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ASSESSMENT OF FOLLICLE-STIMULATING HORMONE (FSH)-, LUTEINIZING HORMONE (LH)-, ESTROGEN (α AND β)- AND PROGESTERONE (PR) RECEPTORS MRNA EXPRESSION IN THE GONADS AND LIVER OF CHICKEN *(GALLUS DOMESTICUS)* EMBRYO*

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Activation of particular signaling pathways in the cell depends on expression of receptors responsible for hormone binding. Expression of gonadotropin and steroid receptors is necessary for growing, development and gonadal tissue differentiation during embryogenesis. The aim of the present study was to analyze the mRNA expression of FSH- (FSHR), LH- (LHR), estrogen- (ER) and progesterone (PR) receptors in male and female gonads as well as in the liver of the 14-day-old chicken embryo. The experiment was carried out on fertilized Hy-Line eggs incubated in standard conditions. The gonads and fragments of the liver were dissected and immediately placed into StayRNA, then the RNA was isolated using TRI-Reagent. After reverse transcription, the cDNA was subjected to real-time qPCR for examination of FSHR, LHR, estrogen receptor α (ERα) and β (ERβ) and PR. The obtained results show that each examined mRNA receptor was expressed in male and female gonads as well as in the liver. The expression level of each analyzed gene was significantly lower in the liver than in male and female gonads. In the ovary, the mRNA expression of LHR was significantly higher than PR, while the expression of FSHR and ERα was much lower; the lowest level of mRNA expression was found in the case of ERβ expression analysis. In the testes, the highest expression was for LHR and PR, however, the level of PR mRNA was higher in comparison to LHR. Relatively lower expression was for FSHR, followed by ERα, and the lowest for ERβ mRNA. In the embryonic liver a different pattern of these receptors expression was noticed. The highest expression was observed for ERα, lower for LHR, ERβ and PR, and the lowest for FSHR. These results indicate that during chicken embryonic development significant alterations in mRNA expression of gonadotropin and steroid hormone receptors appear in both gonadal and liver tissues. They suggest that in comparison to male and female gonads, the liver (as the extragonadal tissue) shows different sensitivity to gonadotropins and steroid hormones.

Key words: chicken embryo, gonads, liver, gonadotropin receptors, steroid hormone receptors

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In the chicken, gonadal sex is bipotential up to day 6 of embryogenesis (ED6). By days 8–10 of incubation, the gonads differentiate and can be morphologically distinguishable. Gonads of females (heterozygotes ZW) develop as asymmetric ovaries. Finally, only left ovary develops and the right ovary regresses during late embryogenesis. Male (homozygotes ZZ) gonads develop as symmetric testes (see Hirst et al., 2018 for review). In the avian species, estrogens play an essential role in sex-dependent differentiation of the ovarian tissue and the blockage of estrogen synthesis leads to phenotypical sex-reversal in the genetic females (Elbrecht and Smith, 1992; Major and Smith, 2016). Synthesis of sex steroids by embryonic gonads is regulated by pituitary gonadotropins: luteinizing hormone (LH) and follicle-stimulating hormone (FSH), while in gonadal tissue the biological action of LH and FSH is mediated by membrane receptors: LHR and FSHR. Previously it was found that gonadotropins play essential role in bipotential gonad differentiation as well as in the hypothalamo-pituitary-gonadal axis development in the chicken embryo (Akazome and Mori, 1999; He et al., 2013). Gonadal expression of LHR and FSHR mRNA was detected at ED4 in males and females (Akazome et al., 2002). Despite receptors binding gonadotropins, crucial role is played by steroid hormone binding receptors. In the gonadal tissue, two isoforms of nuclear receptors: estrogen receptor α (ERα) and ß (ERß) play different physiological roles. The ERß protein is smaller than the ERα but possesses the modular structure of distinct functional domains (A–F) characteristic of the members of the superfamily of nuclear receptors. When compared with ERα, the protein sequence of the ERß demonstrates considerable homology in the DNA and ligand binding domains (Kuiper et al., 1997). Relative binding studies on *in vitro* translated protein have shown that the ERß is able to bind 17ß-estradiol with an affinity similar to that of ERα (Kuiper et al., 1997). ERß is able to mediate the effects of 17ß-estradiol in a dose-dependent manner, although levels of induction were slightly lower than those obtained with ERα (Tremblay et al., 1997). ERα activity may by associated with proliferation, whereas ERß with stimulation of apoptosis and cell differentiation (Zhao et al., 2008; Paterni et al., 2014). Higher mRNA expression of ERα than ERß in the laying hen ovary (Hrabia et al., 2008; Sechman et al., 2020) and in the chorioallantoic membrane of the chicken embryos at ED12, ED15 and ED18 has been previously observed (Grzegorzewska et al., 2016).

Progesterone, another steroid hormone, may be the initiator of germ cell meiotic initiation for both male and female chicken embryo (Mi et al., 2014). Two PR isoforms have been described in chicks: a full-length form (PR-B, 110 kDa) and the N--terminally truncated one (PR-A, 79 kDa), which lacks the amino-terminal 128 amino acids of PR-B (Tora et al., 1988). PR isoforms are derived from a single gene and are generated from alternative translational start sites (Conneely et al., 1987). In oviparous species its role is well exemplified by the chicken oviduct. Progesterone is essential for complete development of the oviduct and for the synthesis of egg white proteins (avidin) in estrogen-primed oviductal cells (Joensuu et al., 1991; Socha et al., 2017). Hepatic vitellogenin is a yolk precursor protein sequestered in follicular oocytes as a nutrient supply for developing embryos in non-mammalian vertebrates. The importance of this nutrient protein declines with placental evolution and viviparity, and it is not expressed in mammals (Callard, 1992). It is well established that hepatic vitello-

genin is up-regulated by estradiol (Perlman et al., 1984) and progesterone (Custodia--Lora and Callard, 2002). In the Japanese quail, progesterone completely inhibited vitellogenin synthesis (Gupta and Kanungo, 1996).

Chicken embryo is a useful model in analysis of angiogenesis and metastasis and the level of estrogen and progesterone receptor expression is used as a marker in breast cancer. Receptors able to bind hormones regulating gonadal function are present not only in gonads, but also in some other target tissue. Therefore, the aim of this study was to analyze mRNA expression of *LHR*, *FSHR*, *ERα*, *ERβ* and *PR* in male and female gonads, as well as in the liver of chicken embryos at ED14 (i.e. at the stage following gonadal differentiation).

Material and methods

Animals and experimental procedure

Experiments were carried out on fertilized Hy Line eggs (n=20) purchased from a local breeder (Tarnów, Poland) and incubated in a Brinsea 190 Advance automatic incubator under standard conditions (37.5°C, 55% humidity). The experiment was conducted according to a research protocol approved by the First Local Animal Ethics Committee in Kraków, Poland. The gonads (i.e. the left ovary and both testes) and the liver were isolated after decapitation of 14-day-old chicken embryos (n=6 of male and female embryos). Tissue samples were immediately placed into StayRNA and stored at –20°C until total RNA isolation.

RNA extraction, reverse transcription, and real-time PCR

RNA isolation and reverse transcription were carried out as described previously (Grzegorzewska et al., 2020) and EvaGreen real-time PCR analyses were made according to manufacturer instruction. Briefly, total RNA (2 mg from each tissue extracted with TRI-reagent) was reverse-transcribed with a High-Capacity cDNA Reverse Transcription Kit including random primers. Samples were incubated in a thermocycler (Mastercycler Gradient; Eppendorf, Hamburg, Germany) in accordance with the profile recommended by the manufacturer (25°C for 10 min, 37°C for 120 min, and 85°C for 5 min). The cDNA was subjected to real-time qPCR for examination of *FSHR*, *LHR*, *ERα*, *ERβ* and *PR* with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as a housekeeping gene. Real-time qPCR was performed in a 96-well thermocycler (StepOnePlus; Applied Biosystems, USA) according to the recommended cycling program: 15 min at 95°C, 40 cycles of 15 s at 95°C, 20 s at 62°C, and 20 s at 72°C. The singleplex real-time qPCRs were performed in a 10 mikro volume containing 2 µl 5x HOT FIREPol EvaGreen qPCR Mix Plus, 0.12 µl Primer Forward (10 pmol/µl), 0.12 µl Primer Reverse (10 pmol/µl), 1 µl cDNA (10x diluted sample after the RT template, and H_2O up to a final volume of 10 µl). The characteristics of primers are described in Table 1. Each sample was run in duplicate, and a no-template control was included in each run. Relative quantification was calculated after normalization with the GAPDH transcript by using the $2^{-\Delta\Delta Ct}$ method. Quantification was performed using StepOne integrated software.

Table 1. Positions of oligonucleotide primers in mRNA sequence GenBank Accession Numbers, sequences of amplified gene primers, annealing temperature of PCR reaction and product size

Gene	Primer sequence (5'-3')	Position (bp)	GenBank Accession Number	Product size (bp)
FSHR	*ATGGAACCTGCCTGGATGAG* *CTTGTATGTAGACCTCGCTCTTAG*	627-646 785-808	NM_205079	182
LHR	*ATTGTGCTCCTCGTCCTC* *GTCTATGGCGTGGTTGTAG*	1273-1290 1416-1434	AB009283	162
ERα	*TGCGAGCTCCAACCCTTTGGACA* *GGAGCGCCACACTAAGCCGATCA*	1037-1059 1343-1365	MN_205183	329
ERβ	*TCCTGCATGCTGAATTACAAC* *GGCTCTTAGGCTGCTCTG*	299-420 548-565	AB036415.1	167
PR	*GGAAGGGCAGCACAACTATT* *GACACGCTGGACAGTTCTTC*	2067-2086 2130-2149	MN_205262.1	83
GAPDH	*GTGTGCCAACCCCCAATGTCTCT* *GCAGCAGCCTTCACTACCCTCT*	752-774 827-848	MN_204305	97

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase (housekeeping gene): FSHR – follicle stimulating hormone receptor; LHR – luteinizing hormone receptor; ERα – estrogen receptor α; ERβ – estrogen receptor β; PR – progesterone receptor.

Reagents

The chemicals used in tissue preparation and real-time PCR analysis were purchased from the following companies: StayRNA (A&A Biotechnology, Gdynia, Poland); TRI-Reagent (MRC Inc., Cincinnati, OH, USA); High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA); primers (IBB PAN, Warsaw, Poland); 5x HOT FIREPolEvaGreen qPCR Mix Plus (ROX) (Solis BioDyne, Tartu, Estonia).

Statistical analysis

The data were analyzed by one-way ANOVA followed by Duncan's multiple range test. Log transformations were performed as needed to maintain homogeneity of variance and normality. The values are expressed as mean ± SEM and considered significantly different at $P<0.05$. Calculations were performed using SigmaStat (Systat Software Inc., CA, USA).

Results

The mRNA expression of each selected gene was detected in the ovary, testes and the liver of the chicken embryo (Table 2). The expression level of *FSHR*, *LHR* and *PR* was significantly (even more than 100-fold) lower in the liver than in male and female gonads. The mRNA expression of *ERa* was 2-fold lower in the liver than in male and female gonads, whereas *ERβ* mRNA in the liver was 2-fold lower than in ovary and 3-fold lower than in testes.

Table 2. Expression of *FSHR*, *LHR*, *ERα*, *ERβ* and *PR* mRNAs in gonads and the liver of chicken embryo (each RQ value was counted in relation to expression of FSHR in the ovary)

Gene	Examined tissue		
	Ovary	Testes	Liver
FSHR	1.04±0.09 d	1.36±0.04 d	0.01±0.001 a
LHR	10.08±0.58 f	3.34±0.28 e	0.08±0.04 b
ERα	0.69±0.08 c,d	0.65±0.002 c,d	0.31±0.04 c
ERβ	0.06±0.004 b	0.09±0.006 b	0.03±0.005 a,b
PR	3.82±0.29 e	4.40±0.22 e	0.03±0.007 a,b

The data were analyzed by one-way ANOVA (F value for effect of tissue at P<0.001) followed by Duncan's test. Means marked with different letters are significantly different at P<0.05.

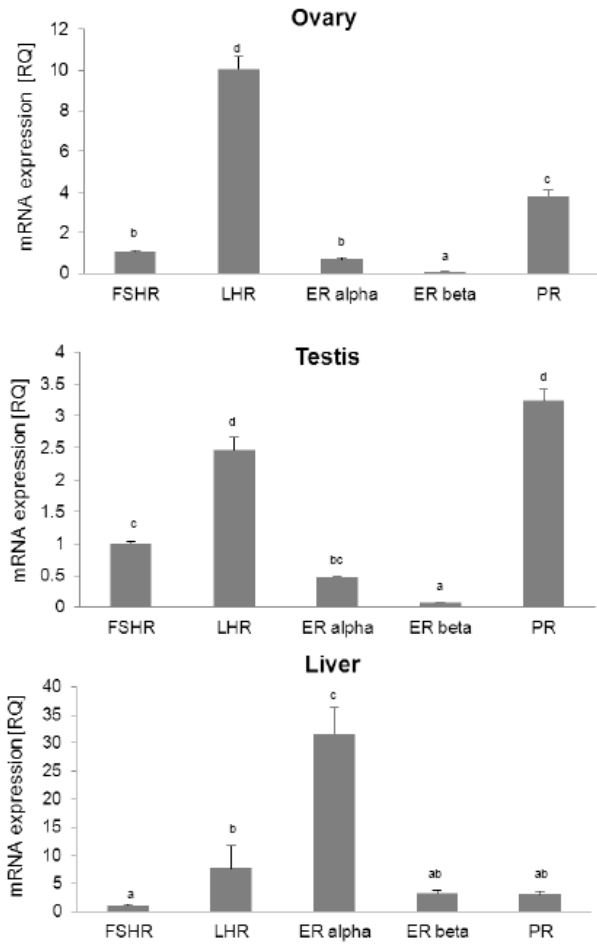

Figure 1. Expression of *FSHR*, *LHR*, *ERα*, *ERβ* and *PR* mRNAs in the ovary, testis and liver of the 14-day-old chicken embryo. Each value represents the mean ± SEM from 6 (six) animals. Data of gene expression represent the mean of relative quantity (RQ) standardized to expression of *FSHR* in the tissue (RQ = 1). Values marked with different letters differ significantly (P<0.05)

Comparison of mRNA expression of all analyzed genes within only one tissue (ovarian, testicular or liver) is presented in Figure 1. In the ovary, *LHR* expression was significantly higher than *PR*, and the mRNA expression of *FSHR* and *ERα* was much lower, and the lowest level of expression was observed with respect to *ERβ* expression. In the testes, the same as in the ovary, the highest expression was for *LHR* and *PR*. In comparison with these receptors, a significantly lower mRNA expression was found in the case of *FSHR, ERα* and *ERβ*, with the expression of these genes arranged in the following order: *FSHR* > *ERα* > *ERβ*. In the embryonic liver a different pattern of these receptors expression was noticed. The highest expression was found for *ERα*, lower for *LHR, ERβ* and *PR*, and the lowest for *FSHR*.

Discussion

In the present study mRNA expression of *LHR* was significantly higher in the ovary than in the testes, while the *FSHR* achieved a similar value in female and male gonads. In the chicken embryo, the expression of FSHβ mRNA in the hypophysis (Grzegorzewska et al., 2009) and plasma FSH concentration (Rombauts et al., 1993) was higher in males than in females. In all examined tissues, *LHR* expression was higher than *FSHR* expression. Similar results were obtained by Grzegorzewska et al. (2009) using semiquantitive PCR in the chicken embryonic gonads at ED11 and ED17. LH stimulates estradiol synthesis and secretion from the left ovary *in vivo* (Teng et al., 1982) and *in vitro* (Pedernera et al., 1999). González-Morán (2007) showed that LH *in ovo* injection inhibits oogonial proliferation and induces its meiotic prophase, as well as follicle formation in the ovary of a newly-hatched chicken.

In the present study, mRNA expression of *FSHR* and *LHR* was also found in the liver of the chicken embryo, but the expression was very low. In mouse liver, FSH enhances gluconeogenesis, suggesting an essential role of FSH in the pathogenesis of fasting hyperglycaemia (Qi et al., 2018), whereas in an elderly human population it was found that the development of nonalcoholic fatty liver disease (NAFLD) may be associated with lower FSH level (Wang et al., 2016; Li et al., 2018). Additionally, FSHR was expressed by the endothelium of blood vessels in the majority of metastatic tumors in human (Siraj et al., 2013). These results suggest that the liver is a target tissue for FSH and the role of this hormone may be fundamental for protein synthesis and embryonic development.

This study demonstrated mRNA expression of *ER*s in both the gonadal and liver tissues. In the ovary of 14-day-old chicken, the *ERα* mRNA expression was at a similar level as in the testes. Surprisingly, the high expression of mRNA for *ERα* was observed in the liver; it was comparable to the expression in the gonads. Previously, it was shown that in yak *(Bos grunniens)* the mRNA expression of *ERα* was the highest in the mammary gland, followed by the uterus, oviduct, and ovary, and the lowest in the liver, kidney, lung, testis, spleen, and heart. The *ERβ* mRNA level was the highest in the ovary; intermediate in the uterus and oviduct; and the lowest in the heart, liver, spleen, lung, kidney, mammary gland, and testis and the expression level of *ERα* was higher than that of *ERβ* in all tested tissues, except testis (Fu et al., 2014). In our study,

in male, as well as in female gonads and the liver, *ERα* mRNA expression was also higher than *ERβ*. The differences between literature data and results obtained in the present study may be due to the fact that only one developmental stage of the chicken embryo was studied and it does not represent a full expression profile occurring during development. Knowledge of the distribution of *ERβ* in various tissues is limited at this time. In the rat, the highest level of *ERβ* mRNA was reported in the ovary as well as in the prostate epithelium (Kuiper et al., 1996). *ERβ* mRNA was detected in several regions of the anterior hypothalamus of the female rat (Shughrue et al., 1996), whereas in humans, *ERβ* transcripts were detected in the testis, ovary, and thymus (Mosselman et al., 1996). In the mouse, *ERβ* transcripts were not detected in the liver, heart, kidney, skeletal muscle, thymus, spleen, and brain (Tremblay et al., 1997) and the highest level of *ERβ* mRNA was found in the ovary of the female, the prostate and epididymis of the male, followed by the hypothalamus and lung in both sexes.

In the current investigation, the highest level of mRNA expression of PR was found in the gonadal tissue, especially in the testes, but the detectable mRNA levels were also detected in the liver. These results suggest that also gestagens (including progesterone) may have physiological effects on many organs outside the reproductive system not previously known as steroid-target tissues. It has been revealed that PR is under dual regulation of estrogen and progesterone, and the amount of PR is increased by estrogen in most target cells (Nardulli et al., 1988). On the other hand, there is evidence of constitutive expression of PR in certain cell types independent of these two steroid hormones (Salomaa et al., 1989; Joensuu et al., 1991). In addition to the expression of PR in the chicken oviduct and ovary (Gasc et al., 1984; Joensuu et al., 1991), it has also been detected in immunological organs such as the chicken bursa of Fabricius (Ylikomi et al., 1987) and the mammalian thymus (Nilsson et al., 1990). PR distribution in the brain has been documented in the hypothalamus, forebrain and pituitary gland (Sterling et al., 1987; Gasc and Baulieu, 1988). PR-positive stromal cells were identified in the colon and cloaca of immature chickens (Salomaa et al., 1989). Pasanen et al. (1997) reported constitutive PR expression in stromal cells of the lung, abdominal and urogenital organs, but not in the liver and kidney. Moreover, Salomaa et al. (1989) found expression of PR in the peritoneal and smooth muscle cells of the lower gastrointestinal tract and suggested the possible role of progesterone in regulation of muscular contractions. In non-reproductive organs like the gastrointestinal canal, lung and liver the steroid receptor studies have mainly focused on malignancies (Singh et al., 1993; Ishizu et al., 1994). Based on these studies hormonal etiology and hormonal therapies have been proposed.

Progesterone and estradiol are involved in the regulation of ovulation (for review see: Rangel and Gutierrez, 2014) and sexual and nesting behaviors in birds (Gahr, 2001). Camacho-Arroyo et al. (2007) determined PR and ERα isoforms content in the brain of chicks of both sexes on days 8 and 13 of embryonic development as well as on the day of hatching. PR isoforms protein content increased during embryonic development in both female and male chick brain. The highest PR isoforms content was observed on the day of hatching in both sexes. Interestingly, PR-A content was higher in the brain of chick males than in that of females on day 8 of embryonic development. PR-A/PR-B ratio was higher in the brain of males than in that of females

at all ages. There were found two ERα isoforms of 66 and 52 kDa; the content of both isoforms was higher in the brain of females than in that of males on days 8 and 13 of embryonic development. An opposite pattern of ERα isoforms content was observed. In males, ERα content increased during embryonic development whereas in the females it decreased during this process. These results indicate that the content of PR and ERα isoforms is related to the degree of brain development in chicks, and suggest that PR and ERα isoforms should exhibit sexual dimorphism in the brain of chicks during embryonic development (Camacho-Arroyo et al., 2007). ERα may be relevant in the development of hepatic steatosis and diffuse expression of ERα would appear to impede disease progression, including hepatic fibrosis (Choi et al., 2018). However, the presence and role of steroid receptors in normal non-reproductive tissues is not precisely established and additional research is needed not only to understand the mechanism of physiological function regulation by steroid hormones, but also to unravel their probable role in pathological states.

In summary, results of this study indicate that significant alterations in mRNA expression of gonadotropin and steroid hormone receptors exist in both the ovary and testes, as well as in the liver on day 14 of embryonic development. They suggest that in comparison to male and female gonads, the liver (as the extragonadal tissue) shows different sensitivity to gonadotropins and steroid hormones.

Conflict of interest

Author declares no conflict of interest regarding the publication of this article.

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AGNIESZKA GRZEGORZEWSKA

Ekspresja mRNA receptorów hormonu folikulotropowego (FSH), hormonu luteinizującego (LH), estrogenu (α i β) i progesteronu (PR) w gonadach i wątrobie zarodka kurzego (*Gallus domesticus*)

STRESZCZENIE

Aktywacja poszczególnych szlaków sygnalizacyjnych w komórce zależy w dużym stopniu od ekspresji receptorów odpowiedzialnych za wiązanie hormonów. Ekspresja receptorów gonadotropin i steroidów jest niezbędna do wzrostu, rozwoju i różnicowania gonad podczas embriogenezy. Celem pracy była analiza ekspresji mRNA receptorów FSH (FSHR), LH (LHR), estrogenów (ER) i progesteronu (PR) w męskich i żeńskich gonadach, a także w wątrobie 14-dniowego zarodka kury domowej. Eksperyment został przeprowadzony na zapłodnionych jajach kur Hy-Line inkubowanych w standardowych warunkach. Gonady i fragmenty wątroby wypreparowano i natychmiast umieszczono w odczynniku StayRNA, a następnie wyizolowano RNA przy użyciu odczynnika TRI-Reagent. Po odwrotnej transkrypcji cDNA poddano reakcji amplifikacji (qPCR) w celu analizy ekspresji FSHR, LHR, receptora estrogenowego α (ERα) i β (ERβ) oraz PR. Uzyskane wyniki pokazują, że mRNA każdego badanego receptora wykazuje ekspresję w gonadach oraz wątrobie zarówno w męskich, jak i żeńskich zarodkach kury domowej. Poziom ekspresji każdego analizowanego genu był istotnie niższy w wątrobie niż w męskich i żeńskich gonadach. W jajniku ekspresja mRNA LHR była istotnie wyższa niż PR, podczas gdy ekspresja FSHR i ERα była znacznie niższa; najniższy poziom ekspresji mRNA stwierdzono w przypadku ERβ. W jądrach najwyższa ekspresja występowała w przypadku LHR i PR, jednak poziom mRNA PR był wyższy w porównaniu z LHR. Wykazano relatywnie niższą ekspresję FSHR, następnie ERα, a najniższą ekspresję stwierdzono w przypadku ERβ. W wątrobie zarodków kury najwyższą ekspresję zaobserwowano w przypadku ERα, niższą dla LHR, ERβ i PR, a najniższą dla FSHR. Wyniki te wskazują, że w trakcie rozwoju embrionalnego kury znaczące różnice w ekspresji mRNA receptorów gonadotropin i hormonów steroidowych obecne są zarówno w tkankach gonad, jak i wątroby. Wzajemne relacje poziomu ekspresji mRNA receptorów poszczególnych hormonów są odmienne w gonadach i wątrobie. Sugeruje to, że w porównaniu z gonadami męskimi i żeńskimi, wątroba (jako tkanka pozagonadalna) wykazuje odmienną wrażliwość na gonadotropiny i hormony steroidowe.

Słowa kluczowe: zarodek kurzy, gonady, wątroba, receptory gonadotropinowe, receptory hormonów steroidowych