

mRNA Expression and Immunocytochemical Localization of Leptin Receptor in the Oviduct of the Laying Hen (*Gallus domesticus*)*

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The role of leptin in female reproduction is fairly well established in mammals, whereas reports concerning leptin action in birds are scarce. The aim of the present study was to detect leptin receptor (LEP-R) mRNA and to localize the leptin receptor protein in the oviduct of laying hens 2h after ovulation by the RT-PCR method and immunocytochemical staining. The RT-PCR reaction demonstrated expression of the long form of leptin receptor mRNA in all examined oviductal parts (infundibulum, magnum, isthmus and shell gland) and the weakest level was found in the isthmus. The expression of the short isoform was lower than the long form in all examined tissue samples and no differences between oviductal parts were observed. Immunostaining specific for leptin receptor was found in the walls of all examined oviductal parts. The intensity of the immunopositive reaction was the strongest in the epithelium of all examined parts of the oviduct and in the endothelium and muscles of blood vessels. The weakest immunopositive reaction was observed in tubular glands, the connective tissue layer and in circular and longitudinal muscles. The results obtained in this experiment suggest that the oviduct may be a target tissue for leptin, where this polypeptide hormone may participate in egg formation and/or its transport through the oviduct of the domestic hen.

Key words: Leptin receptor, oviduct, chicken.

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Leptin has been shown to play an important role in the regulation of food intake, energy metabolism and reproduction (FRIEDMAN and HALAAS, 1998). In mammals leptin is produced mainly in the adipose tissue (ZHANG *et al.* 1994) and also in reproductive organs such as placenta, uterus (KAWAMURA *et al.* 2003), oviduct (KAWAMURA *et al.* 2002) and in embryonic tissue (TRAYHURN *et al.* 1999). Leptin receptor is a member of the class I cytokine receptor family (FRIEDMAN & HALAAS 1998). In mammals as a result of alternative splicing six isoforms of leptin receptor exist. They have an identical extracellular domain and differ only in the length of the intracellular domain.

Reports concerning the action of leptin in birds are unsatisfactory. Leptin in birds is produced mainly by the liver (TAOUI *et al.* 1998; ASHWELL *et al.* 1999). The genes encoding the long form of leptin receptor have been cloned in chicken (HOREV *et al.* 2000; OHKUBO *et al.* 2000) and turkey (RICHARDS

& POCH 2003). The short isoform of leptin receptor has also been identified (LIU *et al.* 2006). It has been demonstrated that in laying hens exogenous leptin attenuates the negative effects of fasting on ovarian function (PACZOSKA-ELIASIEWICZ *et al.* 2003) and the expression of mRNA of the long form of leptin receptor has been found in the hypothalamus (HOREV *et al.* 2000; PACZOSKA-ELIASIEWICZ *et al.* 2003), the pituitary (PACZOSKA-ELIASIEWICZ *et al.* 2003) and the ovary (OHKUBO *et al.* 2000; PACZOSKA-ELIASIEWICZ *et al.* 2003).

The oviduct of the hen develops rapidly after 16 weeks of age. It consists of five distinguishable regions: infundibulum, magnum, isthmus, shell gland and vagina. Subsequent to ovulation, the ovum is engulfed by the infundibulum, where it resides for approximately 15-30 min. The fertilization of the ovum and the production of the first layer of egg white occur in the infundibulum. Next, the ovum passes to the largest portion of the

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oviduct, the magnum, where the majority of egg white proteins are formed. The ovum remains in the magnum for approximately 2-3h. Both inner and outer shell membranes are formed in the next portion of the oviduct, the isthmus, during 1.5h. Calcification of the egg occurs in the shell gland where the egg stays for about 18h. When this process is finished, the expulsion of the egg commences through the vagina (see GILBERT 1979).

The mRNA expression of the long form of leptin receptor has been obtained in mammalian ovaries, uterus and testes (ZAMORANO *et al.* 1997; GONZALES & LEAVIS 2003; KAWAMURA *et al.* 2003; ZERANI *et al.* 2004). Leptin receptor has also been detected in the oviduct of mammals (LIU *et al.* 2003; ZERANI *et al.* 2005), but so far it has not been shown in the avian oviduct. Therefore, the aim of the present study was to determine whether mRNA of the short and long form of leptin receptor is expressed in the oviduct of the laying hen and to ascribe their relative contributions if both isoforms are expressed. The next purpose of this experiment was to detect and immunocytochemically localize the leptin receptor in the oviductal tissue.

Material and Methods

The experiment was carried out in accordance with the principles and procedures of the Local Animal Ethics Committee in Kraków on 34-week-old Hy-Line Brown laying hens (n=8), caged individually with free access to food and water, under a photoperiod of 14L:10D. The time of ovulation was determined by checking oviposition at 0.5h intervals between 7.00 a.m. and 4.00 p.m. for at least 2 weeks before selection for the test. Ovulation was considered to occur 5 min after oviposition of the previous egg in the series. Birds were decapitated 2h after oviposition/ovulation.

RNA isolation and RT-PCR reactions

Fragments of the middle part of each oviductal segment (infundibulum, magnum, isthmus and shell gland) were kept frozen in liquid nitrogen until isolation of total RNA by using the TRI-reagent (Molecular Research Center, USA), according to the method described by CHOMCZYNSKI and SACCHI (1987). RNA was dissolved in pure RNase free water (Promega, USA) and kept at -80°C. The density of RNA was measured spectrophotometrically using a Biophotometer (Eppendorf, Germany). After DNase treatment using RQ1 RNase-Free DNase (Promega, USA) the first strand complementary DNA (cDNA) synthesis from total RNA was catalysed. Each 20 µl RT reaction mixture contained 5 µg of total RNA, 200U M-MuLV reverse transcriptase (Fermentas, Lithuania), 0.5 µg oligo(dT)₁₈ as a primer, 1mM of each dNTP, 20U Ribonuclease Inhibitor (Fermentas, Lithuania). The resulting first strand cDNA was used for PCR reaction with primers (IBB PAN, Poland) designed to amplify the regions of the chicken leptin receptor encoding the extracellular and intracellular domains of the long form of leptin receptor, and a further pair to amplify the intracellular region of the chicken leptin receptor short form (LIU *et al.*, 2006). As a reference gene 18S rRNA was used (Table 1). The 25 µl PCR reaction mixture contained 1 µl of cDNA, 0.2 mM of each dNTP, 0.2 mM of each primer, 1.5 mM MgCl₂ and 0.625U of TaqDNA Polimerase (Fermentas, Lithuania). The PCR reactions were performed in a Thermocycler Gradient (Eppendorf, Germany). PCR products were resolved on 1.5% agarose gels containing ethidium bromide. The PCR products were semi-quantitatively determined from the density of the gel band using the Scion Image for Windows (Scion Corporation, Maryland, USA). The relative density of each gene was compared with 18S rRNA products, and expressed as the mean of 8 samples.

Immunocytochemical localization of leptin receptor

Fragments of the middle part of each oviductal segment were fixed in 4% formaldehyde in

Table 1

Parameters of primers used in RT-PCR reactions and product size

Gene	Position	GenBank accession number	Sequences: (5'-3')	Primers sequences according to:	Size of product
Extracellular domain of leptin receptor	1502-1523 2054-2073	AF169827	F: ATGGTCTGCAAACCCAAACGCA R: AGGGAGCTTGGATATCTTTT	LIU <i>et al.</i> 2006	572 bp
Intracellular domain of long form of leptin receptor	2749-2768 3607-3626	AF169827	F: GAATGAAGAAACTGCTCTGG R: ACAGTCTGTTCTCTGTGGTCT	LIU <i>et al.</i> 2006	878 bp
Intracellular domain of short form of leptin receptor	1-18 197-216	AY348719	F: GAATGAAGAAACTGCTCTGG R: TACAACTCATGTGCTGCATT	LIU <i>et al.</i> 2006	216 bp
18S rRNA	160-179 308-326	AF173612	F: CGCGTGCATTTATCAGACCA R: ACCCGTGGTCACCATGGTA	PACZOSKA-ELIA-SIEWICZ <i>et al.</i> 2003	167 bp

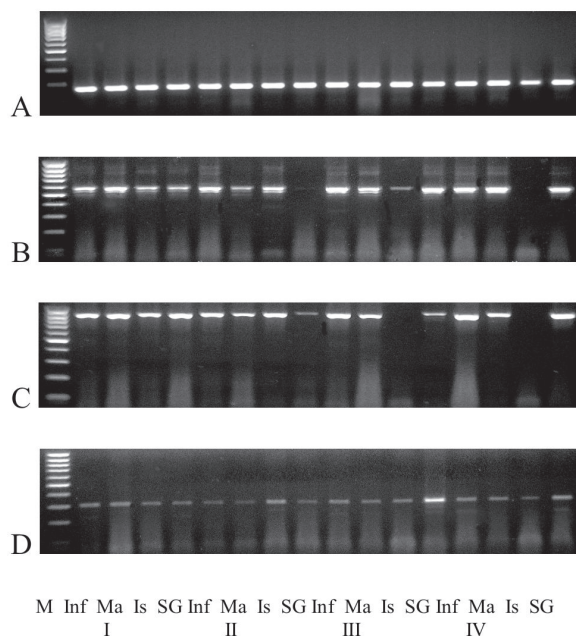


Fig. 1. Expression of mRNA of leptin receptors in the oviduct of the laying hen (4 series were shown). A) reference gene 18S rRNA, B) extracellular domain of long form of leptin receptor, C) intracellular domain of long form of leptin receptor, D) short form of leptin receptor. M-Molecular marker (100-1000bp), Inf-Infundibulum, Ma-magnum, Is-Isthmus, SG-Shell gland.

phosphate-buffered saline (pH 7.4) and embedded in paraffin by routine procedure. The 6 μ m thick sections of the examined oviductal parts were stained by routine haematoxylin-eosin technique or the avidin-biotin-horseradish peroxidase (HRP) complex technique. The sections were dewaxed in xylene and hydrated through a series of graded ethanol and microwaved in 10mM citric acid (pH 6.0) for antigen retrieval. Next, they were incubated in 1% H_2O_2 to inhibit the endogenous peroxidase activity. Afterwards, sections were incubated with normal goat serum and then with primary antibody rabbit antiserum raised against recombinant leptin-binding domain (LBD; aminoacids 419-624) of chicken leptin receptor purified by affinity chromatography. The sections were then incubated with the secondary antibody (biotinylated goat anti-rabbit, 1:350 in PBS; Vector Laboratories) and with the avidin-biotin-peroxidase complex StreptABComplex/HRP (DakoCytomation, Denmark). The colour reaction was developed by incubation with the DAB- H_2O_2 mixture. The negative control was incubated without the primary antibody. To verify the specificity of the primary antibody, it was preincubated with recombinant chicken LBD before incubation with tissue sections. The resulting immunocytochemical staining was the same as in the negative control, confirming the specificity of primary antibody against leptin receptor.

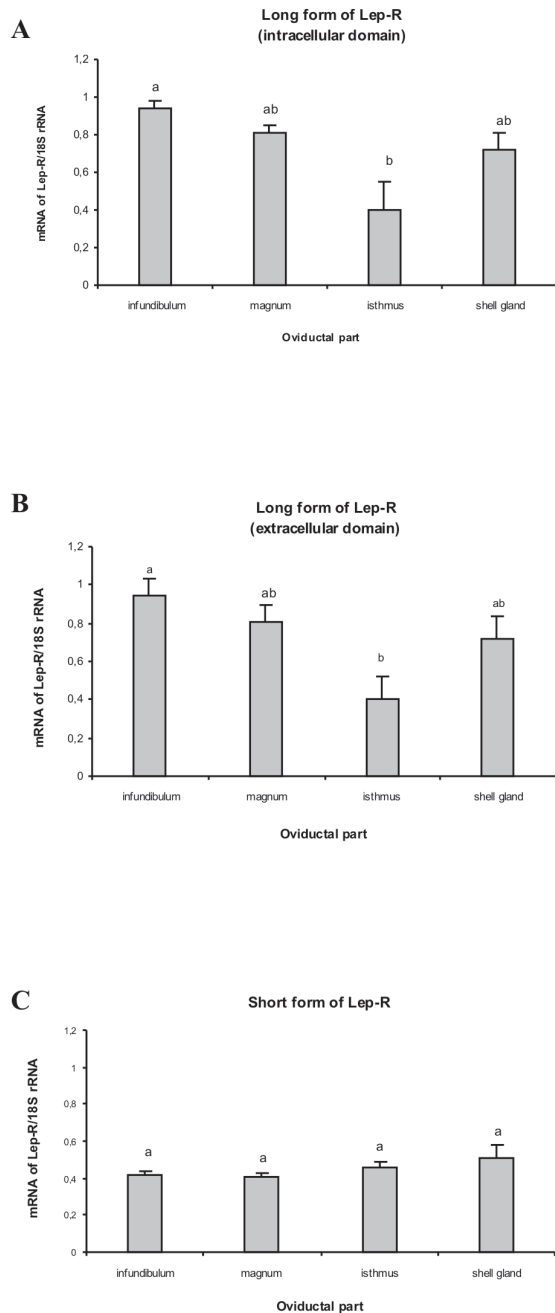


Fig. 2. Semiquantitative analysis of expression of intracellular domain (A), extracellular domain (B) and short form (C) of leptin receptor mRNA in the chicken oviduct 2h after ovulation (n=8, mean ± SEM). a,b,c - means with different letter differ significantly ($P < 0.05$).

Statistical analysis

Data were statistically analyzed by one-way analysis of variance ANOVA followed by Student's *t*-test. Values are expressed as the mean ± SEM from 8 determinations and considered significantly different at $P < 0.05$.

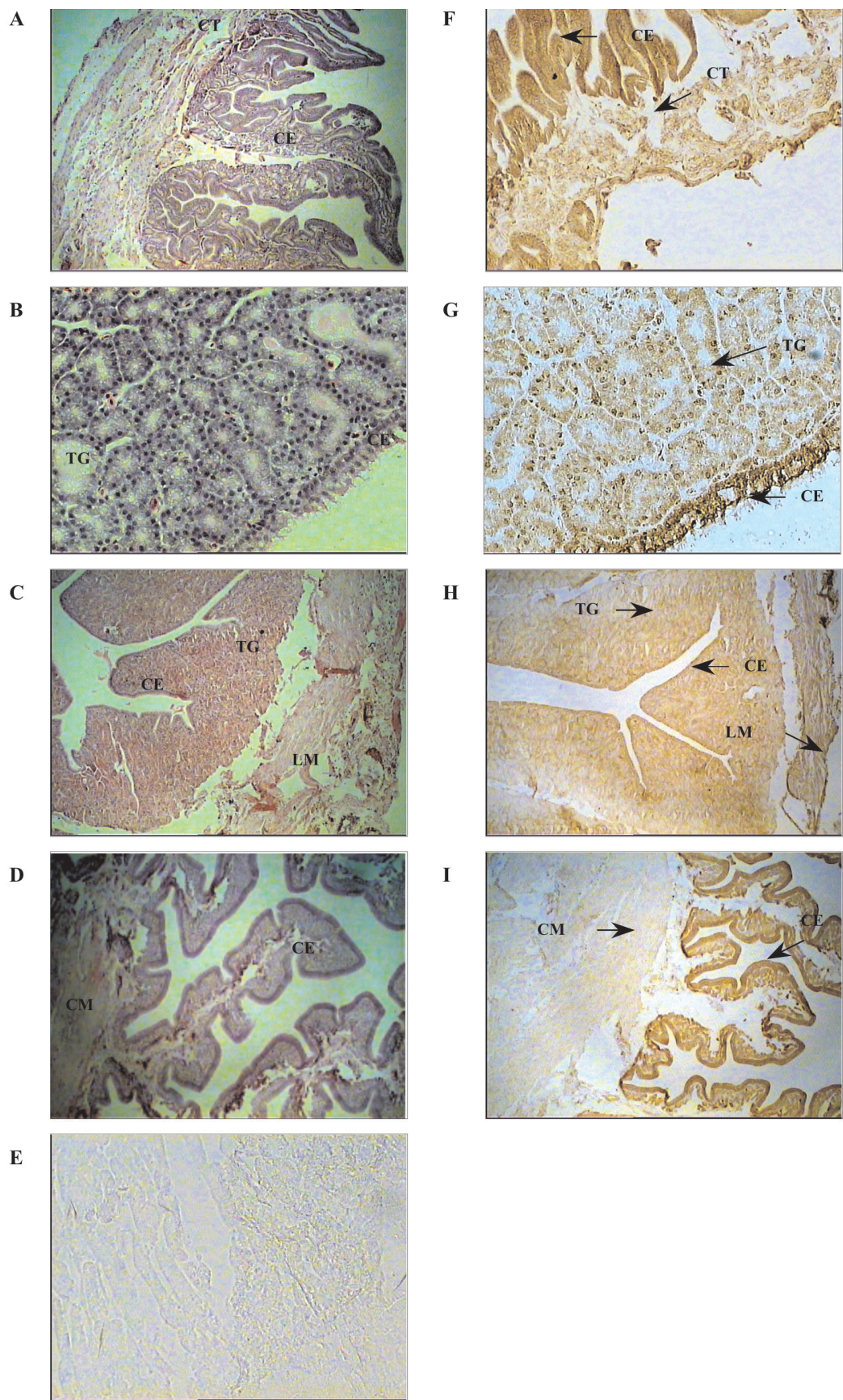


Fig. 3. A-D Haematoxyllin and eosin staining of hen oviductal parts: infundibulum (A), magnum (B), isthmus (C) shell gland (D), F-I Immunocytochemical localization of leptin receptor in hen oviductal segments: infundibulum (E), magnum (F), isthmus (G), shell gland (H), E negative control incubated without specific 1° antibody against leptin receptor. CE – cilliated epithelium, CM – circular muscles, CT – connective tissue, LM – longitudinal muscles, TG – tubular glands.

Results

RT-PCR reactions demonstrated that the mRNA of leptin receptor long and short isoforms are expressed in all examined oviductal parts (Fig. 1).

High mRNA expression of the long form of receptor was found in the infundibulum, the magnum and the shell gland and was lowest in the isthmus. (Fig. 2 a, b).

The expression of short isoform mRNA was lower than the expression of long isoform of leptin receptor in the hen oviduct (Fig. 2c). There were no differences in expression of mRNA for short form of leptin receptor between the examined parts of the oviduct.

Immunostaining specific for leptin receptor was found in the wall of all examined oviductal parts. The most intense immunostaining was observed in ciliated cells of the epithelium of all examined parts of the oviduct. In the infundibulum a strong colour reaction was also obtained in connective tissue and muscles. In the magnum, apart from the ciliated cells, a very intense reaction was observed in tubular gland cells, goblet cells, muscles and connective tissue. In the isthmus an intense reaction was also observed in glandular tissue and muscles. In the shell gland the staining was hardly visible in muscles and connective tissue. In the wall of the examined oviductal parts, an intense reaction was observed in epithelium, both in the outer mucous membrane and inner ciliated epithelium and also in endothelium and muscles of blood vessels localized in the connective tissue. Immunoreactivity in the tubular glands, connective tissue and circular and longitudinal muscles was less intense (Fig. 3).

Discussion

To our knowledge, the present study is the first concerning mRNA expression of both long and short isoform of leptin receptor in the chicken oviduct. Previously, in mammalian oviduct, mRNA expression of long form of leptin receptor was detected (LIU *et al.* 2003; ZERANI *et al.* 2005), but there were no data on other isoforms of leptin receptor. Moreover, so far there were no reports on the detection of leptin receptor in the oviductal tissue in birds. ZERANI *et al.* (2005) detected positive immunohistochemical staining of leptin receptor in the cytoplasm of the secretory cells of the rabbit oviduct.

The differing expression of mRNA of two isoforms of leptin receptor obtained in this study is striking. The expression of the short form of leptin receptor was obtained in all examined tissues,

whereas there are no reports concerning the short form of leptin receptor expression in the oviduct of any animal. According to suggestions of BAUMANN *et al.* (1996) and TARTAGLIA (1997), this truncated form exhibits partial or no signalling capabilities and it may have alternative functions such as uptake, transport or clearance of leptin from circulation, but BRANN *et al.* (2002), and HEGYI *et al.* (2004) have shown that some signalling events can be initiated by the short isoforms and/or involve other pathways, such as mitogen-activated protein kinase (MAPK). In the laying hen, expression of the short form of leptin receptor mRNA has been previously detected in pituitary, liver, kidney, ovary, lungs, but interestingly, it has not been detected in hypothalamus (LIU *et al.* 2006).

The expression of the long form of leptin receptor was higher than the expression of mRNA of the short form of receptor. Statistically significant differences in expression of mRNA of the long form of receptor among oviductal segments were detected. In the isthmus the expression was lower than in other parts of the oviduct.

ZERANI *et al.* (2005) suggested that leptin may modulate prostaglandin synthesis in the oviduct of rabbit. Prostaglandins in the mammalian oviduct affect muscular activity and enable oocyte transport. It is now recognized that different regions and types of muscle respond differently to prostaglandins. In birds, as in mammals, prostaglandins are synthesized in the oviduct (RZASA 1978, 1981) and they are involved in smooth muscle contractility and ovum transport (TALO & KEKALAINEN 1976; RZASA 1978). The presence of prostaglandins was detected in each oviductal segment (RZASA 1978, 1981). It has been shown that intrauterine administration of several prostaglandins caused premature oviposition in quail, chicken, and turkey (HERTELENDY 1974; RZASA 1978; HAMMOND *et al.* 1981). Participation of prostaglandins in the regulation of oviductal motility (OLSON *et al.* 1978; WECHSUND & HOUVENAGHEL 1978; OLSON *et al.* 1981; RZASA 1981) may suggest the possible cooperation of leptin with prostaglandins in the regulation of the activity of avian oviduct.

Another possible explanation of the role of leptin, mediated by its receptors present in the oviduct, may be modulation of immune response as was previously detected in birds (LOHMUS *et al.* 2004) as well as in mammals (GAINSFORD *et al.* 1996; SANCHEZ-MARGALET *et al.* 2002; CALDEFIE-CHEZET *et al.* 2003). Sperm storage and fertilization occurs in the oviduct, but it is also a site of bacterial colonization. The predominant colonization sites of *Salmonella enteritidis* in the oviduct of the laying hen are tubular glands of the isthmus

(DE BUCK *et al.* 2004). WITHANAGE *et al.* (2003) showed an increase in lymphocyte and macrophage subpopulations in the oviducts of infected hens. These reports and results obtained in this experiment, suggest that leptin in the oviduct of the laying hen may play a role in stimulation of immune response in case of infection.

There is no data concerning the exact time of the onset of leptin synthesis in chicken embryo and it cannot be excluded that leptin is secreted into the egg during its formation. Leptin mRNA expression was detected on the 5th day of embryogenesis in the whole chicken embryo and on the 17th day in the liver and yolk sac (ASCHWELL *et al.* 1999). The expression of the long form of leptin receptor mRNA was detected in the chorioallantoic membrane of chicken embryo on day 11 of incubation (GRZEGORZEWSKA *et al.* 2005) and on the 14th day of embryogenesis in turkey embryonic brain, liver and yolk sac (RICHARDS & POCH 2003). Leptin may also stimulate angiogenesis during chicken embryogenesis (BOULOUMIE *et al.* 1998; RIBATTI *et al.* 2001) and in the Japanese quail *in ovo* injection of recombinant leptin accelerated embryonic and postembryonic development (LAMOSOVA *et al.* 2003). These results suggest the role of this polypeptide hormone in the development of the chicken embryo.

In the oviduct of the laying hen, leptin may also participate in calcium absorption and storage. During calcification of the egg shell 2-2.5g of calcium is deposited per day into the egg shell, representing over 10% of the whole body stores of calcium (HODGES 1969; SIMKISS & TAYLOR 1971). Leptin is known as a regulator of bone formation process (PARHAMI *et al.* 2001; GALUS & WŁODARSKI 2004; FAN *et al.* 2007; GAD 2007; HANDSCHIN *et al.* 2007). Hence, it cannot be excluded that leptin takes part in shell building in the hen oviduct.

Unlike mammals, the oviduct of a laying hen is characterized by a high rate of metabolic processes connected with egg formation during its passage through the oviductal segments. During each ovulatory cycle hen lays an egg weighing 50-70g, representing 1.5% of its body weight (GILBERT 1971). Egg formation is a very energy-consuming process, and different metabolic pathways are involved in it. Leptin, a metabolic hormone, may stimulate expression of genes encoding egg white proteins.

In conclusion the results of this study, demonstrating the presence of leptin receptors in oviductal segments of the hen (i.e. infundibulum, magnum, isthmus, shell gland), indicate that the oviduct is a target tissue for leptin. This polypeptide hormone can exert its physiological effect on chicken oviduct functions, such as formation and

passage of the egg. Further experiments are needed in order to explain the role of different isoforms of leptin receptor in the oviduct, the mechanism of signal transduction and to propose the mechanism of leptin action during the production of the egg.

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