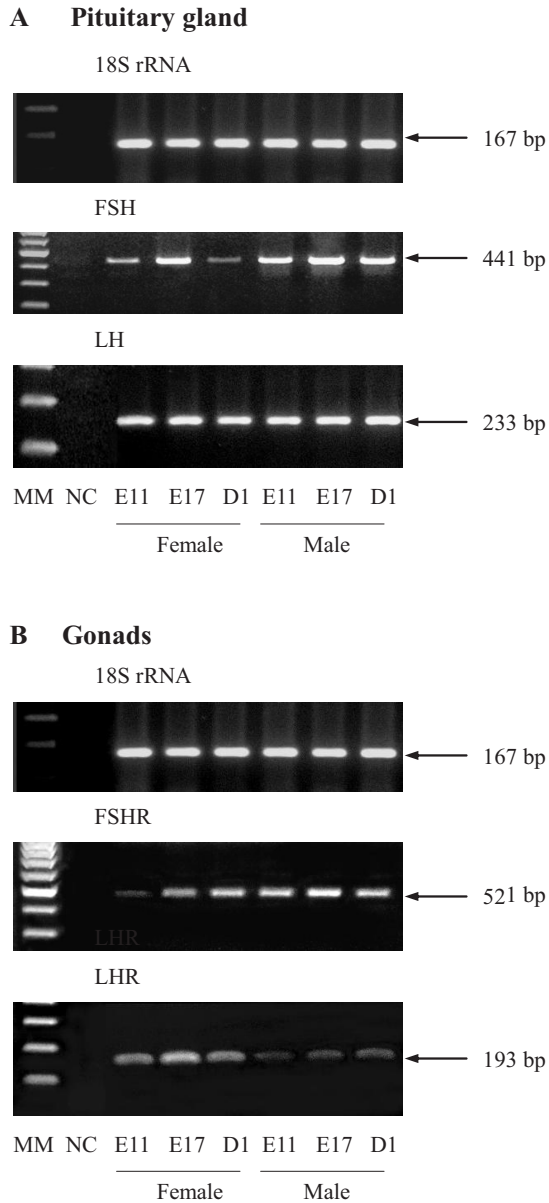


Figure 1. Exemplary expressions of mRNA encoding: FSH β and LH β in the pituitary gland (A) and FSH receptor (FSHR) and LH receptor (LHR) in the gonads (B) of the chicken embryo. E11, E17: embryonic days 11 and 17; D1: day after hatching; 18S rRNA: housekeeping gene; NC-negative control without cDNA; MM-molecular marker (100-1000 bp)

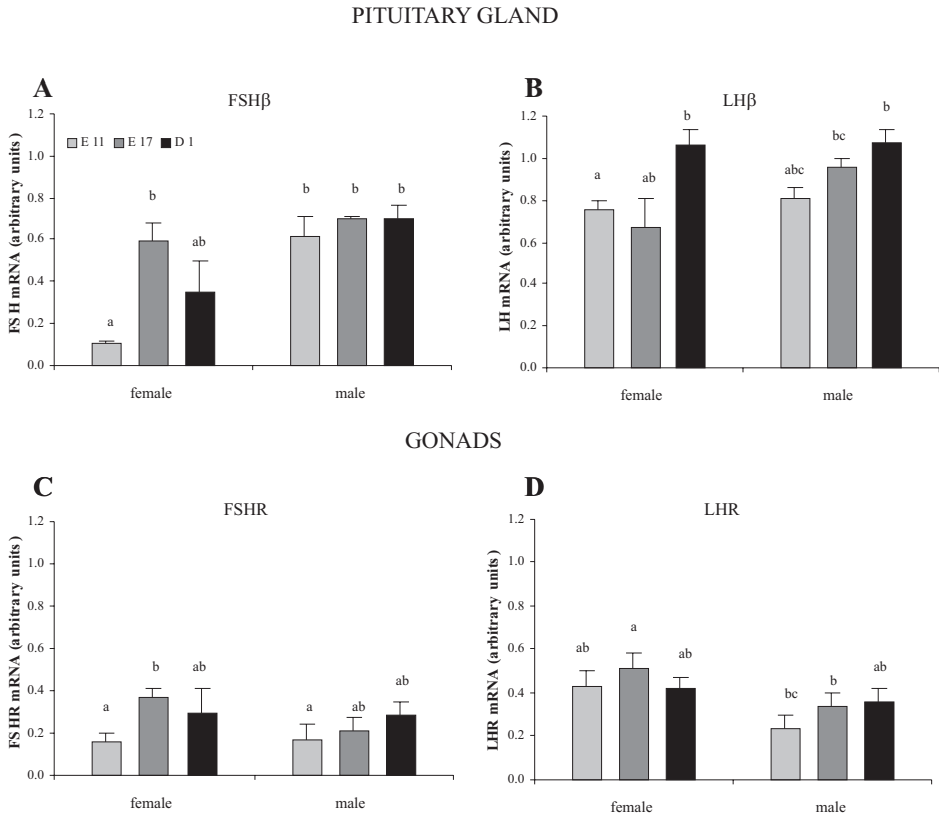


Figure 2. Semiquantitative analysis of mRNA expression (means \pm SEM) of FSH β (A) and LH β (B) in the pituitary gland as well as FSH receptor (FSHR; C) and LH receptor (LHR; D) in the gonads of the chicken embryo. The relative density of each gene product was compared with 18S rRNA product; n=6 independent experiments; means with different superscripts designate significant ($p<0.05$) differences.

DISCUSSION

The chicken embryo is an excellent model for studying developmental changes in the expression of hormones as well as their receptors during the growth and differentiation of the endocrine system. In the present study, we used this model to investigate LH β and FSH β gene expression in the pituitary gland and FSHR and LHR in gonads of female and male embryos. Our most significant results included: 1/ positive correlation between pitu-

Table 2. Coefficients of correlation (r) between the expression of FSH β or LH β mRNA in the pituitary gland and their appropriate receptors in the gonads of the female and male embryos.

	FSH β		LH β	
	Female	Male	Female	Male
FSHR	r=0.630 p<0.01, n=18	r=-0.133 NS, n=18	---	---
LHR	---	---	r=0.196 NS, n=18	r=0.654 p<0.01, n=18

NS: not significant

itary expression of FSH β mRNA and ovarian expression of FSHR mRNA, and 2/ positive correlation between pituitary LH β mRNA and testicular LHR mRNA. These data suggest that FSHR and LHR expression in the ovary and testes, respectively, is regulated by the appropriate gonadotropins. Moreover, they indicate the pivotal role of FSH in the ovarian and LH in testicular development. On the other hand, the lack of correlation between e.g. pituitary LH β mRNA and ovarian LHR mRNA in female embryos may be explained by the possibility of *trans*-activation of the receptor i.e. without requirement of the ligand binding, which is an alternative mechanism to *cis*-activation associated with activation of liganded receptor [12, 13].

It has been established that the HPG axis in chicken embryos appears to be functional between 11.5 and 14.5 days of egg incubation [40]. During this phase of embryonic development the pituitary expresses both LH β and FSH β subunits at the protein level [16, 24]. The pituitary expression of LH β mRNA in the present study was greater than that of FSH β in both female and male embryos. Similar results were shown in chickens by Maseki et al. [16] who demonstrated that LH β and FSH β transcripts started to be expressed on day 4 and day 7 of incubation, respectively, then, gradually increased during embryonic development reaching the maximal values on day 20 [16]. We observed a similar increase in LH β mRNA expression in female embryos.

Pituitary expression of FSH β mRNA in the female embryo on E17 was greater than that on E11. Higher expression of FSH β mRNA was observed on E11 in the hypophysis in males than in females. In agreement with our results, Rombauts et al. [26] found that starting from day 10 of egg incubation, FSH plasma concentrations were significantly higher in male than female embryos. In male embryos, the FSH plasma concentrations quickly rose after day 10 to reach a peak on day 13. Thereafter, they progressively decreased reaching the lowest values on day 20. In female embryos, plasma concentrations of FSH remained low throughout the incubation period [26]. In our opinion, the lack of relationship between FSH mRNA expression in the pituitary gland and plasma FSH level in male embryos suggests that during the second part of chicken embryogenesis, synthesis of FSH seems to be post-transcriptionally regulated by sex steroids.

The increase (1.4-fold) in pituitary LH β mRNA level was observed in females between E11 and D1. Woods [38] demonstrated that plasma LH is present in the male and female embryos on day 10.5. Plasma LH was detected on days 10 and 12 of egg incubation in female and male embryos, respectively [32]. Moreover, Gonzalez et al. [10] found that plasma LH in females increased through development to reach a plateau near hatching and that serum LH shows a maximum on day 19 of incubation. These results are consistent with data obtained in our experiment.

The present study showed that on E17 FSHR mRNA expression in the ovary was higher than in the testes. We observed a significant increase in ovarian FSHR mRNA expression on E17 in comparison with the E11. These results are in agreement with data of Mao et al. [15] who by means of Northern hybridization found that the levels of FSHR mRNA in the left embryonic ovaries remained very low from days 10 to 16 of egg incubation, and then increased after day 17 reaching a plateau on days 1-14 after hatching. The authors showed a similar increase in FSHR mRNA expression in the testes. We did not observe any significant changes in mRNA expression of FSHR in male gonads.

Several experimental approaches revealed that FSH acting *via* FSHR plays a crucial role in the gonadal development in chicken. FSH stimulates cell proliferation and sex steroid synthesis in the left ovary [21, 34, 36]

and the testes [17, 18, 21, 22]. Woods et al. [41] demonstrated that there was a significant increase in the numerical density of the FSH positive cells of the whole ovary between days 12.5 and 19.5. This implies that the increase in FSH receptor mRNA expression is probably related to some physiological changes within the gonads at the time approaching hatching. Velazquez et al. [36] showed that *in vitro* treatment of the left ovary of a chicken embryo with FSH resulted in a dose-dependent stimulation of DNA synthesis in the immature ovary. Pedermera et al. [21] observed the proliferative effect of FSH in the left ovary on days 15 and 18 of chicken embryogenesis. In the same ovary, estradiol and testosterone secretion were stimulated after FSH treatment on days 8-18 of chicken embryo development. Gómez et al. [9] demonstrated that the effects of FSH are associated with the induction of aromatase and 3 β -hydroxysteroid dehydrogenase activity. The testicular tissue displayed a FSH response as an increment in cell proliferation [21, 22, 18]. The *in vitro* effect of FSH on cell proliferation and steroid hormone secretion differed in the gonads with the age of the embryo [21].

Woods et al. [40] detected the LH-binding cells in the ovary and testes as early as on day 6.5 of the chicken embryogenesis. Akazome et al. [2] using *in situ* hybridization demonstrated that cells expressing LHR mRNA started to appear in chicken embryos on day 7 and 14 in the ovary and testis, respectively. Using the quantitative PCR, they were able to detect LHR mRNA in the gonads of both sexes between days 4 and 6 of embryogenesis [3]. The early ovarian expression of LHR mRNA is related to the earlier sex steroid production in females.

In the present study, the LHR mRNA expression was found on E11 in male and female gonads. This expression was higher in the ovary than in the testes only on E17. This difference was not as spectacular as that shown by Akazome et al. [3] who found on day 12 of egg incubation a sevenfold higher expression of LHR mRNA in the ovary than in the testes. Mao et al. [15] established that the expression of LHR mRNA was higher in the ovary than in testes until day 18 of incubation. LHR mRNA expression in testes started to increase from day 20 of incubation. Surprisingly, in 7-day-old chicks it was higher in the testes than in the ovary possibly reflecting the gonadal steroidogenic activity.

The results of the present study showed that gonadal LHR mRNA expression in males was lower than in the females and showed a tendency to increase as embryogenesis advanced. The presence of LHR and its changes in the chick embryonic gonads indicate that in both the ovary and testes, LH acts as an important modulator of processes associated with gonadal growth including steroidogenesis and cell proliferation. LH stimulates estradiol synthesis and secretion from the left ovary *in vivo* [33, 34, 39] and *in vitro* [10] as well as testosterone synthesis by embryonic testes *in vitro* [4, 6, 21]. Moreover, recent studies have shown that LH injected during embryonic development inhibits oogonial proliferation and induces meiotic prophase and follicle formation in the ovaries of newly-hatched chicks [11].

To summarize, results of this study showed that mRNA expression of LH and FSH in the pituitary gland and their receptors in gonads changes during egg incubation. Sexual dimorphism in the gene expression indicates a sex- and time-dependent role of FSH and LH during gonadal development. To our knowledge, this is the first report concerning the simultaneous determination of mRNA expression of gonadotropin and their receptors in female and male chicken embryos during egg incubation. The increase in mRNA expression of FSH β in female hypophysis and FSHR in the ovary on day 17 suggests that the transcription of FSHR mRNA might be directly regulated by FSH. A similar interrelationship probably exists between pituitary LH β mRNA expression in males and LHR mRNAs in the testes. These assumptions are supported by the positive correlation between FSH β and FSHR mRNA expression in female and LH β and LHR mRNA expression in male embryos. Further investigations are needed to explain the interrelationship between pituitary and gonadal function in developing chicken embryos.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Polish Committee of Scientific Research (KBN, Warsaw) No. 2 PO6D 020 29 and DS 3243/KFZ. The results of this study were previously partly presented at the 15th Interna-

tional Symposium of Polish Network of Molecular and Cellular Biology UNESCO/PAS “Molecular and Physiological Aspects of Regulatory Processes of the Organism”, Krakow, 2006.

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