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Clinical endocrinology of leptin in ruminants

Ph.D. dissertation

Written by
Margit Kulcsár

Supervisor: **† Prof. Péter Rudas**

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A jelölt témavezetője: **† Prof. Dr. Péter Rudas, DSc**
Témabizottsági tag: **Prof. Dr. Szabó József, DSc**

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Kulcsár Margit dr.
tud. munkatárs
egyéni tanrendű felkészülő

Dr. Solti László
DSc, az MTA levelező tagja
tv. egyet. tanár

Introduction

Leptin, the long-sought, cytokine-like protein hormone of adipocytes was identified by Zhang et al. (1994). Its *production rate* and actual *plasma level* are in positive relation with the triglyceride content of producer cells, and reflect the actual energy balance of organism. Leptin is one of the signal proteins of the white adipose tissue: its circulating level informs the hypothalamic region of central nervous system on degree of lipid saturation in the periphery (visceral and subcutaneous fat stores), playing important role in long-time (homeorhetic; syn. teleophoretic) regulation of feed intake and reproduction (reviewed by Houseknecht et al., 1998; Bokori, 2000; Schneider, 2004; Chilliard et al., 2005; Zieba et al., 2005). Leptin plays an important role in signaling nutritional status to the central regulation of reproduction (hypothalamic GnRH-producing neurons), and appears to be a permissive factor especially in the initiation of cyclicity, e.g. in modulation of ovarian function shifting from anovulatory-acyclic to ovulatory-cyclic (puberty, resumption of cyclicity after parturition and at the beginning of the breeding season). On the other hand studies in lab rodents and Primates have revealed that plasma leptin is influenced also by the age, gender and physiological status (puberty, pregnancy, lactation / postpartum period), furthermore by the health condition: intravenous endotoxin challenge or Gram-negative sepsis, and some diseased conditions with intensive cytokine release evoke an increase in plasma leptin, which is thought to depress the subsequent feed intake. So since its discovery leptin has been in the focus of interest of nutritionists, reproductionists and clinicians both in the human and veterinary medicine.

Although increasing body of information is available nowadays, but – comparing to that one in lab rodents and Primates – our knowledge on factors influencing the plasma leptin level in farm mammals (mostly in ruminants), as well as on the diagnostic and prognostic value of this hormone in care of reproduction is still rather limited and sometimes contradictory, predominantly due to the analytical difficulties. Due to these uncertain-

ties the clinical (diagnostic, prognostic) application of leptin has failed so far.

Studies on plasma leptin level for any forms of practice-related application require high-performance, sensitive and specific assay techniques (¹²⁵I-RIA or ELISA). Due to the species-based differences in its amino acid sequence (Zhang et al., 1997; Blache et al., 2000), its low immunogenicity (Chilliard et al., 2005), and the technical difficulties of *in vitro* production of this protein molecule (Gertler et al., 1998), the progress in assaying plasma leptin of domestic mammals was slow at the beginning, in the first few years of the about 13-year-long leptin history.

Recently a ruminant-specific ¹²⁵I-RIA was adapted in our lab, which is successfully used for quantification of leptin in bovine, ovine and caprine plasma/serum samples. This new method gave us an opportunity to re-analyze and re-evaluate several hundreds of frozen samples collected in our earlier studies, and since than in cooperation with some other teams numerous new experiments have also been conducted. After giving a review of the relevant literature, in this dissertation I summarize the results of 7 series of experiments with using this assay system in sheep and cattle.

MATERIALS AND METHODS

All experiments were conducted within the framework of bilateral (French - Hungarian) or trilateral (French - Uruguayan - Hungarian, French - Finnish - Hungarian or French - Serbian - Hungarian) international cooperations, in Uruguay (Exp. 4a), Serbia (Exp. 5a), Finland (Exp. 6 and 7a) and Hungary (the further experiments). With the exception of some model studies (Exp. 1a, 1c, 1d, 2, 4a, 6 and 7a), housing and feeding conditions of others experiments were the same as general in Hungary.

In order to determine most of the hormones and metabolites, blood samples were taken generally from the *jugular* or *coccygeal* (tail) veins in cattle, and from the *jugular* vessel in sheep, into sodium fluoride containing

tubes (for assaying glucose only), as well as into heparinized tubes (for any other parameters). If another schedule is not indicated, samples were always collected before the morning feeding. All these samples were cooled and centrifuged within 60 min; plasma was harvested immediately, and stored at +4 °C (if assayed <48 h), or -20 °C or -60 °C (if assayed only later). Milk samples for progesterone (P4) determination were taken from the healthy udder quarters into potassium dichromate containing tubes, and were stored at +4 °C until the assay. All milk samples were assayed within 21 days after collection. In *Exp. 2* also feces was collected for assaying progesterone metabolites (P₄-met). For this purpose, fecal samples were taken from the rectum, and frozen and stored at -20 °C until analyzed for P₄-met within some weeks.

Plasma **leptin** concentration was quantified by a locally modified version of the ruminant-specific, homologous, double-antibody, non-equilibrium ¹²⁵I-RIA of *Delavaud et al.* (2000, 2002), which was adapted with the kind personal assistance of *T. Forgách* (National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary) and *Magdy H. G. Ishak* (Atomic Energy Authority, Nuclear Research Center, Cairo, Egypt), and the technical contribution of the Institute of Isotopes Co. Ltd., Budapest, Hungary. The current adaptation of this assay was based on the use of an anti-ovine leptin antibody yielded by *Delavaud et al.* (2000) in rabbit¹. Instead of recombinant ovine leptin, however, in this version a commercially available form of recombinant bovine leptin (_{rb}leptin²) was used for radio-iodination, as well as for preparing the standards. Furthermore the bound and free ligands were separated by a magnetisable immu-

nosorbent suspension³, rather than applying a specific anti-rabbit second antibody. The _{rb}leptin was labeled with ¹²⁵I by Chloramine T method (after *Hunter and Greenwood*, 1962, with modification). The ¹²⁵I-_{rb}leptin was separated in a Sephadex G 50-fine column. The free iodine to ¹²⁵I-_{rb}leptin ratio was determined by thin layer chromatography (DC-Alufolien Kieselgel 60)⁴. The triplicates of standards were prepared from a stock preparation of _{rb}leptin at the following concentrations: 0.0833, 0.125, 0.25, 0.40, 0.75, 2.00, 2.50, 4.00 and 5.00 ng/tube (in 50 µl). As samples and quality control tubes (n=3; with 0.058±0.004, 0.241±0.008 and 0.577±0.023 nmol/l levels, as regular quality control samples with “low”, “medium” and “high” leptin content in each assay run), triplicate aliquots of 100 µl plasma were used. Under routine use the sensitivity of this assay (defined as the lower quantity able to generate a diminution of 5% of the B/B0 ratio) was 0.032 nmol/l. The calculated inter- or intraassay coefficients of variation (CV) were 12.15, 5.61 and 6.13 %, or 10.06, 4.57 and 5.28 % in ranges of quality control samples with “low”, “medium” and “high” leptin content, respectively.

The other hormones were measured with locally developed assay systems or commercially available kits, after a previous validation for the use in ovine and bovine samples (Table 1). Metabolites and acute phase proteins were quantified by our partner labs involved in this activity, with commercial kits.

¹ Raised against recombinant ovine leptin (_{rb}leptin) in rabbit (gifted by *Prof. Y. Chilliard* and *C. Delavaud*, INRA, Saint-Genes-Champagnelle, France)

² DSL-RO 1708-100, Diagnostic Systems Laboratories, Inc., Webster, Texas, USA

³ 1.0 mg/ml; containing magnetisable iron oxide microparticules coated by anti-rabbit goat IgG; particle size: 1-5 µm; minimum binding capacity: 2,5 µg IgG/mg (MIS-4100; Isotope Institute Co. Ltd., Budapest, Hungary)

⁴ Art. No. 5553 (Merck Lab., Darmstadt, Germany)

Table 1: The other endocrine assay systems

		Experiments
Determination of hormones in plasma samples		
Insulin-like growth factor-I (IGF-I)	¹²⁵ I-RIA (local) ^a	3, 4a, 4b, 7a, 7b, 7c
	¹²⁵ I-IRMA (commerc.) ^b	5b, 6
Testosterone	³ H-RIA (local) ^c	1c
Progesterone (P4)	³ H-RIA (local) ^d	1d, 1e, 2, 3
	¹²⁵ I-RIA (commerc.) ^e	4a
Cortisol	³ H-RIA (local) ^f	3
Insulin	¹²⁵ I-RIA (commerc.) ^g	2, 3, 4a, 4b, 7a, 7b, 7c
	¹²⁵ I-IRMA (commerc.) ^h	5a, 5b, 6
Thyroxine (T4)	¹²⁵ I-RIA (commerc.) ⁱ	3, 7a, 7b, 7c
	¹²⁵ I-RIA (commerc.) ^j	4a, 4b, 6
3,3',5' triiodothyroin-ine (T3)	¹²⁵ I-RIA (commerc.) ⁱ	3, 7a, 7b, 7c
	¹²⁵ I-RIA (commerc.) ^j	4a, 4b, 6
17β-oestradiol (E2)	¹²⁵ I-RIA (commerc.) ^k	1e
LH	ELISA (commerc.) ^l	1e
Determination of hormones in milk samples		
Progesterone (P4)	ELISA (local) ^m	4b
Determination of hormones in fecal samples		
Progesterone me- tabolits (P4-met)	ELISA (local) ⁿ	2

^a Nikolic et al., 2001

^b DSL-5600 Active IGF-I Coated-Tube IRMA Kit; Diagnostic Systems Laboratories Inc., Webster, Texas, USA

^c Dahlbom et al., 1995; Fodor et al., 2003

^d Nagy et al., 1998

^e Coat-a-count, DPC Diagnostic Products Co, Los Angeles, CA, USA

^f Nikolic et al., 1998; Jánosí et al., 2003

^g ¹²⁵I-Insulin RIA CT kit; CIS Bio International Ltd, Gif-Sur-Yvette, France

^h BI-Insulin IRMA kit; CIS Bio International Ltd – Subsidiary of Schering S.A., Gif-Sur-Yvette, France

ⁱ ¹²⁵I-T₄-Spec and ¹²⁵I-T₃ MIS RIA kits (Institute of Isotopes Co., Ltd. Budapest,)

^j ¹²⁵I-T₄-Spec and ¹²⁵I-T₃ kötött csöves RIA kiték (Institute of Isotopes Co., Ltd. Budapest,)

^k DSL-39100 3rd Generation Estradiol RIA kit, Diagnostic Systems Laboratories Inc., Webster, Texas, USA

^l LH Detect, INRA, Tours, France

^m Nagy et al., 1998; Huszenicza et al., 1998; Taponen et al., 2002

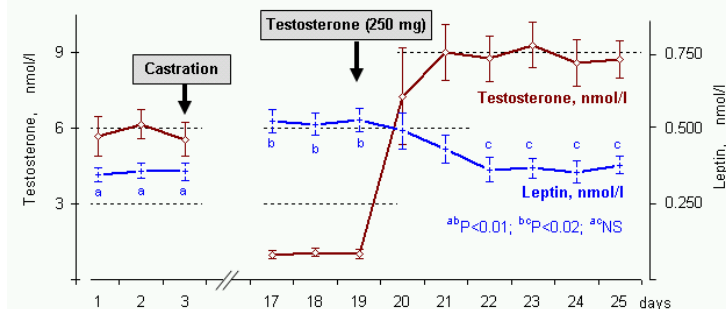
ⁿ Kulcsár et al., 2006

BIOLOGICAL VALIDATION OF THE LEPTIN ASSAY

Using our ruminant-specific ¹²⁵I-RIA, the first responsibility was to check and prove the **biological validity of findings** provided by this laboratory procedure. For this purpose, we planned to reproduce some tendencies known from the literature, such as the effects of (i) 24 h feed deprivation, (ii) reproductive status and lactation (iii) and gender (including the surgical removal of gonads, and the influence of gonadal steroid replacement), as well as the presence or absence of cycle-related and diurnal changes (*Exp. 1a, 1b, 1c, 1d* and *1e*).

With this ¹²⁵I-RIA we could reproduce these expected tendencies (such as the fasting-induced and lactation-related decrease in plasma leptin; the lack of diurnal changes), which proved the biological validity of our results (*Exp. 1a, 1b, 1c* and *1d*).

Fig 1: The changes of plasma leptin compared to testosterone in rams (n=7) before and after castration, and following testosterone supplementation of wethers (mean ± SEM; with repeated measures ANOVA; *Exp. 1c*).



Our further experiences of the first series of experiments (*Exp. 1c, 1d, 1e*) revealed that, despite the similar feeding system, the plasma leptin content is usually higher in ewes than in rams. There are no cycle-related changes in plasma leptin of ruminants, and it remains also unchanged after ovariectomy and estrogen replacement. However, after castration elevated

plasma leptin content was measured, which was reduced again by testosterone replacement (Fig. 1).

Upon these data we think that although the gonadal steroids are not principal regulators of leptin production, testosterone is responsible for the gender dichotomy of plasma leptin.

ORIGINAL TRIALS

Based on promising preliminary experiences, later on 6 series of original trials were conducted.

Exp. 2

Along the gestation elevated plasma leptin levels were reported to occur in ewes (Ehrhardt et al., 2001; Thomas et al., 2001) and nulliparous (but not in lactating primiparous) goats (Bonnet et al., 2005). However, there have not been available data yet showing whether this pregnancy-associated hyperleptinaemia of small ruminants interacts with the number of fetuses and/or with the gestation-related increase of sexual steroids and insulin. In *Exp. 2* our purpose was to study whether during the early and mid pregnancy (i) the number of fetuses, (ii) the gestation-associated continuous gestagen load, and (iii) the plasma levels of insulin interact with the circulating leptin in ewes.

The gestagen load was quantified with determination of plasma P₄ and fecal P₄ metabolite (P₄-met) contents, from samples taken before the insemination (d 0), and again 41, 81 and 101 d later. Compared to their non-pregnant flock mates (n=32), data of ewes conceived from a synchronized/induced ovulation in January, and lambing one (n=12), two (n=6) or 3 to 5 lambs (n=6) after a regular gestation in June, were evaluated. The initial (d 0) levels of leptin and insulin varied between 0.125 and 0.463 nmol/l, as well as between 64.4 and 111.9 pmol/l, respectively, and showed a remarkable positive correlation (r= +0.455; P<0.001). This interrelation-

ship remained existing throughout the study in non-pregnants only (n=32; r= +0.438; P<0.001). The d 0 level of leptin was higher in ewes became pregnant (n=24) than in those remained non-pregnant (n=32), but the mean of body weight in these two groups did not differ. By d 41 the plasma leptin of pregnant had doubled, it showed a moderate further increase on d 81, and decreased slightly thereafter. The plasma insulin levels did not show significant gestation-related changes, and during pregnancy significant correlation was not found between these two hormones. The mean levels of P₄, P₄-met and leptin were slightly lower in ewes bearing single fetus than those with 2 or 3-5 fetuses. According to the number of fetuses the repeated measures ANOVA proved significant differences in P₄ and leptin, but not in P₄-met (P=0.044, 0.042, and 0.059, respectively). On d 41, 81 and 101 the leptin was in mild positive correlation (P<0.01 to 0.05) with P₄ (r = +0.353, +0.389 and +0.329, respectively) and P₄-met (r= +0.297, +0.305 and +0.303, respectively).

It was concluded that the degree of pregnancy-associated hyperleptinaemia is affected by the number of fetuses and level of progesterone in ewes. However, pregnancy stage is a more important regulator than these additional factors.

Exp. 3

Ovine ketosis (syn. gestational or pregnancy toxemia) occurs in late pregnant ewes bearing twins or multiple fetuses, as a consequence of relative undernourishment of these dams during the last 2 to 4 weeks of gestation. The insufficient pancreatic β -cell function is a known factor of pathogenesis. However, there are only quite a few data available on the endocrine aspects of this disease: there have not been available data yet showing how the pregnancy-associated hyperleptinaemia of ewes interacts with hyperketonaemia/gestational toxemia. Furthermore, the subsequent reproductive performance of survivals has not been studied yet, either. We studied the supposed leptin-related and reproductive alterations in spring-

lambing ewes (n=98) in a Merino flock affected by gestational toxæmia. Ewes were sampled on d 140 of gestation, and those with hyperketonæmia (e.g. β OH-butyrate, BHB: ≥ 1.60 mmol/l; Henze et al., 1998) received an antiketogenic therapy. Data on lambing were recorded. The lambs were weaned at 72-75 d of age, and the dams were dried off simultaneously. On d 78-80 postpartum (at the beginning of May) all ewes received a gestagen+eCG treatment to induce their cyclic ovarian function. At the time of AI (on d 92-94 after lambing), and 10 days later again, blood samples were collected to determine the same metabolites and hormones as on d 140 of pregnancy, and to monitor the ovarian response to the cycle-inducing treatment (P4 content in both samples).

On day 140 of gestation the plasma BHB level varied between 0.27 and 1.32 mmol/l (single pregnancy; n=41) and 0.35-3.65 mmol/l (twin pregnancy; n=57). Almost half of the twin-bearing ewes (27 of 57 = 47%), but none of those with single pregnancy were considered to have hyperketonæmia-related elevation in plasma BHB. The circulating leptin level was relatively high in all animals, and was not significantly correlated with the other metabolic and endocrine parameters in normoketonaemic ewes. However, the twin-bearing hyperketonaemic ewes had lower plasma leptin content than the others, which was, however, still elevated comparing to those values measured in the post-lambing samples, and showed some mild significant correlation with insulin, IGF-I, T3 and BHB.

On day 92-94 after lambing the plasma leptin levels were only about half of those found in late pregnancy. The twin-lambing, formerly hyperketonaemic ewes had significantly lower leptin levels than the others. At that time the circulating leptin was positively related to plasma glucose, insulin and IGF-I ($r=+0.414$, $+0.375$ and $+0.425$, respectively; $P<0.001$), and it was in mild negative correlation with NEFA ($r=-0.341$; $P<0.01$). The twin-lambing, formerly hyperketonaemic dams showed poorer ovarian response to the gestagen+eCG based cycle-inducing treatment than the others: only less than 60 % of them ovulated, while the rate of well-responding ewes

were above 80 % in the other two groups. Regardless of the former number of fetuses and/or their earlier metabolic state the overwhelming part of the ovulated animals re-conceived. However, the rate of twin pregnancies was also lower among the formerly hyperketonaemic individuals. The leptin and IGF-I levels of non-responders (n=20) were lower than of those ovulated (n=78).

It was concluded that the subclinical form of ovine ketosis is characterized by complex endocrine alterations reflecting the pregnancy-associated energy imbalance, which include a decrease in plasma leptin. If out of the breeding season (some weeks after lambing, immediately after weaning) the ovarian cyclicity is induced again, the plasma leptin level, furthermore the ovarian response and fertility of formerly ketotic ewes may be depressed.

Exp. 4a and 4b

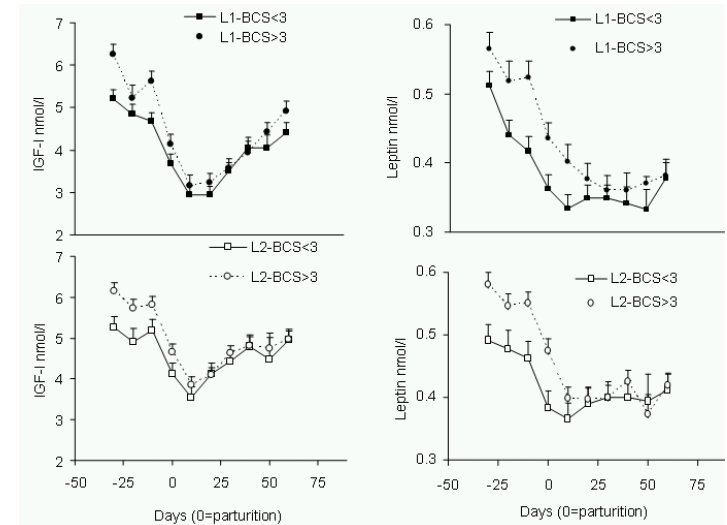
In dairy cows the first postpartum ovulation and resumption of ovarian cyclicity is a milestone on the way to re-conception, which is closely related to the negative energy balance in this period: the time to the beginning of the recovery of energy balance is positively correlated with the time to first ovulation (*Beam and Butler, 1999*). The physiological pathways by which the hypothalamic-pituitary-ovarian axis is informed about the energetic status of the animal are complex, and involve several metabolites and hormones such as the growth hormone - IGF-I system, insulin, thyroid hormones and leptin (*Blache et al., 2007; Wathes et al., 2007*). When our trials were conducted, however, no clear data were available on involvement of leptin. We wished to study that in healthy Holstein Friesian cows what are (i) the peri- and post-parturient tendencies in plasma leptin concentrations along with the BHB, non-esterified fatty acid (NEFA), insulin, IGF-I and thyroid hormone profiles, as well as (ii) the influence of parity and body condition at parturition on endocrine and metabolite patterns and reproductive parameters (*Exp. 4a*); furthermore, (iii) what differences are in

plasma levels of these metabolic hormones and metabolites between cows with already *cyclic* vs. still *acyclic* ovarian function at the desirable time of the first postpartum insemination (*Exp. 4b*).

In *Exp. 4a* multiparous and primiparous Holstein cows (n=21 in each parity group) grazing on improved pastures were sampled regularly for metabolic and endocrine profiles from one month before to two months after parturition: NEFA, BHB, insulin, IGF-I, leptin, T4 and T3 were determined in plasma every 10 days. Body condition score (BCS) and milk production were determined every 2 weeks. The BCS at parturition was determined by using the BCS closest to parturition, and animals were assigned into two groups, e.g. groups of cows calving in relatively *poor* (<3.00: n=20), or *moderate to good* body condition (≥ 3.00 : n=22). Accordingly, terms of LEAN and FAT cows were used thereafter in this study. After parturition plasma samples were taken to assay P4 three times per week, in order to monitor resumption of ovarian cyclicity. Estrus was detected twice a day, and animals were inseminated 12 h after heat detection (voluntary waiting period = 50 days).

Primiparous cows had lower BCS during the early postpartum period and produced less milk than multiparous animals. Primiparous cows had higher NEFA concentrations and they presented more samples with BHB concentrations of >1 mmol/l than multiparous cows. Multiparous cows had higher T3, T4 and IGF-I concentrations, while fat cows had higher leptin and IGF-I concentrations. All hormone concentrations were diminished in the first week postpartum. Primiparous cows and FAT cows presented a steeper decay of IGF-I and leptin around parturition than multiparous cows and LEAN cows. While thyroid hormones and IGF-I showed increasing concentrations from approximately d 30, leptin concentrations remained low until the end of the experimental period (Fig. 2). The initiation of ovarian cyclicity was delayed in primiparous cows and especially in primiparous LEAN cows consistent with longer intervals from parturition to first service and to conception.

Fig 2: Concentrations of insulin-like growth factor I (IGF-I) and leptin in LEAN (L1-BCS<3 n=12) and FAT (L1-BCS \geq 3 n=9) primiparous cows and LEAN (L2-BCS<3 n=8) and FAT (L2-BCS \geq 3 n=13) multiparous cows (mean \pm SEM; *Exp. 4a*).



In *Exp. 4b* (which was conducted in 7 herds) the data of 383 healthy cows were evaluated: 132 of them were young primiparous animals. At the time of blood sampling (day 49-63 after calving) they produced 19-47 kg/day milk, and – regardless of their parity – almost all of them were in poor body condition (BCS: <3.00). As usual during the peak lactation, multiparous cows produced more milk than primiparous individuals (39.6 \pm 0.7 vs. 30.5 \pm 0.4 kg/day; mean \pm SEM, P<0.001). The BHB was <1.00 mmol/l in all animals, and also the plasma concentrations of NEFA, total cholesterol, insulin, IGF-I and thyroid hormones were within the physiological range of lactating dairy cows, with minimal between-herd variability. In accordance with the actual BCS and stage of lactation, the circulating leptin level was low in all cows (varying between the detection limit and 0.288 nmol/l; about 80 % of samples represented the range <0.220 nmol/l), and – although the mean of BCS did not show age-related differences – like the tendencies in

IGF-I and insulin, the leptin content was lower in the multiparous than in primiparous animals

We concluded that in dairy cows the age-related differences must be considered when the peri-parturient metabolic and endocrine changes – including the changes in plasma leptin – are monitored. The endocrine signals that most likely could inform the reproductive axis regarding the postpartum negative energy balance and the level of body reserves, include IGF-I and leptin.

Exp. 5a and 5b

As it was demonstrated also in Exp. 4a, the plasma leptin concentration of non-lactating, late-pregnant cows is high, and starts to decline 2-3 weeks before calving. Despite the pregnancy-related leptin resistance, these high prepartum levels of leptin may be hypothesized to suppress the dry matter intake, which might be an additional factor predisposing for hyperketonaemia, whereas the more intensive form of negative energy balance may result in more rapid and/or more obvious peri/post-parturient decline of plasma leptin in hyperketonaemic individuals. Up to now, however, these ideas have remained poorly documented. In the Exp. 5a and 5b we wanted to study the interaction of BHB with insulin and leptin in multiparous Holstein Friesian cows kept in commercial dairy herds during the peri-parturient period.

Cows were sampled (i) regularly, e.g. at inclusion (on day 259-265 of gestation), again two times 7 days apart, and thereafter once a day until day 7 after calving (in Exp. 5a; n=22), or (ii) only once between day 4-12 postpartum (in Exp. 5a; n=253). All samples (taken before the morning feeding) were analyzed for BHB, leptin and insulin, as well as for NEFA (in Exp. 5a only) and IGF-I (in Exp. 5b only). All cows were sampled; however, data of those with severe mastitis⁵ and/or retained fetal membrane result-

⁵ Inappetance, rectal temperature >40.5 °C

ing in puerperal metritis⁶ were not evaluated. In accordance with *Whitaker et al.* (1999), 1.00 mmol/l of BHB was used as the cut-off value between normo- and hyperketonaemia.

On day 259-265 of gestation all cows of Exp. 5a were in moderate to good condition (mean of BCS: 3.7; range: between 3.00 and 4.25); their plasma leptin content was high (0.623±0.020 nmol/l; range: between 0.448 and 0.782 nmol/l), and the BCS was in close positive correlation with circulating levels of leptin ($r = +0.507$; $P < 0.001$). Seven days after calving poorer BCS (mean of BCS: 3.2; range: between 2.50 and 3.75) and significantly lower plasma leptin levels (0.315±0.018 nmol/l; $P < 0.0001$; range: 0.141 and 0.431 nmol/l) were detected, and only a weak positive correlation existed between these two parameters. The plasma BHB started to increase some days before calving, with wide individual variation. In most of the animals the BHB levels never exceeded the threshold value of 1.00 mmol/l (*normoketonaemic* cows; n=9), or were elevated only on the day of, and/or one day after calving (*transient ketone increase*; n=7). In the further cows (n=6), however, the ketone body elevation became significant 2 days before calving (BHB levels compared to those on day 18-22 before calving: $P < 0.05$), the BHB levels reached threshold of 1.00 mmol/l on day -2 to 0 (day 0 = day of calving), and remained elevated in at least 5 consecutive days (in 4 cows: in all samples collected thereafter; *continuous* form of hyperketonaemia; n=6). Significant time-related changes ($P < 0.001$) were detected by repeated measures ANOVA also in plasma levels of NEFA, insulin and leptin. NEFA concentrations started to elevate earlier than the BHB, peaked at the time of calving, and declined thereafter, and were more elevated in cows with continuous hyperketonaemia than in normoketonaemic animals. The plasma insulin content was high at the beginning, declined until calving and started to increase again after calving in normoketonaemic cows and in those with transient ketone increase, whereas remained very low in animals

⁶ Presence of malodorous, reddish-brown vaginal discharge.

with continuous hyperketonaemia. The plasma leptin showed a schematic decline in the last few days of gestation. This decrease was less overt in normo- than in hyperketonaemic cows. The earliest BHB-related differences were seen only about one to two days before calving, and tended to become more significant during the first week of lactation. However, the leptin profile of cows with transient ketone increase was almost the same as of those with continuous hyperketonaemia.

Almost half of the cows sampled on d 4-12 after calving in *Exp. 5b* had elevated BHB levels (n=122). Both the production level (305-day milk yield kg of the previous year), and the incidence of hyperketonaemia were significantly different between herds. No significant herd-related differences were seen, however, in the plasma leptin content. Neither the number of days elapsed since calving (days postpartum), nor parity was associated with plasma BHB, insulin, IGF-I and leptin concentrations. After pooling the data of cows in different herds, the hyperketonaemic cows showed lower insulin, IGF-I and leptin concentrations than their normoketonaemic mates. Negative correlation ($P < 0.001$) was established between plasma levels of BHB with insulin ($r = -0.38$), IGF-I ($r = -0.83$) and leptin ($r = -0.50$), whereas a positive correlation ($P < 0.001$) existed between plasma levels of insulin and IGF-I ($r = +0.37$), insulin and leptin ($r = +0.69$), and IGF-I and leptin ($r = +0.49$).

These data clearly proved that cows affected by hyperketonaemic decompensation of postpartum negative energy balance show lower levels of plasma leptin than their non-affected herd mates.

Exp. 6.

Fat supplementation of early lactation diets is an attractive alternative to diminish the gap between energy intake and the demand of high milk yield. In this model experiment we wished to study whether a commercially available inert (by-pass) fat source may influence the plasma leptin

levels (and the circulating insulin, IGF-I and thyroid hormones) in high-producing postpartum dairy cows.

Twenty-four cows were fed with 0 (NF), 3.5 (MF) or 7 (HF) % of calcium salts of palm fatty acids in the concentrate⁷, for 8 weeks after calving. The increase of total dry matter intake in group MF tended to be slower than in the other groups during lactation weeks 1 to 4. Fat supplementation increased milk fat content linearly during lactation weeks 1 to 4. Live weight and fat depth changes and concentration of plasma NEFA suggest that tissue mobilization was greater in MF than in other groups. However, these forms of by-pass fat supplementation did not significantly affect the plasma leptin, insulin, T3, T4 and IGF-I levels.

Exp. 7a, 7b and 7c

In high-producing dairy cows perhaps the postpartum *mastitis* – mainly the cases resulted from Gram-negative (GN) infection – and *puerperal metritis* are the two most important poly-etiological inflammatory diseases, which have (besides microbial factors) also metabolic and endocrine elements with the supposed involvement of leptin in the pathogenesis. Postpartum mastitis is caused only by one pathogen, and represents a rapid (usually taking for 24-72 hours, but often robust) endotoxin and cytokine challenge for the host, which sometimes is followed by bacteraemia (*Sandholm et al., 1995*). In contrast, puerperal metritis is a more sustained inflammatory process with multi-bacterial etiology and a risk for toxic and septic complications (taking for 2-3 to 10-15 days, so causing a protracted, but not very intensive release of endotoxin and cytokines in mild cases; *Mateus et al., 2003; Sheldon and Dobson 2004*). We wished to study, how these inflammatory diseases may interfere with circulating levels of leptin and other metabolic hormones.

⁷ Raisio Feed Ltd, Raisio, Finland

In a model experiment (*Exp. 7a*) we studied whether plasma leptin levels is influenced by **experimental endotoxin mastitis** in dairy cows in early vs. late lactation. In a cross-over design, 9 cows were challenged twice with 100 µg of endotoxin⁸ infused intramammarily, each cow serving its own control. The endotoxin challenge resulted in systemic and local signs of mastitis (including some-hour anorexia). The clinical symptoms were coincided with a rapid temporary elevation of cortisol, insulin and NEFA, peaking 2-4 h (cortisol) and 8-12 h (insulin, NEFA) after challenge, furthermore a slower, 24-48 h-long decrease of IGF-I, T4 and T3. Cortisol responses were similar, regardless of the stage of lactation. The clinical symptoms, furthermore the other endocrine (insulin, IGF-I, T4 and T3) and NEFA changes were pronounced only in the early, but not in the late lactation. In spite of these clinical and endocrine alterations the plasma leptin and BHB levels remained unchanged.

In the *Exp. 7b* we followed up the supposed endocrine alterations in cows affected by **natural outbreak of mastitis on d 0-14 after calving**. All cows were milked 3 times a day 8 h apart, and new cases of mastitis diagnosed at morning milking were sampled for bacteriology, and involved in the study if also systemic symptoms including anorexia were observed. Blood samples were taken first when represented the about 7th to 14th h of the clinical course, and again five times 6 h apart. At the beginning all of our cows with *GN mastitis* (n=13) showed moderate to severe clinical symptoms, and 3 of them died shortly after the sampling process. The tendencies in their endocrine and metabolic characteristics fully agreed with those found in the corresponding stage of experimental endotoxin mastitis. Compared to the data of their healthy counterparts, in endotoxin (GN) mastitis the plasma level of cortisol was higher at the beginning and declined thereafter, insulin showed a massive but only short-term temporary increase, whereas IGF-I, T4 and T3 were on the same level in the first

samples and decreased continuously afterwards. These endocrine changes were dramatic in the fatal cases. Our cows affected by *GP mastitis* in this study (n=11) showed only mild to moderate but not severe clinical signs, recovered clinically within 5-7 days, and no significant mastitis-related endocrine or metabolic alterations were observed to occur in them. The plasma leptin content remained almost unchanged, or showed a mild declining tendency in all the mastitic cows, regardless of the identified pathogens and/or of the clinical course.

In *Exp. 7c* conducted in a large-scale dairy herd, development of **puerperal metritis** and the related changes in plasma levels of leptin, some other metabolic hormones and metabolites, as well as of certain cytokines and acute phase proteins were studied in $\geq 2^{\text{nd}}$ parity cows predisposed for this disease (retained placenta: n=10; dystocia: n=31). Body weight (BW) and BCS were determined 3 d after calving and again on d 28-30. Starting on d 3 postpartum repeated vaginoscopy and rectal palpation were performed once a day until d 15, and thereafter at 2-3 d intervals until d ≈ 45 , and also the rectal temperature was taken at the same time. The putrid character of discharge (e.g. foul-smelling, reddish-brown, watery exudate with some necrotic debris) was the main diagnostic criterion of puerperal metritis. All cows showing these pathognostic signs received a repeated intrauterine antibiotic therapy. Those affected by severe (toxic) form of puerperal metritis (PM_S; e.g. at least once between d 3 and 15 after calving: rectal temperature >40.5 °C, with simultaneous anorexia) were treated also with 3 mg/kg of oxytetracycline, intramuscularly. Simultaneously the daily milk yield was recorded, and blood samples were collected once a day until d 15, and thereafter 3 times a week until d ≈ 45 for assaying progesterone, NEFA, BHB, IGF-I, insulin, leptin, T4, T3 (and also for tumor necrosis factor- α levels and some acute phase proteins, not detailed here).

⁸ *E. coli* 0111:B4 LPS, Bacto, Difco Laboratories Inc., Detroit, MI, USA

Severe (**toxic**) form of puerperal metritis (PM_S) with fever (rectal temperature >40.5 °C) and depressed feed intake was observed in 7 cows, a **mild form** (PM_M; with putrid discharge only) developed in 15 animals, whereas 19 cows remained healthy. The vaginal discharge usually becomes fetid soon (on day ≤3-4) after calving in toxic puerperal metritis, but only later (on day 6-10) in the milder cases. Compared to those found on d 3 after calving, during the first four weeks of lactation all cows lost BW and condition (BCS) (changes in all the 3 groups: P<0.05-0.01); however, the loss of BW and BCS was more obvious in the PM_S than in the PM_M and healthy animals. The daily milk yield of PM_M and healthy cows increased steadily throughout the study, whereas showed a marked temporary (about 2 to 3 wk) depression in the PM_S cows. The means of daily milk production, BW and BCS of PM_M and healthy cows were similar. On d 3 after calving hyperketonaemia (BHB >1.00 mmol/l) was detected in 24 cows: within 1 to 6 days the vaginal discharge became fetid in 19 of them, whereas only in 3 of the 17 normoketonaemic animals (P<0.001). However, the plasma ketone content decreased soon in all animals. At the beginning the NEFA, insulin, IGF-I, leptin, T4 and T3 concentrations of PM_S, PM_M and healthy cows did not differ. The insulin, IGF-I, T4 and T3 tended to decline for some days, and increased thereafter, whereas the NEFA increased since the beginning, and remained altered for some weeks. All these changes were more pronounced in the metritis-affected (predominantly in the PM_S) cows. Simultaneously also the plasma leptin decreased, but it remained low (in PM_M and healthy cows) or very low (in PM_S cows) throughout the sampling period.

It was concluded that in postpartum dairy cows inflammatory diseases with intensive endotoxin/cytokine release may influence the circulating levels of metabolic hormones, depressing also the leptin content. However, these changes in plasma leptin are only consequences, rather than the causative elements of anorexia associated with infection-induced inflammatory response in ruminants

NEW SCIENTIFIC RESULTS

The below results are thought to represent remarkable novelty value:

- 1) There are no cycle-related changes in plasma leptin of ruminants. Although the gonadal steroids are not principal regulators of the circulating leptin content, testosterone is responsible for the gender dichotomy of plasma leptin.
- 2) Degree of pregnancy-associated hyperleptinaemia is affected by number of fetuses and level of progesterone in ewes. However, pregnancy stage is a more important regulator than these additional factors.
- 3) The subclinical form of ovine ketosis is characterized by complex endocrine alterations reflecting the pregnancy-associated energy imbalance, which include a decrease in plasma leptin. If out of the breeding season (soon after weaning) the ovarian cyclicity is induced again, the plasma leptin level, furthermore the ovarian response and fertility of formerly ketotic ewes may be depressed.
- 4) In dairy cows the age-related differences must be considered when the peri-parturient metabolic and endocrine changes – including the changes in plasma leptin – are monitored. The endocrine signals that most likely could inform the reproductive axis regarding the negative energy balance and level of body reserves, include IGF-I and leptin.
- 5) During the peri-parturient period and at the beginning of lactation, obvious differences can be demonstrated between the circulating leptin levels of normo- and hyperketonaemic dairy cows, with lower leptin content in plasma of those which have had >1.00 mmol/l βOH-butyrate since calving.
- 6) Consumption of a diet enriched with calcium salts of palm fatty acids does not influence the plasma leptin.
- 7) In postpartum dairy cows also the inflammatory diseases with intensive endotoxin / cytokine release (puerperal metritis, severe forms of clinical mastitis) influence the circulating levels of metabolic hormones, depressing also the leptin content.

THE CANDIDATE'S PUBLICATIONS RELATED TO THE PRESENT DISSERTATION

1. Full-text papers published in peer-reviewed journals in English:

- 1.1. Nikolic, J.A., Nedic, O., Samanch, H., Aleksic, S., Miscevic, B., **Kulcsár, M.**: Peripheral circulating insulin-like growth factor-I and II in cattle. *Acta Vet. Hung.*, 2001. 49. 53-63. (IF: 0.420)
- 1.2. Meikle, A., **Kulcsár, M.**, Chilliard, Y., Fébel, H., Delavaud, C., Cavestany, D., Chilibroste, P.: Effects of parity and body condition score at calving on endocrine and reproductive parameters of the dairy cow under grazing conditions. *Reproduction*, 2004. 127. 727-737. (IF: 2.181)
- 1.3. Huszenicza Gy., Jánosi Sz., Gáspárdy A., **Kulcsár M.**: Endocrine aspects in pathogenesis of mastitis in postpartum dairy cows. *Anim. Reprod. Sci.*, 2004. 82-83. 389-400. (IF: 1.410)
- 1.4. Kokkonen, T., Taponen, J., Tuori, M., Lohenoja, S., **Kulcsár, M.**, Delavaud, C., Chilliard, Y., Tesfa A.T.: Effects of fat supplementation in early lactation dairy cows. *J. Anim. Feed Sci.*, 2004. 13. Suppl. 1. 499-502. (IF: 0.416)
- 1.5. Cavestany, D., Blanc, J.E., **Kulcsár, M.**, Uriarte, G., Chilibroste, P., Meikle, A., Fébel, H., Ferraris, A., Krall, E.: Metabolic profiles of the transition dairy cow under a pasture-based milk production system. *J. Vet. Med. A.*, 2005. 52. 1-7. (IF: 0.756)
- 1.6. **Kulcsár M.**, Jánosi Sz., Lehtolainen T., Kátai L., Delavaud C., Balogh O., Chilliard Y., Pyörälä S., Rudas P., Huszenicza Gy.: Feeding-unrelated factors influencing the plasma leptin level in ruminants. *Domest. Anim. Endocrin.*, 2005. 29. 214-226. (IF: 1.559)
- 1.7. **Kulcsár M.**, Dankó G., Magdy, H.G.I., Reiczigel J., Forgach T, Proháczik A., Delavaud, A., Magyar K., Chilliard, Y., Solti L., Huszenicza Gy.: Pregnancy stage and number of fetuses may influence maternal plasma leptin in ewes. *Acta Vet. Hung.*, 2006. 54. 221-234. (last known IF in 2005: 0.530)
- 1.8. **Kulcsár M.**, Dankó G., Delavaud, C., Mircu, C., Nikolic, A.J., Gáspárdy A., Cernescu, H., Chilliard, Y., Cseh S., Rudas P., Huszenicza Gy.: Endocrine characteristics of late pregnant hyperketonaemic ewes and their reproductive performance following the induction of ovarian cyclicity out of the breeding season. *Acta Vet. Hung.*, 2006. 54. 235-249. (last known IF in 2005: 0.530)
- 1.9. Földi J., **Kulcsár M.**, Pécsi A., Huyghe, B., de Sa, C., Lohuis, J.A.C.M., Cox, P., Huszenicza Gy.: Bacterial complications of post-

partum uterine involution in cattle. *Anim. Reprod. Sci.*, 2006. 96. 265-281. (last known IF in 2005: 2.136)

2. Full-text papers published in peer-reviewed journals in Hungarian:

- 2.1. Huszenicza Gy., **Kulcsár M.**, Kátai L., Balogh O.: A nagy tejtermelésű tehén takarmányozásának, tejtermelésének és szaporodóképességének kapcsolata. Irodalmi áttekintés. 2. A petefészkek működése az ellés utáni időszakban. *Magy. Áo. Lapja*, 2003. 125 (58). 75-82. (IF: 0.089)
- 2.2. Kátai L., **Kulcsár M.**, Kiss G., Huszenicza Gy.: A nagy tejtermelésű tehén takarmányozásának, tejtermelésének és szaporodóképességének kapcsolata. Irodalmi áttekintés. 3. Az újravemhesülés zavarai. *Magy. Áo. Lapja*, 2003. 125 (58). 143-146. (IF: 0.089)
- 2.3. Huszenicza Gy., **Kulcsár M.**, Dankó G., Balogh O., Gaál T.: A nagy tejtermelésű tehén takarmányozásának, tejtermelésének és szaporodóképességének kapcsolata. Irodalmi áttekintés. 4. A ketonanyag-képződés fokozódása és annak klinikai következményei. *Magy. Áo. Lapja*, 2003. 125 (58). 203-208. (IF: 0.089)
- 2.4. Faigl V., Marton A., Keresztes M., Novotniné Dankó G., Csatári G., Antal J., Nagy S., Árnási M., **Kulcsár M.**, Cseh S., Huszenicza Gy.: Az anyajuhok szaporodási teljesítményének növelésével összefüggő egyes újabb élettani kérdések és ezek technológiai vonatkozásai. Irodalmi áttekintés. *Magy. Áo. Lapja*, 2005. 127 (60). 586-593. (IF: 0.114)
- 2.5. Pécsi A., Földi J., **Kulcsár M.**, Pécsi T., Huszenicza Gy.: Az involúció bakteriális eredetű szövödményei szarvasmarhában. Irodalmi áttekintés. 1. rész. *Magy. Áo. Lapja*, 2006. 128. 721-730. (last known IF in 2005: 0.114)

3. Abstracts published in peer-reviewed journals in English:

- 3.1. Huszenicza Gy., **Kulcsár M.**, Jánosi Sz., Lehtolainen, T., Dankó, G., Kátai L., Delavaud, C., Magdy H.G.I., Balogh O., Chilliard, Y., Pyörälä, S., Rudas, P.: Feeding-unrelated factors influencing the plasma leptin level in ruminants. Abstract. *Biotechnology, Agronomy, Society and Environment*, 2004. 8. 22.
- 3.2. **Kulcsár M.**, Jánosi Sz., Kátai, L., Kóródi P., Balogh O., Delavaud, C., Chilliard, Y.: Mastitis-related endocrine alterations in postpartum dairy cows. Abstract. *Reprod. Dom. Anim.*, 2004. 39. 289.

- 3.3. **Kulcsár M.**, Lehtolainen, T., Balogh O., Delavaud, C., Chilliard, Y., Pyörälä, S.: Experimental endotoxin mastitis fails to alter the plasma leptin level in dairy cows. Abstract. *Reprod. Dom. Anim.*, 2004. 39. 290.
- 3.4. Huszenicza Gy., **Kulcsár M.**, Jánosi Sz, Lehtolainen T., Delavaud C, Chilliard Y, Pyörälä S: Effects of Gram-negative mastitis on plasma leptin level in dairy cows. Abstract. *Tierärztliche Praxis*, 2004. 32(G). 107.
- 3.5. Huszenicza Gy., Jánosi Sz., **Kulcsár M.**: Effects of Gram-negative mastitis on ovarian activity in postpartum (pp) dairy cows. Abstract. *Tierärztliche Praxis*, 2004. 32. 107.
- 3.6. **Kulcsár M.**, Kátai L., Balogh O., Pécsi A., Dalevaud, C., Földi J., Hirvonen, J., Faigl V., Chilliard, Y., Huszenicza, Gy.: Metabolic and endocrine changes, inflammatory proteins and ovarian activity in dairy cows with acute puerperal metritis. Abstract. *Reprod. Dom. Anim.*, 2005. 40. 407.

4. Full-text papers published in conference proceeding books

- 4.1. Huszenicza, Gy., Kátai, L., **Kulcsár, M.**, Pécsi T., Kiss G., Delavaud, C., Fébel H., Chilliard, Y., Driancourt, M.A., Butler, W.R.: Metabolic factors influencing fertility at the first postpartum insemination in high-producing dairy cows. *Proc. of 15th European A.I. Vets' Meeting* (8-11 October, 2003, Budapest, Hungary), pp. 16-25.
- 4.2. Huszenicza, Gy., **Kulcsár, M.**, Kátai, L., Balogh, O., Meikle, A., Fébel, H., Delavaud, C., Pécsi, A., Földi, J., Cavestany, D., Chilliard, Y., Fekete, S.: Metabolic factors influencing the onset of cyclic ovarian function and fertility in postpartum dairy cows. *Proc. of the 8th Internat. Conf. of the European Society of Veterinary and Comparative Nutrition* (Sept. 23-25, 2004, Budapest, Hungary), pp. 17-37.
- 4.3. **Kulcsár M.**, Jánosi Sz., Lehtolainen T., Delavaud, C, Chilliard, Y., Pyörälä, S., Huszenicza Gy.: Effects of Gram-negative mastitis on plasma leptin level in dairy cows. *Proc. 3. Leipziger Tierärztekongress: Endotoxikosen bei Rinden.* (22 Jan. 2005, Leipzig, Deutschland), pp. 133-151.

FURTHER LEPTIN-RELATED PUBLICATIONS OF THE CANDIDATE

1. Full-text papers published in peer-reviewed journals:

- 1.1. Nikolic, J. A., **Kulcsár, M.**, Kátai, L., Nedic, O., Jánosi, Sz., Huszenicza, Gy.: Periparturient endocrine and metabolic changes in healthy cows and in those affected by various forms of mastitis. *J. Vet. Med. A*, 2003. *50*. 22-29. (IF: 0,558)
- 1.2. Sayed-Ahmed, A., **Kulcsár, M.**, Rudas, P., Bartha, T.: Expression and localization of leptin and leptin receptor in the mammary gland of the dry and lactating non-pregnant cow. *Acta Vet. Hung.*, 2004. *52*. 97-111. (IF: 0,566)
- 1.3. Cavestany, D., **Kulcsár, M.**, Crespi, D., Chilliard, Y., La Manna, A., Balogh, O., Keresztes, M., Delavaud, C., Huszenicza, Gy., Meikle, A.: Effect of prepartum energetic supplementation on production and reproductive characteristics, and metabolic and hormonal profiles in dairy cows under grazing conditions. *J. Dairy Sci.*, submitted for publ. (last known IF in 2005: 2.240)

2. Further full-text leptin-related papers

- 2.1. Huszenicza, Gy., **Kulcsár, M.**, Nikolic, J. A., Schmidt, J., Kóródi, P., Kátai, L., Dieleman, S., Ribiczei-Szabo, P., Rudas, P.: Plasma leptin concentration and its interrelation with some blood metabolites, metabolic hormones and the resumption of cyclic ovarian function in postpartum dairy cows supplemented with Monensin or inert fat in feed. In: Diskin, M.G. (ed.): Fertility in the high-producing dairy cow. British Society of Animal Science (Edinburgh, United Kingdom), Occasional publications, 2001., No. 26., Vol. 2., pp. 405-409.
- 2.2. Huszenicza Gy., Kulcsár M., Kátai L., Balogh O., Balogh-Pál B.: Az endokrin ritmusok összhangjának példája a szaporodásbiológiában: a ciklikus petefészek-működés újraindulása az ellés utáni időszakban nagy tejtermelésű szarvasmarhában. In: Csernus V., Mess B.: Biológiai órák: ritmikus biológiai folyamatok az élővilágban. Akadémiai Kiadó, Budapest, 2006. pp. 137-150.

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