Szent István University
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Clinical endocrinology of leptin in ruminants

Ph.D. dissertation

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Content

List of abbreviations

ACTH	adrenocorticotrop hormone
AGRP	agouti-related protein
AI	artificial insemination
AMP	adenosine monophosphate
ANOVA	single trait analysis of variance
AST	aspartate-aminotransferase
α-MSH	melanocyte stimulating hormone
BCS	body condition score
BHB	βOH-butyrate
bp	boiling point
BW	body weight
Ca-PFA	calcium salts of palm oil fatty acids
CL	corpus luteum
CRH	corticotrophin releasing hormone
CoA	coenzyme A
CV	coefficients of variation
d	day
5'D	5'-deiodinase (enzyme for outer-ring deiodination of T_4)
db	mutant version of leptin receptor gene
db/db mice	homozygote carriers of the mutant leptin receptor *(db/db)* gene (genetically leptin receptor-deficient animals, producing normal leptin, but biologically inactive LR-s)
DM	dry matter
DMI	dry matter intake
DX	dexamethasone
E_2	17β-estradiol
EB	estradiol benzoate
E. coli	Escherichia coli
eCG	equine chorionic gonadotropin (syn. pregnant mare serum gonadotropin, PMSG)
EL	early lactation in *Exp. 7a*
ELISA	enzyme-linked immunosorbent assay
FAT	cows calving in *moderate to good* (BCS $\geq$3.00) body condition in *Exp. 4a*
FSH	follicle-stimulating hormone
GH	growth hormone (syn. somatotrop hormone, STH)
GHRH	growth hormone hormone
GN	Gram-negative
GN mastitis	mastitis caused by Gram-negative pathogens
GnRH	gonadotrop releasing hormone
GP	Gram-positive
GP mastitis	mastitis caused by Gram-positive pathogens
h	hour
HF	cows receiving 7 % fat supplementation in *Exp. 6*
IGF-I	insulin-like growth factor-I
IGFBP	IGF-I binding proteins (IGFBP-1 to 5)
IL	interleukin; IL-1 (IL-1α, IL-1β), IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and IL12: subclasses of IL
IRMA	immunoradiometric assay
IU	international unit
i.m.	intramuscular
i.v.	intravenous
K-EDTA	ethylene-diamine tetraacetic acid, potassium salt
LH	luteotrop hormone
L1	primiparous cows in *Exp. 4a*
L2	multiparous cows in *Exp. 4a*
LEAN	cows calving in *poor* (BCS<3.00) body condition in *Exp. 4a*
LL	late lactation cows in *Exp. 7a*
LPS	lipopolysaccharides, e.g. cell wall component of GN bacteria (endotoxin)
LR	leptin receptor
ME	metabolizable energy
MF	cows receiving 3.5 % fat supplementation in *Exp. 6*
MCH	melanin-concentrating hormone
mRNA	messenger RNA
NDP mastitis	mastitis with no detected pathogens
NEB	negative energy balance
NF	cows receiving 0 % fat supplementation in *Exp. 6*

NEFA	non-esterified fatty acids (syn.: free fatty acids, FFA)
NPY	neuropeptide Y
ob	obese gene
ob/ob mice	homozygote carriers of the mutant "obese (*ob*) gene" (genetically leptin-deficient animals, producing biologically inactive leptin, but normal LR-s)
P_4	progesterone
P_4-met	fecal P_4 metabolite
PM_m	puerperal metritis, mild form (rectal temperature <40.5 °C)
PM_S	puerperal metritis, severe, toxic form (at least once between d 3 and 15: rectal temperature >40.5 °C, with simultaneous anorexia)
PC	post challenge (e.g. time elapsed since endotoxin challenge)
POMC	proopio-melanocortin
pp	postpartum
$_{rb}$leptin	recombinant bovine leptin
$_{rh}$leptin	recombinant human leptin
$_{ro}$leptin	recombinant ovine leptin
$_{rb}$TNF-α	recombinant bovine tumor necrosis factor-α
RIA	radioimmuno assay (^{3}H-RIA, ^{125}I-RIA: ^{3}H- or ^{125}I-labelled version of this assay)
RP	rectal palpation
rT_3	reverse-triiodothyronine (3,3',5'-triiodothyronine)
S. aureus	Staphylococcus aureus
SCC	somatic cell count
SD	standard deviation
SEM	standard error of the mean
SOCS	cytokine signaling proteins
Str.	Streptococcus
T_3	triiodothyronine (3,3',5-triiodothyronine)
T_4	thyroxine
TCH	total cholesterol
TNF-α	tumor necrosis factor-α
TMR	total mixed ration
TRH	thyrotropin releasing hormone
TRIS	tris-(hydroxymethyl)-aminomethane base
TSH	thyroid stimulating hormone
wk	week
vs.	versus
WAT	white adipose tissue

Conversion of traditional and SI units

(for hormones used at the experimental activity only;
after *Feldman* and *Nelson*, 2004; with completion)

	Units			
	Traditional	**SI**	**Trad. ⇒ SI***	**SI ⇒ Trad.***
17β-estradiol (E_2)	pg/ml	pmol/l	3.67	0.273
Cortisol	ng/ml	nmol/l	2.76	0.360
Insulin	μU/ml	pmol/l	7.18	0.139
Insulin-like growth factor-I (IGF-I)	ng/ml	nmol/l	0.13	7.69
Leptin	ng/ml	nmol/l	0.0625	16
Progesterone (P_4)	ng/ml	nmol/l	3.18	0.315
Testosterone	ng/ml	nmol/l	3.47	0.288
Thyroxine (T_4)	ng/ml	nmol/l	1.287	0.78
Triiodothyronine (T_3)	ng/ml	nmol/l	1.536	0.65

*Factor to multiply for conversion from one unit to other

Összefoglalás

A leptin egyike a nagyrészt a fehér zsírszövet által termelt citokinszeru protein hormonoknak. A testzsír-depók triglicerid telítettsége, illetve a takarmányozás szintje (energetikai egyensúly) az a két legfontosabb tényezo, ami az adipocytákban meghatározza a leptin génexpressziójának a mértékét, illetve ezzel szoros összefüggésben a plazma leptinszintjét. A leptin egyik fontos élettani szerepe, hogy a metabolikus szignálmechanizmus részeként informálja a tápláltsági állapotról az ivari muködést centrálisan szabályzó, a hypothalamusban lokalizált GnRH-termelo neuronokat. E szerepe megengedo jellegunek tunik, és elsosorban a petefészek-muködés ciklikussá válásakor jelentos, azaz mindazokban az állapotokban (pubertás, ellés utáni idoszak, tenyészszezon kezdete), amikor egy anovulációs-acikliás idoszak multával az állat ovulál, majd ezt követoen petefészkének muködése ciklikussá válik. Másrészt laboratóriumi rágcsálókon és foemlosökön szerzett tapasztalatok arról tanúskodnak, hogy a plazma leptin szintjét az életkor, az ivar, a szaporodásbiológiai státusz (ivarérés, vemhesség, laktáció, ellés utáni idoszak), továbbá az egészségi állapot is befolyásolja. E fajokban intravénás endotoxin terhelés nyomán, Gram-negatív baktériumok okozta szepszisben, valamint egyes intenzív citokin-felszabadulással járó megbetegedésekben a plazma leptin szintje emelkedik, aminek jelentosége lehet a táplálékfelvétel ezt követo csökkenésében. A házi emlosök (mindenek elott a kérodzok) plazma leptin szintjét befolyásoló tényezokre vonatkozó ismereteink napjainkban folyamatosan gyarapodnak ugyan, de – elsosorban az analitikai nehézségekbol következoen – a laboratóriumi rágcsálókon és foemlosökön nyert tapasztalatokhoz képest még mindig nem kelloen szélesköruek, és nem egyszer ellentmondásoktól sem mentesek. Mindezek a bizonytalanságok napjainkig meghiúsították a leptin klinikai diagnosztikai célú felhasználását.

Laboratóriumunk újabban egy kérodzokbol származó vérszérum/plazma minták leptin tartalmának a meghatározására szolgáló, fajcsoport-specifikus mérorendszert (^{125}I-RIA) adaptált, amelyet sikerrel használunk szarvasmarhában és kiskérodzo fajokban. A vonatkozó irodalom rövid áttekintését követoen értekezésemben ismertetem hét, e módszer alkalmazásával végzett kísérletsorozat eredményeit.

Elso lépésként módszerünkkel sikerrel reprodukáltunk néhány, az irodalmi adatok alapján várható tendenciát (pl. a leptin szintjének a 24 órás táplálékmegvonást követo, vagy a laktáció kezdetén tapasztalható csökkenése; a diurnális ingadozás hiánya), ezzel is bizonyítva eredményeink élettani megbízhatóságát (*1a*, *1b*, *1c* és *1d kísérlet*).

További, új tudományos eredménynek tekintheto tapasztalataink az alábbiakban összegezhetoek: (1) A plazma leptin szintje anyajuhokban magasabb, mint kosokban.

Kérodzokben a plazma leptin szintje a ciklus tüszo- és sárgatest-fázisában, továbbá ovariectomiát követoen, illetve ösztrogén-visszapótlás nyomán nem változik; ezzel szemben kasztráció nyomán emelkedik, a tesztoszteron kezelés nyomán pedig ismét csökken. Ennek alapján megállapítható, hogy – a szexuálszteroid hormonok nem tekinthetoek ugyan a leptinprodukció elsodleges regulátorának – a leptinnek a hímivarban mérheto alacsonyabb szintjéért a here tesztoszteron termelése a felelos (*1c*, *1d* és *1e kísérlet*). (2) Anyajuhokban a plazma leptinszintjének a vemhességgel kapcsolatos emelkedése összefüggést mutat a magzatok számával, illetve a progeszteron szintjével. A vemhesség egyes szakaszainak a befolyásoló szerepe azonban meghaladja e járulékos tényezok hatását (*2. kísérlet*). (3) Az anyajuhok vemhességi ketózisának szubklinikai formája a vehemépítés energiaszükségletének a kielégítetlenségét tükrözo komplex endokrinológiai következményekkel jár, ami a leptin szintjének a csökkenését is magában foglalja. Ha a biológiai tenyészidoszakon kívül (néhány héttel az ellést, és közvetlenül a választást követoen) ismét ovulációt / ciklikus petefészek-muködést indukálunk, állománytársaikhoz viszonyítva a korábban ketózisos anyajuhokban a plazma leptinszintje alacsonyabb lehet, miközben csökken az ovariális válaszkészségük és fertilitásuk is (*3. kísérlet*). (4) Amikor tejhasznú tehenekben az ellés körüli metabolikus és endokrinológiai változásokat – ide értve a plazma leptinszintjének a változásait is – kívánjuk nyomon követni, tekintettel kell lennünk az egyes életkor-csoportok közötti különbségekre is. Az endokrin szignálmechanizmus, ami tejhasznú tehenekben az ellés utáni negatív energetikai egyensúlyról, illetve a testzsír-raktárak telítettségérol tájékoztatja az ivari muködés központi szabályozását, magában foglalja a plazma IGF-I és a leptinszintjét (*4a* és *4b kísérlet*). (5) Az ellés körüli idoszakban, továbbá a laktáció elso heteiben a normo- és hyperketonaemiás tehenek plazma leptinszintjében jelentos különbségek igazolhatóak: azok az állatok, amelyekben az ellést követoen emelkedett ketonanyag-szintek (βOH-vajsav: >1.00 mmol/l) fordultak elo, alacsonyabb plazma leptin-koncentrációkkal jellemezhetoek (*5a* és *5b kísérlet*). (6) Védett zsírforrásként a pálmaolaj zsírsavainak Ca-sóit (7,0 és 3,5 ill. 0 %, abraktakarmányhoz kevert koncentrátum formájában, a laktáció elso 8 hetében) etetve tejhasznú tehenekben nem tapasztaltunk jelentos különbségeket a plazma leptin, inzulin, T_3, T_4 és IGF-I szintjében. (7) Tejhasznú tehenekben a laktáció kezdetén gyakran eloforduló, intenzív endotoxin / citokin-felszabadulással járó gyulladásos kórképek (purerperalis metritis, súlyos általános tünetekkel is kísért mastitis) számos metabolikus hormonnak a vérplazmában mérheto szintjét befolyásolják, így a leptin koncentrációját is csökkentik. A leptin szintjének a változása azonban inkább következménye, mintsem oka az e megbetegedések során tapasztalható takarmányfelvétel-csökkenésnek (*7a*, *7b* és *7c kísérlet*).

Úgy gondoljuk, eredményeink hasznos alapként szolgálhatnak a leptin diagnosztikai és prognosztikai értékének a megítéléséhez a termelés- és reprodukció-orientált, illetve a klinikai jellegu kutatómunkában.

Summary

Leptin is one of the cytokine-like protein hormones of the white adipose tissue. The triglyceride content of lipid depots associated with the current feeding level (energy balance) is the primary determinant of leptin gene expression in adipocytes, and the circulating leptin level. 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. 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 uncertainties the clinical (diagnostic, prognostic) application of leptin has failed so far.

Recently a ruminant-specific ^{125}I-RIA was adapted in our lab, which is successfully used for quantification of leptin in bovine, ovine and caprine plasma/serum samples. After giving a brief 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.

With this ^{125}I-RIA we could reproduce some tendencies known from the literature (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*). Our further experiences revealed the followings: (1) The plasma leptin content is 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. 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 (*Exp. 1c, 1d, 1e*). (2) 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. 2*). (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 (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. 3*). (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 postpartum negative energy balance and the level of body reserves, include IGF-I and leptin (*Exp. 4a* and *4b*). (5) During the peri-parturient period and at the beginning of lactation, obvious differences were 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 (*Exp. 5a* and *5b*). (6) Consumption of a diet enriched with calcium salts of palm oil fatty acids (7.0 and 3.5 vs. 0 % of Ca-PFA; in concentrate fed for the first 8 weeks of lactation) did not influence the plasma leptin, insulin, T_3, T_4 and IGF-I levels in dairy cows (*Exp. 6*). (7) In postpartum dairy cows inflammatory diseases with intensive endotoxin / cytokine release (such as puerperal metritis, severe forms of clinical mastitis) 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 (*Exp. 7a*, *7b* and *7c*). We think these experiences represent remarkable contribution to the successful use of leptin in further production- reproduction- and clinical-oriented research.

1. 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 (WAT): 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). So since its discovery leptin has been in the focus of interest of nutritionists, reproductionists and clinicians both in the human and veterinary medicine. Between 1994 and 2001 knowledge of leptin physiology progressed impressively in rodents and humans, but less rapidly in farm mammals and other species, due to difficulties encountered for the development of specific tools to study leptin gene expression and plasma leptin in them.

Studies on plasma leptin level for any forms of practice-related application require high-performance, sensitive and specific assay techniques (^{125}I-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. As a first promising step, a "multispecies" leptin ^{125}I-RIA[1] was developed only in the late nineties. Since than several data have been published, assaying leptin with this method in bovine (*Chilliard* et al., 1998; *Akerlind* et al., 1999; *Kawakita* et al., 2001; *Maciel* et al., 2001; *Soliman* et al., 2002; *Accorsi* et al., 2005), ovine (*Soliman* et al., 2001), porcine (*Estienne* et al., 2000; *Barb* et al., 2001a), equine (*Fitzgerald* and *McManus*, 2000; *McManus* and *Fitzgerald*, 2000; *Gentry* and *Thomson*, 2002; *Gentry* et al., 2002; *Bruce*, 2004; *Cartmill*, 2004**;** *Ferreira-Dias* et al., 2005; *Waller* et al., 2006), rabbit (*Corico* et al., 2002) and feline plasma (*Backus* et al., 2000).

[1] XL-85K Multi-Species Leptin RIA kit, Linco Research, St. Luis, USA

In 1999 this technique was adapted and validated also in our lab. Our first experiences were introduced is some papers (*Huszenicza* et al., 2001; *Nikolic* et al., 2003), and partly in a DSc dissertation (*Huszenicza*, 2003). Since the beginning of our leptin research we have been working in close cooperation with the team of Profs. *P. Rudas* and *T. Bartha* (Szent István University, Faculty of Veterinary Science, Dept. of Physiology and Biochemistry, Budapest): their activity is focused on some molecular aspects, e.g. the intramammary leptin and leptin receptor (LR) gene expression (papers: *Sayed-Ahmed* et al., 2003 and 2004; *Bartha* et al., 2005; PhD dissertation: *Sayed-Ahmed*, 2004). Their results are considered, as a remarkable contribution to our current leptin-related knowledge, and appreciated very much by the competent international scientific community.

With this "multispecies" leptin ^{125}I-RIA in ruminants, however, unexpectedly high levels (not correlating with the actual body fat content and energy balance) were measured in samples of about 4-20 % of individuals (*Kulcsár* et al., unpubl. data; *Butler* et al., personal com.; *Delavaud* et al., 2000, 2002 and 2004, *Chilliard* et al., 2005). Due to this uncertainty in sheep and cattle, currently this method is offered for assaying leptin content in porcine, equine and feline plasma, rather than in samples from ruminants. Ruminant-specific leptin assays have been available only since 2000: currently some local versions of about 6 assay systems (*Blache* et al., 2000; *Delavaud* et al., 2000; *Ehrhardt* et al., 2000; *Kauter* et al., 2000; *Thomas* et al., 2001; *Sauerwein* et al., 2004) are used all over the world. Unfortunately, up to our current knowledge none of these ruminant-specific methods are commercially available in form of a ready-to-use diagnostic kit.

Using a specific anti-ovine leptin antibody gifted us by *Chilliard* and *Delavaud*[2], in 2001-2002 we developed and validated a local version of the ruminant-specific ^{125}I-RIA of *Delavaud* et al. (2000 and 2002). 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. In the current dissertation I wish to summarize and evaluate the experiences of the first 7 of these studies.

[2] Herbivore Research Unit, Adipose Tissue and Milk Lipids Group, INRA, Saint-Genes-Champanelle, France; risen in rabbits against recombinant ovine leptin of *Gertler* et al. (1998).

2. Aims

Although increasing body of information is available nowadays, comparing to that one in lab rodents and Primates our current knowledge on factors influencing the plasma leptin level in *ruminants* is still rather limited and sometimes contradictory. Due to these uncertainties the clinical (diagnostic, prognostic) application of leptin has failed so far.

Using our ruminant-specific ^{125}I-RIA, the first responsibility was to check and improve 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*, *1.d* and *1e*). Later on 6 series of **original trials** were conducted, in order to study the followings:

(1) Whether in prolific Merino ewes, 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 may interact with the circulating leptin content (*Exp. 2*).

(2) In spring-lambing Merino ewes affected by gestational toxaemia (i) what kind of endocrine alteration – including changes in plasma leptin level – may occur, and (ii) what may be the reproductive consequences of this disease in a large-scale flock, when ovulation and ovarian cyclicity is induced soon after weaning, out of the breeding season (*Exp. 3*).

(3) In healthy dairy (Holstein Friesian) cows what are (i) the peri-parturient and postpartum changes of plasma leptin concentrations along with the βOH-butyrate (BHB), non-esterified fatty acid (NEFA), insulin, insulin-like growth factor-I (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) is there any difference in plasma levels of these metabolic hormones and metabolites in cows with already *cyclic* vs. still *acyclic* ovarian function at the desirable time of the first postpartum insemination (*Exp. 4b*).

(4) In cows kept in large-scale dairy herds, are there any interrelationships between the BHB profile and insulin and leptin during the peri-parturient and postpartum period (*Exp. 5a* and *5b*)?

(5) May supplementation with a commercially available inert (by-pass) fat source influence the plasma leptin levels (and the circulating insulin, IGF-I and thyroid hormones) in postpartum dairy cows (*Exp. 6*).

(6) May inflammatory diseases with intensive endotoxin/cytokine release (such as *puerperal metritis* and *mastitis*, which occur frequently in postpartum dairy cows), interfere with circulating levels of leptin and other metabolic hormones (*Exp. 7a*, *7b*, and *7c*).

3. Physiological aspects of clinical application: a review of literature

3.1. Leptin: the "voice of adipocytes"

The endocrine-like activity of WAT has been supposed for a long time, since in the early fifties a genetic form of obesity with excessive feed intake and infertility was observed in mice. Breeding data revealed that this phenomenon had to be the result of a recessive mutation of a responsible gene, which was called "obese (*ob*) gene" (*Ingalls* et al., 1950)[3]. *Kennedy* (1953) postulated that the amount of body fat and feeding is regulated by the central nervous system through a blood-born product, which signals via the hypothalamus by a negative feed-back mechanism ("lipostasis theory"). However, the *ob* gene, and its 167-amino-acid-long protein product called **leptin** (*Fig. 3.1.1.*) were discovered only in 1994 (*Zhang* et al., 1994).

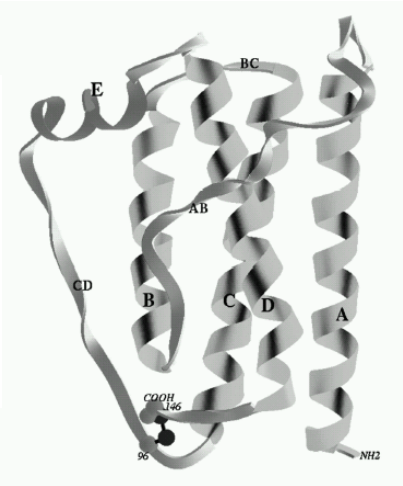

Fig. 3.1.1. The spherical structure of leptin molecule: it forms a four-helix bundle (A-B-C-D) with one disulphide bond (between the two cysteines located at the 96th and 146th position), which is essential for its stability. This structure is consistent with a classification as a cytokine four-helix bundle (*Zhang* et al., 1997)

[3] Up to our current knowledge the homozygote carriers of the mutant "obese (*ob*) gene" – so-called ***ob/ob* mice** – are genetically leptin-deficient animals, producing biologically inactive leptin, but normal LR-s. In them a cytosine to thymidine change at codon 105 changes the amino acid arginine to a stop codon. That causes premature termination of transcription of the leptin gene, resulting in synthesis of a non-functional protein.

Leptin is a 16 kDa, four-helix protein (A-B-C-D; *Zhang* et al., 1997). Leptin and its receptor are structurally and functionally related to the interleukin (IL-6) cytokine family (*Tartaglia* et al., 1995). It contains a single disulphide bond binding two cysteine residues within the C and D helices (*Fig. 3.1.1.*), and this bond has been proven critical for the structural integrity and stability of the molecule (*Rock* et al., 1996; *Zhang* et al., 1997). The first 21 amino acids of leptin function as a signal peptide, and are cleaved off before the 146 amino acid protein is released into the blood as a circulating protein. Leptin has 67% sequence identity among diverse species (human, gorilla, chimpanzee, orangutan, rhesus monkey, dog, cow, pig, rat and mouse; *Zhang* et al., 1997). Bovine and ovine leptin differs from each other by only two amino acids (*Blache* et al., 2000). Leptin binds to its receptor at the interface of α-helices A and C (*Hiroike* et al., 2000).

The WAT and other adipocyte-containing tissues are considered, as the main sites of leptin production (*Kershaw* and *Flier*, 2004). However, leptin gene is also expressed, at much lower levels, in several tissues and organs, such as placental and fetal tissues, mammary gland, stomach / rumen and abomasums, duodenum, anterior pituitary, muscles and brown adipose tissue (in rodents and Primates: *Houseknecht* et al., 1998; *Fried* et al., 2000; *Vernon* et al., 2001 and 2002; *McCann* et al., 2003; in ruminants: *Chilliard* et al., 2001; *Bonnet* et al., 2002a and 2002b; *Ehrhardt* et al., 2002; *Yuen* et al., 2002; *Ingvartsen* and *Boisclair*, 2001; *Chelikani* et al., 2003a; *Leury* et al., 2003; *Muhlhausler* et al., 2003; *Sayed-Ahmed* et al., 2003 and 2004; *Sayed-Ahmed*, 2004; *Bartha* et al., 2005; *Chilliard* et al., 2005). With a few exception (in some species: pregnancy?), however, the contribution of leptin produced by these tissues / organs to the circulating leptin content may be secondary or negligible. The molecular aspects of leptin and LR gene expression, as well as of leptin-related intracellular signal transduction are out of the scope of the current work (for details, please, see some recent reviews: *Chilliard* et al., 2001 and 2005; *Sayed-Ahmed*, 2004; *Bartha* et al., 2005).

Some most recent studies have revealed that leptin is a member of the adipocyte-driven **adipokine** family, rather than the only cytokine/hormone-like signal protein of these cells: the WAT yields also adiponectin, resistin, adipsin and visfatin (*Fantuzzi*, 2005; *Roh* et al., 2006). One of them, the adiponectin is exclusively produced by adipocytes, and – in contrast to leptin – it stimulates energy expenditure without any effect on feed intake when it is infused into the

cerebral ventricle of the rat (*Ahima* 2005). In male rat pituitary cells in culture, adiponectin reduces the expression of GnRH receptor and decreases the secretion of LH (*Malagon et al.* 2006). However, an effect of adiponectin on the activity of the GnRH neurons has not been demonstrated yet. Production and involvement of adipokines in the inflammatory/allergic reaction, immune modulation and metabolic response have already been clearly demonstrated in lab rodents and Primates (*Kershaw* and *Flier*, 2004; *Ahima*, 2005; *Fantuzzi*, 2005; *Chilliard* et al., 2005), and recently also in ruminants (*Roh* et al., 2006). However, our current knowledge is still far from talking about their application in veterinary medicine and animal husbandry.

3.2. Leptin in the circulation

Plasma concentration of leptin is affected by variation in adiposity and nutrition (*Schneider*, 2004; *Chilliard* et al., 2005), by changes in physiological stages like pregnancy and lactation (*Chilliard* et al., 2005; *Zieba* et al., 2005), and – at least in rodents and Primates – by presence of specific binding proteins.

In rodents and humans, leptin circulates in both free and bound form (*Houseknecht* et al., 1996): the soluble isoform ("E" form) of leptin receptor accounted for a major fraction of the leptin-binding capacity present in plasma. In rats, 88% of circulating leptin was present in the bound form (*Hill* et al., 1998), whereas only 24% of bound leptin was reported for humans (*Diamond* et al., 1997). Kinetic studies in rat proved that free leptin had a size of 16 kDa and a biological half-life of 3.4 min, whereas bound leptin had a size of 66 kDa with a half-life of 71 min. This indicated that bound leptin was protected from proteolytic degradation (*Hill* et al., 1998). In humans, the half-life of plasma leptin (bound and free together) was estimated to be 25 min (*Klein* et al., 1996). Presence of binding proteins is supposed also in plasma of ruminants (*Chilliard* et al., 2005; *Zieba* et al., 2005), but up to our knowledge it has not yet been proven undoubtedly. In a study of *Ehrhardt* and *Boisclair* (unpublished results, cited by *Leury* et al., 2003) the leptin binding activity of plasma taken from non-pregnant and pregnant ruminants was negligible. Up to now in farm animal species the half-life of leptin has not yet been determined, either.

In humans (*Licinio* et al., 1997 and 1998; *Sinha* and *Caro*, 1998; *Bergendahl* et al., 2000), pre-pubertal gilts (*Barb* et al., 2001a) and ruminants (intact rams: *Blache* et al., 2000;

Marie et al., 2001; Holstein steers: *Kawakati* et al., 2001; mature, non-lactating ewes: *Daniel* et al., 2002a) concentrations of leptin in the circulation varied in an **episodic** manner. In women leptin levels related inversely to pituitary-adrenal function, with a lack of correlation between mean 24 h levels and pulsatility (*Licinio* et al., 1997 and 1998), and short-term fasting depressed both the circulating concentration of leptin and leptin pulse amplitudes (*Bergendahl* et al., 2000). Similarly, also in gilts (*Barb* et al., 2001a) and ewes (*Daniel* et al., 2002a) feed deprivation reduced the circulating leptin concentration and its pulsatility. Despite the episodic character the leptin levels of *thin-fed* and *fat-fasted* ewes (varying in the intermediate range; 5-10 ng/ml[4]) differed clearly from those of *fat-fed* (varying in the highest range; 12-20 ng/ml) and *thin-fasted* animals (varying in the lowest range; 1-3 ng/ml); in thin-fasted ewes the plasma leptin was very low and almost non-pulsatile (*Daniel* et al., 2002a). In humans (*Licinio* et al., 1997; *Sinha* and *Caro*, 1998), rodents (Cha et al., 2000) and horses (*Cartmill*, 2004) plasma leptin levels showed also a clear **diurnal** variation, with maximum levels between midnight and early morning, and a nadir at noon to afternoon. In dogs serum leptin content changed diurnally in association with feeding-fasting cycles, and was much higher in fat than in thin animals (*Ishioka* et al., 2005; *Jeusett*e et al., 2005). In these monogastric species also a slight postprandial increase was reported to occur. In ruminants, however, the absorption of a wide variety of nutrients and other compounds (including volatile fatty acids) is permanent from the forestomach, and the outflow of ruminal juice into the duodenum is almost continuous (*Dziuk*, 1990), which influences also the (endocrine and exocrine) pancreatic functions (*Martin* and *Crump*, 2003). Perhaps due to the same mechanisms, the diurnal and postprandial variations of leptin are missing in ruminants. In ewes, profiles of plasma leptin were episodic in nature, but did not differ in a circadian manner (*Daniel* et al., 2002a). In a recent study (*Kadokawa* et al., 2006) in postpartum dairy cows neither pulsatile, nor diurnal changes were seen (although samples were taken for assaying leptin in this study only once an hour for 8 h, which may not be frequent and long enough for clear detection of pulsatile and/or circadian rhythms. The plasma leptin level was in a low range, <1.5 ng/ml[5], during the early weeks of lactation). Leptin pulsatility is missing also in lactating rodents (rat: *Pickavance* et al.,

[4] If cited, the absolute values are given, as those were published in original, e.g. in traditional form (ng/ml) or in SI units (nmol/l). Our own data are always given in nmol/l. Conversion: 1 ng/ml = 0,0625 nmol/l; 1 nmol/l = 16 ng/ml.

[5] Technical note: Due to the different assay procedures the absolute values measured in different labs are incomparable. The Australian-Japanese method of *Blache* et al. (2000) always produces the lowest absolute values.

1998) and Primates (women: *Fried* et al., 2000; *Henson* and *Castracane*, 2003). In conclusion, the pulsatile and/or diurnal rhythms represent only relatively moderate variability in plasma leptin, which may be missing in certain species (e.g. diurnal changes in ruminants) and/or conditions (e.g. pulsatility during starvation or lactation). The effects of body fat content, nutrition (short-time fasting or long-time feed restriction), reproductive status (lactation; in some species: pregnancy) and also certain diseased conditions are more robust, and can be clearly recognized (further details: see later).

In lab rodents and Primates leptin is synthesized and released into the circulation in proportion to the amount of **body fat**, reflecting primarily the triglyceride content of lipid depots, furthermore the current balance of energy metabolism (*Houseknecht* et al., 1998; *Morio* et al., 1999; *Bokori*, 2000; *Vernon* et al., 2001 and 2002). Similar tendencies were reported to occur in monogastric farm mammals (pig: *Barb* et al., 2001b; horse: *Bruce*, 2004; *Cartmill*, 2004), and also in ruminants (ewe: *Delavaud* et al., 2000; cattle: *Delavaud* et al., 2002 and 2004; *Lents* et al., 2005). In adult ovariectomized ewes with total body fat content of 20-40 % and fed at different levels for 8 weeks, leptinaemia linked positively to individual body fatness explaining 35 % of leptin variations, whereas 17 % of leptin variations were attributed to the feeding level of animals (*Delavaud* et al., 2000). However, the plasma leptin response to body fatness was non-linear: in ewes it seemed stable between 2 and 4 ng/ml up to a threshold of about 20 % total body lipid content, and increased exponentially thereafter (*Fig. 3.2.1*; *Chilliard* et al., 2005). Furthermore, during the early weeks of lactation plasma leptin level failed to correlate with body condition score (BCS) in dairy cows (*Holtenius* et al., 2003; *Wathes* et al., 2007).

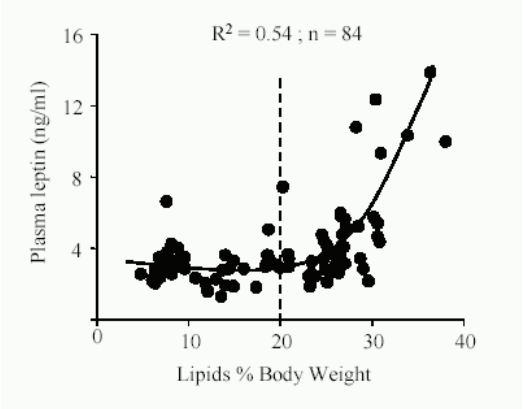

Fig. 3.2.1. Relationship between plasma leptin and body fatness in dry, not pregnant adult ewes (after *Chilliard* et al., 2005)

3.3. Factors modifying the circulating leptin level

In mammals the effects of nutrition on circulating leptin level may be combined with consequences of reproductive status (pregnancy, lactation), and also the gender-related and genetic differences may be significant.

Acute or long-term changes in feed composition or feed restriction caused changes in plasma leptin in ruminants (*Amstalden* et al., 2000; *Chelikani* et al., 2003a; *Chilliard* et al., 2005; *Lents* et al., 2005). In pregnant ewes and adult rams, the concentration of plasma leptin elevated within 5 days after increasing the dietary intake from low to high (*Blache* et al., 2000; *Thomas* et al., 2001). Complete feed deprivation caused a rapid fall in plasma leptin (sheep: *Marie* et al., 2001; cattle: *Amstalden* et al., 2000; *Chelikani* et al., 2004). In cattle, this fall in leptin level coincided with an immediate, apparent decrease of insulin, and increase of non-esterified fatty acids (NEFA), furthermore a less rapid, but also remarkable elevation of growth hormone (GH), and depression of insulin-like growth factor-I (IGF-I). All these changes were obvious after 24 h fasting, and were more pronounced in milking cows (on day 55±8 of lactation) than in late-pregnant non-lactating cows, or post-pubertal heifers. The simultaneous decrease in blood glucose level was less evident, but significant in milking cows and heifers, whereas no change was seen in late-pregnant animals (*Chelikani* et al., 2004). Long-term feed restriction decreased plasma leptin concentration in sheep (*Delavaud* et al., 2000; *Ehrhardt* et al., 2000; *Morrison* et al., 2001). Acute changes in plasma leptin were the result of changes in leptin mRNA expression in adipose tissue: in prepubertal heifers 48 h of total feed deprivation markedly reduced the leptin mRNA content in adipose tissue (and the circulating concentrations of leptin and insulin, whereas neither mean levels nor secretory dynamics of GH were affected; *Amstalden* et al., 2000).

In accordance with those found in lab rodents (*Horlick* et al., 2000; *Vernon* et al., 2001 and 2002; *Abizaid* et al., 2004) and Primates (*Fried* et al., 2000; *Henson* and *Castracane*, 2003; *Mann* and *Plant*, 2003), in adult dairy cattle the highest levels of leptin were observed in well-fed post-pubertal heifers and non-lactating late-pregnant cows, whereas the lowest values were measured in the early weeks of lactation (*Kadokawa* et al., 2000; *Block* et al., 2001; *Geary* et al., 2003; *Holtenius* et al., 2003; *Leury* et al., 2003; *Liefers* et al., 2003; *Chelikani* et al., 2004; *Accorsi* et al., 2005; *Kadokawa* et al., 2006; *Wathes* et al., 2007), when cows are in negative energy balance (NEB), and their body condition is decreas-

ing. Although the NEB of postpartum cows by itself is a physiological phenomenon, it induces a wide variety of endocrine changes, including a sharp reduction in plasma leptin content. In a study of *Block* et al. (2001) the plasma leptin level was reduced by approximately 50% after calving, and remained depressed during lactation, despite a gradual improvement of energy balance. To determine whether NEB caused this reduction in circulating leptin, cows were either milked or not milked after parturition: absence of milk removal eliminated the NEB, and doubled the plasma concentration of leptin in postpartum cows. During late pregnancy and early lactation the same tendency was found also in ewes (*McFadin* et al., 2002), sows (*Estienne* et al., 2000) and mares (*Heidler* et al., 2000). Contrary, however, in a study of *Kokkonen* et al. (2005), plasma leptin concentration was associated with body fatness, but not with estimated energy balance. Furthermore, in many species including cattle, these lactation-dependent peri-parturient changes may be influenced by several, still undefined feeding- and management-related factors and disease conditions, show age/parity-associated variability, furthermore seasonal, genetic and perhaps also breed-connected differences (*Bocquier* et al., 1998; *Chilliard* et al., 1998 and 2001; *Reist* et al., 2003; *Nikolic* et al., 2004; *Delavaud* et al., 2004; *Liefers* et al., 2004; *Chilliard* et al., 2005; *Accorsi* et al., 2005; *Zieba* et al., 2005; *Wathes* et al., 2007). The further pregnancy- and lactation-associated differences in circulating leptin level are discussed elsewhere in this dissertation (see Chapters 3.6. and 5.2.).

Acting on the level of hypothalamus-anterior pituitary axis, and directly on gonads, leptin is deeply involved in regulation of reproduction (details are discussed in Chapters 3.5.3. and 3.6.). It is less known, however, that at least in lab rodents and Primates, after accounting for body fat the second most important determinant of plasma leptin is gender, resulting in more circulating leptin in females than males of equivalent body fat (*Rosenbaum* and *Liebel*, 1999; *Rosenbaum* et al., 2001). This tendency has been conformed recently also in rabbit (*Corico* et al., 2002), cattle (*Geary* et al., 2003) and horse (*Cartmill*, 2004). Testosterone therapy reduces the plasma leptin in hypogonadal men (*Sih* et al., 1997), and simultaneously with the testosterone increase leptin levels have been observed to decline, as boys progress through puberty (*Horlick* et al., 2000). Human *in vitro* adipose tissue cultures of female origin produced leptin at a significantly higher rate than samples taken from men. Androgens inhibited, whereas 17ß-estradiol (E_2) stimulated the *in vitro* leptin release, but both the androgen-related inhibition and E_2-dependent stimulation were restricted on samples of female origin

(*Shimizu* et al, 1997; *Casabiell* et al., 1998; *Pineiro* et al., 1998). 8 weeks after operation the plasma leptin was significantly lower in ovariectomized rats than in controls, and the treatment with E_2 prevented this decrease (*Chu* et al, 1999). In intact female rats E_2 given for 2 days increased the leptin mRNA content of adipose tissue between 2 and 6 h, and the plasma leptin at 12 h after the injection (*Brann* et al., 1999). Experiences with intact, cyclic women demonstrate a trend for an increase in circulating leptin values towards the late follicular phase and higher values in the luteal than in the follicular phase (*Messinis* et al., 1998; *Ludwig* et al., 2000; *Phipps* et al., 2001), which was not influenced by isoflavonic phytoestrogen intake (*Phipps* et al., 2001). In contrast, only little cycle-dependent variation was seen in another study (*Stock* et al., 1999). Ovariectomized women showed significantly lower plasma leptin level 4 days after operation (*Messinis* et al., 1999), but the influences of anesthetic drugs and reduced food intake could not be excluded in the postoperative period. However, transdermal administration of E_2 plus progesterone (P_4) – but not E_2 alone – could prevent this decrease (*Messinis* et al., 2000). When intact, cyclic women were treated, E_2 alone was unable to induce any change in circulating leptin, while during E_2 plus P_4 administration a significant increase in leptin values occurred in the early follicular phase (*Messinis* et al., 2001). In cyclic women superovulated with FSH the plasma leptin concentrations increased gradually from early to mid follicular phase to levels that were significantly higher than in spontaneous cycles of the same persons. In the first half of the follicular phase a significant positive correlation existed between the leptin and E_2 concentrations. However, leptin values did not increase further during the late follicular phase (*Messinis* et al., 1998; *Stock* et al., 1999). After menopausa leptin values are still relatively high, but lower than in pre-menopausal persons (*Shimizu* et al, 1997). However, there are also many conflicting results published, because not all studies used the correction for body mass index or for fat mass. It is still also unclear, whether the synthetic analogues of sexual steroids widely used for contraception in humans, and induction/synchronization of ovarian cyclicy (in moderate dose; in farm mammals), or suppression of ovulatory activity (in large dose; in population control of pets, zoo and sometimes of wild animals) may influence the gene expression and/or plasma levels of leptin.

In cattle also a missense mutation of the leptin receptor gene was reported to influence the circulating leptin level (*Liefers* et al., 2004): the plasma leptin content associated with the genotype during late pregnancy, but not during the early weeks of lactation. In horses an idio-

pathic form of hyperleptinaemia with unspecified (genetic?) origin was observed (*Cartmill*, 2004; *Waller* et al., 2006). The affected mares had continuously higher (but not supraphysiological) plasma leptin levels, than their stud mates with the same body condition, and receiving the same diet. They were also hyperglycaemic and hyperinsulinaemic, had elevated T_3 concentrations, and displayed exaggerated insulin and glucose responses to a standard synthetic glucocorticoid (dexamethasone, DX) treatment (*Cartmill*, 2004). After a standard-dose glucose challenge these "high leptin" mares had greater insulin response, and a faster rate of glucose clearance (*Waller* et al., 2006).

3.4. Regulation of leptin production

There are several (neuro)endocrine and other factors regulating the leptin synthesis. It is generally accepted (*Halleux* et al., 1998; *Houseknecht* et al., 1998 and 2000; *Bokori*, 2000; *Flier* et al., 2000; *Fried* et al., 2000; *Glasow* and *Bornstein*, 2000; *Kieffer* et al., 2000; *Vernon* et al., 2001 and 2002; *Sweeney*, 2002; *Considine*, 2003; *McCann* et al,. 2003; *Fantuzzi, 2005*) that in lab rodents and Primates the leptin gene expression in adipocytes and/or circulating level of leptin are stimulated by insulin, glucocorticoids, bacterial endotoxin and pre-inflammatory cytokines [such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β (IL-1β)], and suppressed by adrenergic stimulation, whereas production and plasma concentration of insulin and glucocorticoids are decreased, as well as of catecholamines are increased by leptin. The circulating leptin may interact also with plasma levels and/or experimental administration of GH, IGF-I, prolactin, glucagon and thyroid hormones, as well as with genital steroids. In monogastric farm mammals (*Fitzgerald* and *McManus*, 2000; *McManus* and *Fitzgerald*, 2000; *Barb* et al., 2001a and 2001b; *Bruce*, 2004; *Cartmill*, 2004) and ruminants (*Chilliard* et al., 1998 and 2001; *Leury* et al., 2003; *Accorsi* et al., 2005; *Chilliard* et al., 2005; *Lents* et al., 2005; *Wathes* et al., 2007) these endocrine interactions are still less known, despite the recently conducted extensive studies.

Glucocorticoids and leptin interact on different levels of regulation. Via its receptors in the hypothalamus, as well as on various adrenal cell populations leptin modulates both the hypothalamic-pituitary-adrenal axis and the systemic sympathetic/adrenomedullary system, which are closely linked to the regulation of energy balance and body weight (*Gaillard* et al., 2000; *Considine*, 2003). Leptin decreased the ACTH-stimulated release of steroid hormones in

vitro without any effect on cell proliferation (*Glasow* and *Bornstein*, 2000). Near term a significant positive correlation was found between plasma concentrations of leptin and cortisol, and fetal adrenalectomy prevented the ontogenic rise in plasma leptin in ovine fetuses (*Forehead* et al., 2002).

In Primates cortisol (*Fried* et al, 2000; *Gaillard* et al., 2000), and also treatment with synthetic glucocorticoids (DX; *Papaspyrou-Rao* et al., 1997; *Casabiell* et al., 1998; *Halleux* et al., 1998), in synergism with insulin, directly stimulate leptin synthesis in adipocytes both *in vitro* and in *vivo*, although the details and clinical relevance of this mechanism have not been fully understood yet. Nevertheless, cortisol does not appear to have a direct role in the serum leptin increase of obese human subjects (*Considine*, 2003). In the adipose tissue 11β-hydroxysteroid dehydrogenase modulates the glucocorticoid concentrations by re-activating glucocorticoids from inactive metabolites, which may be an important local regulator of leptin synthesis and release (*Sandeep* and *Walker*, 2001). Insulin, DX and their combination increase the leptin production by ovine adipose tissue explants. The effects of these two hormones are additive and largely independent. Maximal leptin production was seen after adding 100 nmol DX in the incubation medium (*Faulconnier* et al., 2003). Also bovine adipocytes are sensitive (but perhaps less responsive than human adipocytes) to the stimulatory effects of glucocorticoids on leptin production: in cultured human adipocytes 50 nmol of DX increased the leptin secretion (*Halleux* et al., 1998), whereas in bovine adipose tissue culture only double (100 nmol) concentrations of DX stimulated the leptin mRNA level (*Housecknecht* et al., 2000). DX treatment increased the plasma leptin levels in human subjects at 24-48 h (*Papaspyrou-Rao* et al., 1997), and also in dogs (*Ishioka* et al., 2002) and horses (*Cartmill*, 2004). In contrast, in multiparous non-lactating cows the 10-day administration of DX (44 μg/kg per day) increased the glucose and insulin levels, and decreased the IGF-I and IGF-II concentrations, but failed to alter with plasma leptin (*Maciel* et al., 2001).

3.5. The regulatory role of leptin

As hypothesized by *Kennedy* (1953), leptin plays a central role in regulation of energy homeostasis (appetite, energy expenditure, nutrient partitioning among tissues) and body composition, furthermore of hormone secretion by several endocrine glands, reproduction, immune and renal functions, hematopoiesis, angiogenesis, cell differentiation and proliferation (reviewed

by *Houseknecht* et al., 1998 and 2000; *Bokori*, 2000; *Fantuzzi* and *Faggioni*, 2000; *Vernon* et al., 2001; *Barb* et al., 2001b; *Sweeney*, 2002; *Considine*, 2003; *Lado-Abeal* and *Norman*, 2003; *McCann* et al., 2003; *Spicer*, 2003; *Waddell* and *Smith*, 2003; *Barb* et al., 2004; *Ahima*, 2005; *Chilliard* et al., 2005; *Zieba* et al., 2005).

3.5.1. Leptin and the hypothalamus-hypophysis axis

Within the central nervous system, the hypothalamus is the main site of leptin action with respect to controlling feed intake, energy expenditure and reproduction (*Ahima*, 2005). Unlike those in most other tissues, in the central nervous system the capillary endothel cells are joined by tight junctions and devoid of intercellular spaces and transendothelial channels. Due to its molecular weight leptin enters from the blood to the brain through a specific saturable mechanism: the "C" and perhaps "A" isoforms of LR were thought to act as a "leptin transporter" (*Smith* et al, 2002). As reviewed recently by *Ahima* (2005), in normal animals, leptin transport to brain is partially saturated over a wide physiological range, from low levels associated with fasting to high levels in obesity. In rodents, blood-brain leptin transport is decreased in diet-induced obesity and aging, and might contribute to leptin resistance, excess adiposity and glucose intolerance. In humans, concentrations of leptin are 100-1000-fold higher in plasma than in cerebrospinal fluid, and correlate positively with total body fat mass. The cerebrospinal fluid to plasma leptin ratio is lower in obesity, suggesting a reduction in efficiency of leptin uptake.

Numerous studies evaluated the localization of LR messenger RNA (mRNA) within the hypothalamus in several species (rodents, primates: *Ahima*, 2005; ruminants: *Dyer* et al., 1997; *Ren* et al., 2002): LR is enriched in the arcuate, dorsomedial, ventromedial and ventral premamillary hypothalamic nuclei. Moderate levels of LR mRNA are detectable in the periventricular region and posterior hypothalamic nucleus, whereas low levels are found in the paraventricular nucleus and lateral hypothalamic area. Among them the arcuate nucleus, medial preoptic area, and median eminence are rich also in GnRH neurons (*Dyer* et al., 1997). The hypothalamus transduces leptin signals into neural responses, which cause alterations in feed intake, and reproduction (*Tang-Christensen* et al., 1999; *Ahima*, 2005). LR mRNA has been co-localized with neuropeptides involved in energy homeostasis, as well as in growth hormone releasing hormone (GHRH) and/or gonadotropin releasing hormone (GnRH) pro-

duction. Neuropeptide Y (NPY) and agouti-related protein (AGRP), which stimulate feeding, are present in the same neurons in the medial arcuate nucleus. An increase in leptin directly suppresses the signaling of NPY and AGRP, thus inhibits feed intake (*Kotz* et al., 1998; *Jang* et al., 2000; *Ahima*, 2005). Other orexigenic peptides, such as melanin-concentrating hormone (MCH) and orexins, are synthesized in the lateral hypothalamic area, and are inhibited indirectly by leptin. Because MCH and orexin neurons project to the cerebral cortex, they might provide a channel for transducing the effect of leptin to higher centers to coordinate feeding with sleep-wake cycles and other complex functions. Leptin increases the levels of anorectic peptides, α-melanocyte stimulating hormone (α-MSH) derived from proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript, in the lateral arcuate nucleus. Second order neurons that synthesize corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and oxytocin in the paraventricular nucleus, are controlled indirectly by leptin, and mediate the inhibitory effects of leptin on feed intake, stimulation of thermogenesis and neuroendocrine secretion (*Ahima*, 2005).

Leptin administration stimulated the production of the gonadotrophins (LH and FSH) from the hypophysis mainly via GnRH-neurons in the hypothalamus (*Woller* et al., 2001; *Watanobe*, 2002; *Amstalden* et al., 2003). Also direct hypophyseal effects of leptin on secretion of FSH and LH may exist, since full-length LR mRNA was present in the anterior pituitary of sheep (*Dyer* et al., 1997) and pigs (*Lin* et al., 2000). Leptin also directly affected basal and GHRH-mediated GH secretion from the hypophysis (*McMahon* et al., 2001; *Zieba* et al., 2003a).

3.5.2. Leptin and energy homeostasis

When plasma leptin is elevated, the appetite and dry matter intake may be reduced. Leptin decreases insulin and glucocorticoids, and stimulates GH, catecholamine and thyroid hormone secretions. It could act not only as an endocrine signal in the brain and/or in the large number of peripheral tissues in which LR is expressed, but also as an autocrine/paracrine regulatory factor within tissues where it is produced (*Barb* et al., 2001b; *Kershaw* and *Flier*, 2004; *Chilliard* et al., 2005; *Zieba* et al., 2005). Due to its effects on the central nervous system and endocrine glands, and/or to its direct peripheral role, leptin (i) increases the insulin sensitivity, glucose utilization and energy expenditure (in muscles), (ii) enhances the fatty acid

oxidation (in muscles and liver), (iii) stimulates the lipolysis (in WAT), and (iv) inhibits the lipogenesis (in hepatocytes and/or WAT). This stimulation of fatty acid oxidation is probably the key event for the tissue lipid lowering and insulin-sensitizing effects of leptin (*Ahima* et al., 1996; *Havel*, 2004). This was demonstrated recently to occur through a direct or indirect (via either the central nervous system, or a putative inhibition of stearoyl-CoA desaturase-1 activity) stimulation of AMP kinase, which inactivates the acetyl-CoA carboxylase and decreases the malonyl-CoA concentration, thus stimulating the intra-mitochondrial carnitine palmitoyl transferase-1-mediated fatty acid oxidation (*Cohen* et al., 2002; *Unger*, 2003). This complex physiological role of leptin identified at the beginning in lab rodents, has already been demonstrated in Primates (*Blücher* and *Mantzoros*, 2003; *Henson* and *Castracane*, 2003; *Messinis* and *Domali*, 2003), and recently also in monogastric farm mammals (pig: *Spurlock* et al., 1998; *Barb* et al, 2001b, *Barb* and *Kraeling*, 2004; horse: *Fitzgerald* and *McManus*, 2000; *McManus* and *Fitzgerald*, 2000) and ruminants (*Chilliard* et al., 1998, 2001 and 2005; *Ingvartsen* and *Boisclair*, 2001; *Vernon* et al., 2001 and 2002; *Zieba* et al., 2005).

Injections of leptin caused a rapid decrease in feed intake and body weight in mice (*Campfield* et al., 1995; *Halaas* et al., 1995), monkeys (*Tang-Christensen* et al., 1999) and pigs (*Barb* et al., 1998). When genetically obese (*ob/ob*) mice were pair-fed with leptin-treated *ob/ob* animals, they lost 30 % less weight than the leptin treated *ob/ob* mice (*Campfield* et al., 1995). This data suggested that besides its effect on feed intake via hypothalamic NPY neurons, leptin could also play an important role in regulating fat mobilization (*Halaas* et al., 1995). Using an ovariectomized ewe model, the satiety effect of leptin was also observed in ruminants by administration of recombinant human leptin in ewes for 3 days. This treatment caused a decrease in voluntary dry matter intake to approximately a third of the normal intake (*Henry* et al., 1999). However, this effect was lost when the ewe lambs were underfed and leptin was administered (*Morrison* et al., 2001).

3.5.3. Leptin and reproduction

The involvement of an adipocyte-yielded hormone in regulation of reproduction was supposed by the first studies with homozygote carriers of the mutant "obese (*ob*) gene": these *ob/ob* mice were infertile with atrophized genitals (*Ingalls* et al., 1950), due to the compete lack of their gonadotrophin production. In later experiments with *ob/ob* mice (*Barash* et al.,

1996; *Chehab* et al., 1996) leptin treatment increased serum LH and FSH concentrations, ovarian and testicular weight and sperm counts. Furthermore, repeated administration of leptin to female *ob/ob* mice resulted in ovulation, and after copulation also pregnancy and parturition. Leptin administration stimulated GnRH producing neurons in the hypothalamus, and directly stimulated the hypophysis to produce LH and FSH. In rodents, in the arcuate nucleus leptin binding increased during fasting (*Baskin* et al., 1999), and fasting for 48 h on d 13 and 14 postpartum prolonged lactational anestrus, a response that is eliminated by central or peripheral administration of leptin (*Abizaid* et al., 2004). In ruminants less information is available, but leptin seems to be responsible for changes in LH secretion in animals that are suffering severe energy shortage. In ruminants, recombinant ovine leptin administration to fasted mature beef cows stimulated LH secretion (*Amstalden* et al., 2002), and in fasted ovariectomized dairy cows leptin affected LH secretion in a dose-dependent manner (*Zieba* et al., 2003b). However, also contradictory findings were published. In ovariectomized food-restricted ewes, and in well-fed and undernourished ewe lambs, intracerebro-ventricular infusions of recombinant ovine leptin did not affect plasma concentrations of LH or FSH, LH pulse frequency or amplitude (*Henry* et al., 1999; *Morrison* et al., 2001). Furthermore, intravenous administration of leptin did not affect LH secretion in growing pre-pubertal ewe lambs (*Morrison* et al., 2002). Despite these inconsistencies, which may result from differences (in nutrition, body condition, reproductive status and/or gender) of animals used in various studies, the concept on permissive role of leptin modulating the central regulation of reproduction is fully accepted nowadays (*Smith* et al, 2002; *Williams* et al., 2002; *Barb* et al., 2004; *Zieba* et al., 2005).

Leptin acts also directly in the ovary, and is supposed to influence the cell proliferation and steroidogenic activity there. However, our related knowledge is limited. In an *in vitro* cell culture study, high doses of leptin, equivalent with elevated (supraphysiological: 10 to 300 ng/ml) plasma concentrations, could both increase the insulin-induced proliferation of thecal cells, and inhibit steroidogenesis in bovine ovarian tissues (*Spicer* et al., 2000; *Spicer*, 2003). These supraphysiological levels may occur in obese women suffering from type-2 diabetes, and are thought to have relevance in pathogenesis of polycystic ovary syndrome (*Magoffin* et al., 2003). [However, the correspondingly high plasma levels of leptin are exceptional in farm mammals: in our lab we have never measured yet leptin levels of about >0.850 nmol/l (= 13.6 ng/ml) and >1.250 nmol/l (= 20.0 ng/ml) in samples taken from lactating and non-lactating

ruminants, respectively.] In ewes with ovarian autotransplant the passive immunization against leptin increased the E_2 secretion, whereas the direct ovarian arterial infusion of low dose leptin decreased the E_2 and stimulated the P_4 production (*Kendall* et al., 2004).

The first ovulation and the subsequent onset of cyclic ovarian function at **puberty**, in the **postpartum period** and – in seasonal breeders – at the **beginning of the breeding season** are strictly influenced by nutrition and body condition (*Senger*, 2003; *Schneider*, 2004). The key driver of ovarian transition is the increase in GnRH / LH pulse frequency. Since the late eighties metabolic hormones (insulin, IGF-I, and recently also leptin) have been postulated as modulators in this mechanism (in rodents and Primates: *Blücher* and *Mantzoros*, 2003; *Mann* and *Plant*, 2003; *Abizaid* et al., 2004; pig: *Barb* et al., 2001a, 2001b and 2004; horse: *Gentry* and *Thomson*, 2002; *Gentry* et al., 2002; *Heidler* et al., 2002; *Waller* et al., 2006; ruminants: *Chilliard* et al., 1998; *Smith* et al., 2002; *Williams* et al., 2002; *Diskin* et al., 2003; *Foster* and *Jackson*, 2003; *Zieba* et al., 2005; *Blache* et al., 2007).

The clearest involvement of these metabolic hormones has been demonstrated in **postpartum dairy cows**: during the early weeks of lactation the NEB is associated with reduced plasma concentrations of insulin, IGF-I, and leptin, and increased secretion of GH (*Beam* and *Butler*, 1997, 1998 and 1999; *Kadokawa* et al., 2000; *Block* et al., 2001; *Huszenicza* et al., 2001; *Butler* et al., 2003). Also thyroid hormones are involved in the metabolic adaptation to early lactation (*Blum* et al., 1983; *Pethes* et al., 1985; *Capuco* et al., 2001; for further details please, see our recent review: *Huszenicza* et al., 2002). These hormones are thought to act at hypothalamic, pituitary, and ovarian levels to link changes in reproductive activity to changes in energy balance. The earliest data of *Kadokawa* et al. (2000) showed that BCS was still elevated when leptin concentrations began to decrease several days before parturition, and were still reduced when leptin concentrations started to increase before the postpartum first ovulation. This tendency in leptin-ovary interaction was confirmed soon by others (*Huszenicza* et al., 2001; *Liefers* et al., 2003). In a recent trial of *Kadokawa* et al. (2006) multiparous cows (n=18) were studied on d 14 postpartum, before the first ovulation, and during the period of NEB. Blood (plasma) samples were taken at 10-min intervals for 8 h. All samples were assayed for LH and GH, and hourly samples for NEFA, insulin, IGF-I and leptin. Frequency of LH pulses was correlated positively with energy balance (r= +0.51) and plasma leptin concentrations (r= +0.73). Amplitude of the LH pulses was correlated only with leptin (r= +0.53).

Frequency of GH pulses was not correlated with any measure of LH secretion, but was correlated negatively with plasma concentrations of insulin (r= -0.62) and IGF-I (r= -0.61). First ovulation was observed 34±4 d after parturition. These observations reveal an important linkage between pulsatile LH secretion and blood leptin concentrations during the early postpartum period in dairy cows, when their energy balance is negative, and may explain the delay in ovulation. Thus, it seems likely that leptin and IGF-I interact in controlling the resumption of cyclic ovarian function, which confirms the proposed permissive role of leptin in modulation of ovarian function shifting from anovulatory-acyclic to ovulatory-cyclic (*Smith* et al, 2002; *Williams* et al., 2002; *Zieba* et al., 2005; *Blache* et al., 2007).

At the **beginning of the breeding season** (during the late winter - early spring transitional period), mares in poor body condition, and receiving restricted daily ration exhibited lower leptin levels and more prolonged anovulatory season, compared to those in high body condition, and affected by the same feed restriction (*Gentry* et al., 2002). However, the ovarian pattern of mares with continuously high plasma leptin levels (idiopathic hyperleptinaemia; see also Chapter 3.3.) was the same, as of their stud mates with the same body condition, and receiving the same diet, but showing normal (lower) plasma leptin (*Waller* et al., 2006). It proves that the excessive (but not supraphysiological) leptin concentrations – given by a constant, good body condition – do not modulate the time of first ovulation.

In a most recent study interaction of insulin, IGF-I and leptin has been demonstrated also in modulation of male puberty (*Brito* et al., 2007).

3.6. Leptin in pregnancy and lactation

According to the experiences in lab rodents and Primates (*Ashworth* et al., 2000; *Christou* et al., 2003; *Henson* and *Castracane*, 2003; *Islami* and *Bischof*, 2003; *Waddell* and *Smith*, 2003; *Yoon* et al., 2005; *Zieba* et al., 2005), both leptin and LR mRNA are present in the endometrium and placenta, and also in the fetus. So – simultaneously with a metabolic effect of maternal leptin (see below, and also in Chapter 3.5.2.) – the local intrauterine activity of leptin-LR system may be an important regulator of implantation, as well as of the placental and fetal growth and development.

Maternal leptin levels may vary depending on the stages of pregnancy and lactation (*Bonnet* et al, 2002a; *Vernon* et al., 2002). During pregnancy the plasma leptin increases

significantly in several species, due to either the enhanced expression of placental leptin mRNA (in *Primates* and rat), or a dramatic elevation in plasma content of soluble LR, which serve as binding proteins in circulation, and decrease the renal leptin clearance (in mice) (*Ashworth* et al., 2000; *Henson* and *Castracane*, 2003; *Islami* and *Bischof*, 2003; *Waddell* and *Smith*, 2003). In ruminants the degree of placental leptin gene expression is negligible (*Ehrhardt* et al., 2001; *Vernon* et al., 2002), and the current knowledge is uncertain concerning the presence or absence of circulating leptin binding proteins in the blood (Chapter 3.2.). Despite of these facts, 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). It is not known, however, whether this pregnancy-related elevation in ovine leptin is associated with number of fetuses, circulating level of insulin and gestagens, and – during the latest days of gestation – with hyperketonaemia / pregnancy toxaemia. In cattle, no study has been performed yet to measure leptin levels during early and mid pregnancy in non-lactating animals. During late pregnancy the plasma leptin was high in not milked, dry cows, and it declined to a nadir towards parturition (*Kadokawa* et al., 2000; *Block* at al., 2001; *Liefers* et al., 2003). Not only in cattle but in all mammalian species which have been investigated, these peri-parturient changes may be age/parity-related, associated with the feed intake and health of the animal, and also influenced by the management and feeding system/technology. However, further studies are required to reveal the details.

The increase in leptin concentration during pregnancy seems to be paradoxical as this is a period of increased nutritional demands, thus not a period in which the actions of leptin are expected to increase. However, simultaneously with this pregnancy-associated elevation of plasma leptin level, in rodents and Primates (and presumably also in ruminants) a leptin resistance state is present (*Mounzih* et al., 1998; *Ashworth* et al., 2000; *Seeber* et al., 2002; *Henson* and *Castracane*, 2003; *Islami* and *Bischof*, 2003; *Waddell* and *Smith*, 2003), which prevents a decrease in feed intake (see also Chapter 3.5.2.). This pregnancy-associated leptin resistance has clear species-dependent characters. (i) In mice, concentration of circulating binding proteins (soluble LR) is elevated, so most plasma leptin is present in a bound, inactive form. (ii) In rat and Primates, decreased hypothalamic expression of the signaling form of the LR, and/or an impaired transport of leptin across the blood brain barrier (see also Chapter 3.5.1.) might be responsible for the leptin resistance during pregnancy (*Garcia* et al., 2000;

Seeber et al., 2002). In pregnant ruminants, no studies on hypothalamic leptin receptor expression, or leptin permeability of blood-brain barrier have been performed yet. Due to this leptin resistance during pregnancy, the negative feedback signal of elevated level of leptin on dry matter intake is not present, and the consequent raise in body condition results in further increase in leptin gene expression. This was confirmed in studies with rodents (*Kawai* et al., 1997), and also with sheep (*Ehrhardt* et al., 2001).

After parturition this resistance state decreases, because more LRs – both long (signaling) and short (transport) forms – are expressed at hypothalamic level (*Garcia* et al., 2000; *Sorensen* et al., 2002), which results in a higher sensitivity to leptin. There is a possibility that a certain level of hypothalamic sensitivity to leptin has to be reached before GnRH neurons are activated and able to stimulate gonadotrophin secretion from the hypophysis (see also Chapter 3.5.3.).

As it was mentioned earlier (Chapter 3.3.), regardless of the species the circulating leptin level starts to decline rapidly towards parturition. Several hypotheses have been proposed for the reasons of this decline. In lab rodents the suckling stimulus by itself did not appear to influence the decrease in leptin concentration (*Brogan* et al., 1999). *Pickavance* et al. (1998) observed that the food intake-induced leptin increase was eliminated during lactation and they speculated that the hypoleptinemia may be an important factor promoting the hyperphagia resulted from lactation. The energetic cost of milk production is a determining factor in falling of circulating leptin levels towards and during lactation. In dairy cows, towards lactation NEB is initiated due to a decline in dry matter intake together with an increased energy demand of the fetus (*Hayirli* et al., 2002). As a result adiposity decreases, which down-regulates the leptin expression. *Block* et al. (2001) showed that there was a large similarity between the periparturient energy balance and plasma leptin curves. This suggests that a possible explanation of the decline in leptin levels towards parturition is the fatty acid mobilization from the adipose tissue. Also the inhibition of leptin expression by adipose tissue in early lactation (*Block* et al., 2001; *Sorensen* et al., 2002) could be one of the reasons of the low leptin levels during lactation. Another option is the regulation of leptin expression by insulin and GH. The onset of NEB around parturition was also associated with decreased plasma insulin and increased plasma GH (*Block* et al., 2001), suggesting that both hormones could mediate a portion of the effect of energy balance on plasma leptin. Evidence supporting this idea exists in

rodents and humans, with insulin increasing and GH decreasing plasma leptin (*Ahima* and *Flier*, 2003). Administration of insulin increased circulating leptin in both late pregnant and early lactating cows, whereas GH treatment did not reduce plasma leptin (*Block* et al., 2003; *Leury* et al. 2003). This suggested that GH has no direct effect on leptin concentrations during the periparturient period. *Sauerwein* et al. (2004) investigated early pregnant (10 weeks) and non-pregnant (100 days postpartum) cows, and found that GH treatment reduced leptin concentrations only during early pregnancy. So it may be concluded, that in ruminants the decline in plasma leptin levels towards parturition is probably caused by several mechanisms. These are the decline in adiposity, the decreased expression of leptin in adipose cells and the decline in insulin concentrations.

3.7. Leptin in pathology of viral and prion-associated diseases

Persistent infection with bovine viral diarrhea virus was reported to cause diabetes mellitus in heifers (*Murondoti* et al., 1999). Diabetes mellitus is an important area of leptin-related research in the human medicine, with clear clinical orientation. To the best of our knowledge, however, there have not been data available on leptin levels in diabetic cattle. Multiple endocrine alterations are common features in prion-associated neurodegenerative disorders. Scrapie-affected ewes exhibited more elevated mean insulin and GH concentrations, higher GH pulse frequencies and lower total thyroxine (T_4) levels than their healthy counterparts, but their plasma IGF-I and leptin remained unchanged (*Viguié* et al., 2004).

3.8. Leptin in the inflammation-challenged cytokine-endocrine cascade

The inflammatory response to bacterial infections, especially that one induced by **Gram-negative (GN) pathogens** and the subsequent endotoxin release, involves a complex cytokine-endocrine cascade, with the multiple implication of leptin (*McCann* et al., 2003). Natural or experimental infection with GN pathogens, like the administration of endotoxin induce intense release of pro-inflammatory mediators, such as *cytokines* (IL-s, TNF-α and others), *eicosanoids* (leukotrienes and other prostanoids) and *oxygen free radicals* (nitric oxide and others), furthermore histamine, serotonin and complement fragments in the blood stream (in GN sepsis) or in the infected organ (in cattle: *Escherichia coli* mastitis, puerperal metritis) (*Giri* et al., 1984; *Cullor*, 1992; *Salyers* and *Whitt*, 1994; *Sandholm* et al., 1995; *Sordillo*

and *Daley*, 1995; *Dohmen* et al., 2000; *Mateus* et al. 2003; pathophysiology of endotoxin loading and neutralization: reviewed by *Jánosi* et al., 1998). Although the outer membrane of **Gram-positive (GP) pathogens** does not contain endotoxin, many layers of a mucopeptide (*peptidoglycan*) are located in their cell wall. Like endotoxin this component of GP pathogens has been supposed to possess the capability for inducing pyretic and cytokine (TNF-α) responses (*Salyers* and *Whitt*, 1994; *Sordillo* and *Daley*, 1995). As a recent improvement, milk-derived cells from affected mammary glands contained increased amounts mRNA for IL-1α, IL-1β, IL-10, IL12 and TNF-α in response to chronic *Staphylococcus aureus* (*S. aureus*) infection (*Riolett* et al., 2001), and enhanced TNF-α, IL-1β and IL-8 release was observed in experimentally induced *Streptococcus uberis* mastitis (*Rambeaud* et al., 2003).

These cytokine-mediated neuroendocrine changes play key role in induction of systemic signs and metabolic consequences of the inflammatory process. When the pro-inflammatory cytokines (TNF-α, IL-s) and other products of activated immune cells (mainly of macrophages) reach the central nervous system, these mediators initiate *pyretic reaction* and marked changes in secretory pattern and/or serum level of numerous *hormones*. The cytokine-induced endocrine alterations have dose- and species-dependent character (*Cullor*, 1992; *Sandholm* et al., 1995; *Sordillo* and *Daley*, 1995), and involve the malfunctions of the anterior pituitary, adrenal (both the cortical and medullar parts) and thyroid glands, the endocrine pancreas, furthermore the GH-IGF-I axis and gonad (further details are given in Chapter 5.7., and also in some of our recent review papers: *Huszenicza* et al., 2004; *Kulcsár* et al., 2005; *Földi* et al., 2006).

There is increasing evidence obtained in lab rodents (*Houseknecht* et al., 1998; *Fantuzzi* and *Faggioni*, 2000; *McCann* et al., 2003) and primates (*Landman* et al., 2003; *Xiao* et al., 2003) showing that leptin exerts numerous effects on inflammatory response and immune system. However, data evaluating the same role of leptin in domestic mammals (pig: *Spurlock* et al., 1998; *Leininger* et al., 2000; *Barb* et al. 2001b; ruminants: *Soliman* et al., 2001 and 2002; *Nikolic* et al., 2003; *Waldron* et al., 2003) are just at the beginning to emerge. Although the seasonal changes of plasma leptin and immune status were studied recently in mares (*Ferreira-Dias* et al., 2005), up to now there have not been data yet on inflammation-related leptin changes in the horse.

The earliest data on leptin suggested that it might be the signal protein by which information regarding the adequacy of peripheral fat stores is communicated to the central nervous system, with decreases in plasma leptin content playing a central role in triggering adaptive responses to starvation (*Ahima* et al., 1996). Leptin regulates both the appetite and the energy expenditure. So it was attractive to postulate that the endotoxin-induced hyperleptinemia suppresses the appetite and prevents the usual compensatory responses to starvation. The primary etiologic role of this mechanism was hypothesized in anorexia and cachexia, syndromes of depressed appetite and weight loss related to endotoxicosis and many chronic diseases including cancer (*Berkowitz* et al., 1998). Both the endotoxin and TNF-α administrations prevented the fasting-induced decrease of leptin mRNA and plasma leptin in hamsters (*Grunfeld* et al., 1996). Increasing doses of *E. coli* endotoxin resulted in dose-dependent elevations of leptin mRNA in fat cells, as well as of plasma leptin level in mice. Changes in fat cell leptin mRNA concentrations were preceded by a threefold increase in adipocyte TNF-α mRNA. Moreover, exogenous TNF-α was sufficient to induce changes in leptin mRNA identical to those seen with endotoxin (*Berkowitz* et al., 1998), suggesting that endotoxin alters the synthesis of this protein in the fat tissue, and this effect is mediated by TNF-α.

Leptin can modulate the inflammatory response: rhesus monkeys with low plasma leptin level (*Xiao* et al., 2003) and genetically leptin-deficient *(ob/ob)* or leptin receptor-deficient *(db/db)* mice (*Faggioni* et al., 1997; *Takahashi* et al., 1999; *Madiehe* et al., 2003) are more sensitive to endotoxin and TNF-α than their counterparts. In normal mice, fasting is associated with a fall in leptin levels and an increased susceptibility to endotoxin shock. Both the fasting-induced and *ob/ob*-related forms of increased endotoxin sensitivity can be reversed by leptin administration (*Faggioni* et al., 1999 and 2000a). In rhesus monkeys both the single-dose (100-200 μg) and repeated-dose (6 injections of 100 μg/occasion over a 4-d period) endotoxin administrations induced leptin release in all animals (*Landman* et al., 2003). However, their pre-treatment with recombinant human leptin ($_{rh}$leptin, at a dose of 50 μg/h) diminished the cytokine and hypothalamic-pituitary-adrenal responses to endotoxin (*Xiao* et al., 2003). There was a significant correlation between the mean baseline leptin level and the percent increase in leptin over baseline on the last day of the multiple endotoxin treatment (*Landman* et al., 2003). Low-dose (2-3 ng/kg) intravenous administration of *E. coli* endotoxin was reported to increase the circulating leptin level in healthy postmenopausal women (*Landman* et

al., 2003). Thus, leptin appears to exert important anti-inflammatory effects. Currently it is still unknown whether increased basal leptin levels are protective during a subsequent episode of sepsis, but these data suggest that suppressed leptin levels during starvation or wasting illness could increase the vulnerability to sepsis. It has been thought since the beginning (*Stouthard* et al., 1995) that circulating inflammatory cytokines (IL-1β and TNF-α), rather than the locally produced TNF-α, are responsible for the observed effects of endotoxin on leptin mRNA in adipocytes and plasma leptin (*Grunfeld* et al., 1996; *Sarraf* et al., 1997; *Faggioni* et al., 1998; *Finck* et al., 1998; Francis et al., 1999). Endotoxin failed to stimulate leptin in IL-1β-deficient mice or in normal mice treated with the soluble IL-1 receptor (*Faggioni* et al., 1998; *Francis* et al., 1999). Systemic cytokines could act directly on the adipocyte, or indirectly, via diverse effects on hormonal secretion and autonomic nervous system activity. In rats ketamine anesthesia decreased the plasma leptin concentration within 10 min to a minimum at 120 min, followed by a rebound at 360 min, and largely blunted the endotoxin-induced leptin release. Also further evidences summarized in *Table 3.8.1.* propose the implication of neurohormonal control mechanisms (*Mastronardi* et al., 2001; *McCann* et al., 2003), suggesting that leptin release is inhibited by the sympathetic nervous system, and this inhibitory effect is mediated principally by α-adrenergic receptors. Dopaminergic neurons may inhibit the basal and endotoxin-induced leptin release by suppression of prolactin release from the anterior pituitary.

As it has been discussed, endotoxin also stimulates the hypothalamus-anterior pituitary-adrenal axis, which may exert secondary effects on leptin release. Glucocorticoids have been reported to block the endotoxin-induced leptin release (*Mastronardi* et al., 2000). The attenuation of the ACTH and cortisol responses to endotoxin in leptin treated monkeys (*Landman* et al., 2003) may be secondary to a less extended release of pro-inflammatory cytokines which are known to mediate the stimulatory effects of endotoxin on the hypothalamus-pituitary-adrenal axis (*Turnbull* and *Rivier*, 1999). Leptin also stimulates the expression of POMC in the hypothalamus which is processed to α MSH (*Mol* and *Rijnberk*, 1997), a neuropeptid which has well-documented anti-inflammatory effects (*Cheung* et al. 1997). α-MSH can modulate the inflammatory response by acting on melanocortin receptors both within the brain and on immune cells in the periphery. It is thus likely that endotoxin affects leptin expression and release by more than one mechanism and that there may be species differences with respect to the regulatory mechanisms of leptin response to endotoxin.

Table 3.8.1. Some data improving the presence of neurohormonal control mechanisms in regulation of endotoxin-induced leptin release in rat models (after *McCann* et al., 2003, with some completion and modification).

Treatment in rats	Effects on	
	Plasma leptin (basal)	Endotoxin-induced leptin release
Non-selective β-adrenergic agonists (isoprotenol)	∅ or slightly ↓ *	↓
β-receptor antagonists (propranolol)	↑	∅
α-adrenergic receptor blockers (phentolamine)	↑	↓
Dopamine D-2 receptor agonists (α-bromoergocryptine)	↓	↓

Notes: ∅ No alterations; ↑ Increased significantly; ↓ Decreased significantly

** In non-pregnant heifers isoprotenol infusion (0.07 nmol/kg body weight per min, for 60 min) decreased the plasma leptin level. Selective β_1- and β_2-adrenergic agonists (dobutamine, terbutaline) failed to alter plasma leptin in the same model. (Leptin was determined by the "multispecies" RIA kit of Linco Research; *Chilliard* et al., 1998).

** Prolactin level was also suppressed simultaneously (*McCann* et al., 2003).

The long form of the LR is expressed by tissues and cells of the immune system, including bone marrow, spleen, monocytes, macrophages and T lymphocytes (*Gainsford* et al., 1996; *Fantuzzi* and *Faggioni*, 2000). Leptin has a specific effect on T lymphocytes, differentially regulating the proliferation of native and memory T cells. Intraperitoneal administration of leptin to mice reversed the immunosuppressive effects of acute fasting, restoring the impaired T-cell responses, which are found during starvation (*Faggioni* et al., 1999 and 2000b). This treatment modulated the T-lymphocyte activation toward the increasing IL-2, TNF-α and interferon-γ and decreasing IL-4, IL-5 and IL-10 production (*Lord* et al., 1998). Leptin acts on T cell-mediated liver toxicity in association with regulatory effects on thymus and peripheral blood cellularity, as well as on production of TNF-α and IL-1β (*Faggioni* et al., 2000b). Thymic atrophy is apparent in mice with defected leptin signaling (*ob/ob* or *db/db*) and during fasting. Leptin administration normalizes thymic cellularity in *ob/ob* mice and prevents fasting-induced thymic changes (*Faggioni* et al., 2000b). Leptin deficiency is associated with increased sensitivity to monocyte/macrophage-activating stimuli, resulting in increased sensitivity to endotoxin (*Faggioni* et al., 1999). Direct effects of leptin on activation of human monocytes have also been demonstrated (*Santos-Alvarez* et al., 1999). Leptin activates signal transduction, like other members of this family, by stimulating the Janus kinase-signal trans-

ducer and activator of transcription pathway. Leptin also induces the expression of SOCS-3, a member of the suppressor of cytokine signaling (SOCS) proteins, which inhibits cytokine signal transduction (*Bjorbaek* et al., 1998; *Auernhammer* et al., 2001). Leptin may modulate the production of pro-inflammatory and anti-inflammatory cytokines and may also affect cytokine signaling by a variety of mechanisms, including activation of SOCS-3. Thus leptin has a role in linking nutritional status to cognate cellular immune function, and provides a molecular mechanism to account for the immune dysfunction observed in starvation.

The course of the endotoxin-induced leptin response is quite different in rodents compared to primates. In mice and rat the experimental administration of endotoxin evoked a rapid and long-lasting increase in plasma leptin concentrations with the first detected elevation within 10 min (due to a endotoxin-stimulated release of stored hormone from the pinocytotic vesicles of adipocytes), and a plateau of its doubled circulating level from 2 to 6 h (accounted for the highly significant increase in leptin mRNA) (*Finck* et al., 1998; *Francis* et al., 1999; *Mastronardi* et al., 2000). In contrast, in human and monkey subjects no effect is seen during the initial 7-h period after endotoxin injection, but a stimulatory effect becomes evident by 16-24-h after injection (*Landman* et al., 2003). The reasons for these species-related differences are currently unknown. We discussed only the short-term effects of endotoxin administration: currently it is still unknown whether long-standing infections are characterized by chronically elevated leptin levels.

3.9. Leptin in the current production- reproduction- and clinical-oriented research

In the human medicine, searching of new principles and drugs for reducing the body fat content / body weight was the main driving force of clinical-oriented leptin research at the beginning. However, leptin administration is suitable to induce weight loss only in patients with non-functional leptin in their circulation, and this genetic aberration is very exceptional. So the physiological limitation of this concept was recognized soon. Since than some leptin-related aspects of type-2 diabetes and some associated problems (polycystic ovary syndrome), hypothalamic form of amenorrhea, pregnancy eclampsia, some inflammatory and autoimmune diseases (asthma, inflammatory bowel disease, rheumatoid arthritis, GN sepsis), as well as of multiple sclerosis have been in the focus of researchers' interest (*Sinha* and *Caro*, 1998; *Magoffin* et al., 2003; *Poston*, 2003; *Warren* and *Dominguez*, 2003; *Fantuzzi*, 2005). Beside

some obesity and diabetes research in pat carnivores and horses, in the veterinary medicine and animal husbandry the physiological role and diagnostic value of leptin are studied in association with nutritional aspects of reproduction, monitoring the effect of some feed additives, and as a parameter of carcass quality.

4. 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. Laboratory and statistical procedures and other methods generally used throughout the studies are described here in this chapter. Some clinical procedures and statistical methods employed only in one experiment are detailed where that trial is introduced.

4.1. Animal experimentation. Farm conditions

Animal experimentation was performed in compliance with local regulations where the trial was conducted, and was permitted and controlled by the competent units of State Veterinary Service (in Hungary), or by the Committee of Bioethics of Veterinary Faculties in Montevideo (Uruguay; *Exp. 4a*), Belgrade (Serbia; *Exp. 5a*) and Helsinki (Finland; *Exp. 6* and *Exp. 7a*).

For *Exp. 2*, *4a*, *6* and *7a* animals of experimental farms were used. Except for these studies, all the other trials were conducted in commercial flocks and dairy herds in Serbia (*Exp. 5a*) or in Hungary (all other trials). Farm conditions and feeding system of three bovine (*Exp. 4a*, *6* and *7a*), and all of the ovine studies (*Exp. 1c*, *1d*, *2* and *3*) are introduced at the description of experiments.

The further studies in cattle were carried out in one to eight of the same 12 large-scale dairy herds in Hungary (*Exp. 1a*, *1b*, *1d*, *4b*, *5b*, *7b* and *7c*) or Serbia (*Exp. 5a*). In all herds the cows were kept and fed in free housing system year round, in groups of 80-100 individuals, which were formed in accordance with the monthly checked average daily milk production. Dry cows were housed separately. The animals calved in continuously used maternity barns. Calves were weaned immediately after calving, or when the dams left this unit at the end of the colostral period. Separated groups were formed for fresh-milking young (parity 1) and

older (parity ≥2) cows. Groups of cows were fed with total mixed ration (TMR) of ensilaged maize and alfalfa products, alfalfa and grass hay, as well as with concentrate containing cereals completed with vitamins and minerals, with no technical facilities for individual feeding. Their daily ration was calculated in accordance with the NRC (2001) recommendations. Free access to drinking water was provided in all experiments. Neither pasturing, nor regular physical exercise was available. The course of uterine involution was regularly controlled with rectal palpation (RP) and vaginoscopy, and the bacterial complications were treated with commercially available antimicrobials and uterotonica. Except for those in *Exp. 1a* and *1e*, all cows showing estrus on day about ≥50 were inseminated (AI), and those not returning to estrus were checked for pregnancy by RP on day 45-60 after AI. The body condition (BCS) of cows was estimated using a scale from 1 (emaciated) to 5 (fat) according to *Edmonson* et al. (1989). Animals were scored to the nearest quarter point.

4.2. Collection, preparation and storage of samples

In order to determine most of the hormones (leptin, IGF-I, insulin, T_3, T_4, testosterone, P_4, E_2, LH) and metabolites (glucose, NEFA, ßOH-butyrate, BHB and total cholesterol, TCH), 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; *Exp. 3*), as well as into heparinized tubes (for any other parameters in all studies). In *Exp. 6* only, the plasma NEFA content was analyzed simultaneously also in plasma samples collected from the *superficial epigastric* (mammary) vein. 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 P_4 determination were taken from the healthy udder quarters into potassium dichromate containing tubes, and were stored at +4 C° until the assay (*Exp. 4b*). All milk samples were assayed within 21 days after collection.

In *Exp. 2*. also feces (5-10 g) was collected for assaying progesterone metabolites (P_4-met). For this purpose, fecal samples were taken from the rectum, put into plastic sacs, and frozen and stored at -20 °C until analyzed for P_4-met within some weeks.

4.3. Laboratory procedures

Laboratory procedures used to assay the various endocrine and metabolic parameters in the different studies are summarized in *Tables 4.3.1*. and *4.3.2*.

4.3.1. Radioimmunoassay for leptin

Plasma leptin concentration was quantified by a modified version of the ruminant-specific, homologous, double-antibody, non-equilibrium ^{125}I-RIA of *Delavaud* et al. (2000, 2002). The current adaptation of this assay was based on the use of an anti-ovine leptin antibody yielded by *Delavaud* et al. (2000) in rabbit. Instead of recombinant ovine leptin, however, in this version a commercially available form of recombinant bovine leptin ($_{rb}$leptin[6]) was used for radioiodination, as well as for preparing the standards. Furthermore the bound and free ligands were separated by a magnetisable immunosorbent suspension[7], rather than applying a specific anti-rabbit second antibody. The $_{rb}$leptin was labeled with ^{125}I by Chloramine T method (after *Hunter* and *Greenwood*, 1962, with modification). Briefly, 5 μg $_{rb}$leptin in 10 μl solvent was reacted with 30 MBq ^{125}I (in form of NaI[8]) and 10 μg Chloramine T[9] in boric acid-borate buffer (0.1 mol, pH 8.6). After one minute mixing the reaction was stopped by adding 20 μg sodium metabisulfite[9]. The ^{125}I-$_{rb}$leptin was separated in a column (20 cm long x

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

7 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)

8 Institute of Isotopes Co. Ltd., Budapest, Hungary

9 Reanal Ltd, Budapest, Hungary

Table 4.3.1. Assay procedures used for quantification of various hormones in plasma, milk and fecal samples

Parameter	Method	Experiment														
		1a	1b	1c	1d	1e	2	3	4a	4b	5a	5b	6	7a	7b	7c
Hormones in plasma samples																
Leptin	Local ^{125}I-RIA[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Insulin-like growth factor-I (IGF-I)	Local ^{125}I-RIA[a]							+	+	+				+	+	+
	^{125}I-IRMA (commerc.)[b]											+	+			
Testosterone	Local ^{3}H-RIA[a]			+												
Progesterone (P_4)	Local ^{3}H-RIA[a]				+	+	+	+								+
	^{125}I-RIA (commerc.)[c]								+							
Cortisol	Local ^{3}H-RIA[a]							+						+	+	
Insulin	^{125}I-RIA (commerc.)[d]						+	+	+	+				+	+	+
	^{125}I-IRMA (commerc.)[e]										+	+	+			
Thyroxine (T_4)	^{125}I-RIA (commerc.)[f]							+						+	+	+
	^{125}I-RIA (commerc.)[g]								+	+			+			
Triiodothyronine (T_3)	^{125}I-RIA (commerc.)[f]							+						+	+	+
	^{125}I-RIA (commerc.)[g]								+	+			+			
17β-estradiol (E_2)	^{125}I-RIA (commerc.)[h]					+										
LH	ELISA (commerc.)[i]					+										
Hormones in milk samples																
Progesterone (P_4)	Local ELISA[a]									+						
Hormone metabolites in fecal samples																
Progesterone metabolites (P_4-met)	Local ELISA[a]						+									

Samples were usually assayed for various hormones in the isotope laboratory of Department of Obstetrics and Reproduction (Faculty of Veterinary Science, Budapest, Hungary), under the guidance of the candidate. The only exceptions were the plasma **IGF-I** (in *Exp. 3*, *4a*, *4b*, *7a*, *7b* and *7c*) and **P4** (in *Exp. 4a* only), which were quantified in partner laboratories (**IGF-I**: Institute for the Application of

Nuclear Energy, Belgrade, Serbia; **P_4**: Department of Biochemistry, Faculty of Veterinary Medicine, Montevideo, Uruguay). In each procedure the **sensitivity** is defined as the lowest hormone quantity, which was able to generate a diminution of 5% of the B/B0 ratio.

[a] All the locally developed assay systems are introduced below (see Chapters 4.3.1. and 4.3.2.).

[b] In *Exp. 5b* and *6* the plasma **IGF-I** content was analyzed with a commercial ^{125}I-IRMA kit developed for human samples (DSL-5600 Active IGF-I Coated-Tube IRMA Kit; Diagnostic Systems Laboratories Inc., Webster, Texas, USA; sensitivity: 0.11 nmol/l; intra- and interassay CV: from 3.4 to 6.6 % and ≤7.0 %, respectively). The assay procedure includes a preceding extraction of IGF-I with an ethanolic HCl solution, and a before-assay neutralization of extracts. The assay was run in accordance with the manufacturer's instruction, with the exception of an overnight incubation of the neutralized extract on +4 ^{o}C (rather than its 3 h incubation on room temperature). The assay was validated for bovine plasma samples. The binding pattern of serially diluted bovine plasma samples was parallel to that of the standard curves. The recovery rates of added known quantity of IGF-I (0.78, 2.60 and 7.80 nmol/sample, given either before or after the extraction) to standard bovine plasma samples (n=3; all with pre-determined low IGF-I content) were the following: 78 - 88 % (IGF-I given before extraction) and 89 - 107 % (IGF-I given after extraction).

[c] In *Exp. 4a* the plasma **P4** was determined with a commercial ^{125}I-RIA kit developed for bovine samples (Coat-a-count, DPC Diagnostic Products Co, Los Angeles, CA, USA). The intra and interassay CV were 6 % and 11%. The sensitivity was 0.1 nmol/l.

[d] In *Exp. 2*, *3*, *4a*, *4b*, *7a*, *7b* and *7c* the plasma **insulin** content was quantified with a commercial ^{125}I-RIA kit (^{125}I-Insulin RIA CT kit; CIS Bio International Ltd, Gif-Sur-Yvette, France), developed for human purpose, and validated (*Huszenicza* et al., 1998; *Nikolic* et al., 2003) for bovine and ovine samples (sensitivity: 3.85 pmol/l, intra- and interassay CV: 5.5 - 8.4 and ≤8.8 %).

[e] In *Exp. 5a*, *5b* and *6* the plasma **insulin** content was quantified as free insulin with a commercial ^{125}I-IRMA kit developed for human samples (BI-Insulin IRMA kit; CIS Bio International Ltd – Subsidiary of Schering S.A., Gif-Sur-Yvette, France; sensitivity: 3.16 pmol/l; intra- and interassay CV: from 1.3 to 5.6 % and ≤8.5 %, respectively). The assay was validated for bovine plasma samples. The binding pattern of serially diluted bovine plasma samples was parallel to that of the standard curves. The recovery rates of added known quantity of insulin (35.9, 89.8 and 179.5 pmol/sample) to standard bovine plasma samples (n=3; all with pre-determined low insulin content) were 94 - 106 %.

[f] In *Exp.3*, *7a*, *7b* and *7c* the plasma **T_4** and **T_3** were determined by ^{125}I-T_4-Spec and ^{125}I-T_3 MIS RIA kits (both produced by Institute of Isotopes Co., Ltd. Budapest, Hungary). Both kits were developed for human purpose: the T_4 kit was modified for animal use, whereas the T_3 kit was used without modification (*Huszenicza* et al., 2000). The sensitivity was 1.46 nmol/l (T_4) and 0.18 nmol/l (T_3). The intraassay CV-s were 6.6 - 8.5 % for T_4 and 6.2 - 8.8 % for T_3. The interassay CV-s were ≤7.7 % and ≤6.7 % respectively. Precedingly both methods were validated for assaying bovine and ovine samples (*Huszenicza* et al., 1998; *Stojic et al., 2001*).

[g] In *Exp.4a*, *4b* and *6* the plasma **T_4** and **T_3** were determined by ^{125}I-T_4-Spec and ^{125}I-T_3 coated tube RIA kits (both produced by Institute of Isotopes Co., Ltd. Budapest, Hungary). The T_3 kit was developed for human purpose, whereas the T_4 kit was constructed for animal use including ruminants. The sensitivity was 0.5 nmol/l (T_4) and 0.19 nmol/l (T_3). The intraassay CV-s were 6.4 - 8.1 % for T_4 and 6.0 - 8.3 % for T_3. The interassay CV-s were ≤5.8 % and ≤6.5 % respectively. Precedingly both methods were validated for assaying bovine and ovine samples (*Nikolic* et al., 2003).

[h] The plasma **E_2** concentration was measured without extraction, with a commercial ^{125}I-RIA kit developed for assaying post-menopausal human samples (DSL-39100 3rd Generation Estradiol RIA kit, Diagnostic Systems Laboratories Inc., Webster, Texas, USA). Sensitivity: 1.90 pmol/l; intra- and interassay CV: 8.5 - 11.4 and ≤15.1 %. The assay was validated for bovine plasma samples. The binding pattern of serially diluted bovine plasma samples was parallel to that of the standard curves. The recovery rates of added known quantity of E2 (5.50 and 18.35 pmol/sample) to standard bovine plasma samples (n=3; all with pre-determined low E2 content) were 89 - 109 %.

[i] The plasma **LH** was analyzed with a commercial ELISA kit developed for assaying bovine plasma / serum samples (LH Detect, INRA, Tours, France). Sensitivity: 0.91 ng/ml; intra- and interassay CV: 6.1 - 10.2 and ≤14.5 %.

Table 4.3.2. Laboratory procedures used to assay the metabolic parameters, acute-phase proteins and tumor-necrosis factor-α in plasma samples

Parameter	Method	Experiment														
		1a	1b	1c	1d	1e	2	3	4a	4b	5a	5b	6	7a	7b	7c
Glucose	Enzymatic[a]							+								
Non-esterified fatty acids (NEFA)	Enzymatic[b]							+	+	+	+		+	+	+	+
βOH-butyrate (BHB)	Enzymatic[c]							+	+	+	+	+		+	+	+
Total cholesterol (TCH)	Enzymatic[d]							+		+						
Aspartate amino-transferase (AST)	IFCC, determination in UV range[e]							+								
Tumor necrosis factor-α (TNFα)	^{125}I-RIA (heterologous, commerc.)[f]															+
Haptoglobin (HP)	After *Elson* (1974)[g]															+
α_1-acid glycoprotein (α_1-AG)	Radial immundiffusion kit[g]															+

[a] GOD-POD reaction (Glucose kit, Kat. # 40841, Diagnosztikum RT, Budapest, Hungary; intaassay CV: ≤1.5% and ≤4.3 %); analyzed in the laboratories of Institute for the Application of Nuclear Energy (Belgrade, Serbia; thereafter: IANE).

[b] NEFA kit, Kat. # FA 115 (Randox Laboratories Ltd, Ardmore, UK; intaassay CV: ≤ 4.1% and ≤ 9.2 %); analyzed in the IANE (for *Exp. 3*) or Research Institute for Animal Husbandry and Nutrition (Herceghalom, Hungary; thereafter: RIAHN), for the further experiments.

[c] D-3-Hydroxybutyrate kit, Kat. # RB 1007 (Randox Laboratories Ltd, Ardmore, UK; intaassay CV: ≤ 5.7 % and ≤ 7.5 %); analyzed in the IANE (for *Exp. 3*) or RIAHN (for the further studies).

[d] CHOD-PAP reaction (Cholesterol-PAP kit, Kat. # 40121, Diagnosztikum RT, Budapest, Hungary; intaassay CV: ≤ 1.5 % and ≤ 3.8 %); analyzed in the IANE (for *Exp. 3*) or RIAHN (for the further studies).

[e] AST Kit, Kat. # 7249 (Reanal RT, Budapest, Hungary; intaassay CV: ≤ 6.2 % and ≤ 7.8 %); analyzed in the IANE.

[f] The plasma **TNFα** content was analyzed in our lab, in close cooperation with the manufacturer, using a commercial ^{125}I-RIA kit developed for quantitative determination of human TNFα in plasma, serum and cell culture supernatant (Necroton ^{125}I-RIA kit, "F. J. C." National Research Institute for Radiobiology and Radiohygiene, Budapest, Hungary; sensitivity: 12 pg/ml; intra- and interassay CV: 9.3 - 15.5 and ≤17.1 %); after a preceding validation (*Hirvonen* et al., 1999a and 1999b).

[g] Analyzed by J. Hirvonen, in the laboratory of the Ambulatory Clinic, Faculty of Veterinary Medicine (Helsinki, Finland; *Hirvonen* et al., 1999a and 1999b). The **Hb** content was expressed as hemoglobin-binding capacity of Hb (unit: g/l). The plasma **α_1-AG** was measured with a single radial immundiffusion kit (Bovine α_1-AG plate, Saikin Kagaku Institute, Sendai, Japan), and the results were given as g/l of α_1-AG.

1 cm diameter) filled with Sephadex G 50-fine[10]. 1-ml fractions were collected, and 1-1 ml glycerol[9] was added to each fraction containing ^{125}I-$_{rb}$leptin. The free iodine to ^{125}I-$_{rb}$leptin ratio was determined by thin layer chromatography on DC-Alufolien Kieselgel 60[11]. The triplicates of standards were prepared from a stock preparation of $_{rb}$leptin (1 µg/ml) 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).

The same assay procedure was carried out with both the bovine and ovine plasma samples: 100 µl horse serum was added to all the standards, as well as to the B0 and NSB tubes to obtain a similar protein matrix as in samples. 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. Standard, quality control and sample tubes were vortexed with 50 µl of diluted leptin antiserum (in a final dilution of 1:10.000), and then were incubated for overnight at +4 °C. After that, 100 µl of ^{125}I-$_{rb}$leptin (12-13 000 cpm; in assay buffer) was added to each tube, and after vortexing the process was continued with a second overnight incubation at +4 °C. Bound and free ligands were then separated by adding 500 µl ice-cold magnetisable immunosorbent suspension[12]. Then, the tubes were incubated for 30 min. at room temperature. After centrifugation (3600 g, 30 min., + 4 °C) all the tubes were put in a magnetic separator[13], and the supernatant was decanted. The remaining radioactivity on the microparticules was quantified by an automatic gamma counter[14]. The leptin content was calculated by 1224-310 MultiCalc v2.7 program[15].

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.

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10 No. 17-0042-01 (Pharmacia, Uppsala, Sweden)

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

12 Institute of Isotopes Co. Ltd., Budapest, Hungary

13 Amersham Lab., Little Chalfont, Buckinghamshire, England

14 Wallac-1470 Wizard (Perkin Elmer, Turku, Finland)

15 Perkin Elmer, Turku, Finland

The binding pattern of serially diluted bovine and ovine plasma samples was parallel to that of the standard curves. The recovery rates of added known quantity of $_{rb}$leptin (0.125, 0.250 and 0.500 nmol/sample) to standard bovine and ovine plasma samples (n=6 in each species) varied between 95 and 106 %.

4.3.2. Other locally developed endocrine assay procedures

Local versions of methods were used for assaying IGF-I, testosterone, cortisol and progesterone (P_4; except for *Exp 4a*) in plasma, P_4 in milk, and P_4 metabolites (P_4-met) in feces.

Neutralized acid-ethanol extracts of bovine plasma were analyzed for **IGF-I** using a heterologous ^{125}I-RIA system, which was developed in cooperation with a lab in Belgrade (*Nikolic* et al., 2001). This method was suitable for assaying ovine plasma without any further changes. Briefly, aliquots of serum or plasma (0.15 ml) were mixed and allowed to stand with 0.85 ml solution containing ethanol (87.5%) and 2 mol HCl (12.5%) for 30 min (*Daughaday* et al., 1982). After centrifugation for 30 min at 3000 x g the supernatant (0.5 ml) was neutralized with 0.855M tris-(hydroxyl-methyl)-aminomethane (TRIS) base and kept for at least 2 h at -18 °C (*Breier* et al., 1991). Following further centrifugation, aliquots of the supernatant were assayed. Blank extracts of 0.15 ml assay buffer were prepared in parallel. In the assay human IGF-I[16] labeled with ^{125}I was used as the tracer, and polyclonal rabbit antibodies to human IGF-I[17] as the reagent. Since bovine IGF-I has the same amino acid composition as human IGF-I (*Simmen*, 1991), recombinant human IGF-I was used as the working standard (0.063 - 6.25 ng/tube). Cross-reactivity with IGF-II at 50% inhibition of tracer binding was 0.054%. Reproducibility was checked by including the reference preparation (WHO 87/518) in each test. The mean sensitivity, intra- and interassay CVs were 0.20 nmol/l, ≤6.2 and ≤12.0 %, respectively, in both the bovine and ovine samples (*Nikolic* et al., 2001).

Plasma **testosterone** concentrations were determined with a direct ^{3}H-RIA developed for assaying human plasma (*Csernus*, 1982), which was adopted and validated for samples of many species including sheep (*Kulcsár*, unpublished; for details: *Table 4.3.3*). The canine and rabbit versions of this assay were published elsewhere (*Dahlbom* et al., 1995; *Fodor* et al., 2003).

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[16] ICN Biomedicals Inc., Aurora, USA

Table 4.3.3. The direct ^{3}H-RIA used to assay plasma testosterone levels

Antibody	Raised against testosterone-3-CMO-BSA in rabbit* Cross-reactivity: 5α-dihydro-testosterone: 45.0 %, 5β-dihydro-testosterone: 9.3 %, androstenedione: 2.2 %, 17α-methyl-testosterone: 0.72 %, 25 other steroids: <0.10 %)
Tracer	1,2,6,7-^{3}H-testosterone**
Assay standard	Testosterone (Δ^{4}androstene-17β-ol-3-one)***, prepared in steroid-free plasma (range: 7.8 fmol to 500.0 fmol per tube)
Separation of antibody-bound and free fractions	By cold (+4 C°) dextran-coated charcoal suspension after an 18-24 hours incubation period
Measuring radioactivity	By a liquid scintillation counter****
Sensitivity	0.22 nmol/l
Intraassay CV	Between 7.55-4.89 %
Interassay CV	Between 9.43-8.37 %

* Provided by *V. Csernus* (University Medical School, Pécs, Hungary)
** RK 402 (Radiochemical Centre, Amersham, UK)
*** No. T-1500 (Sigma Chemical Company, St. Louis, USA)
**** Beckman Instrument Typ LS 1701

Except for *Exp. 4a*, the plasma **cortisol** and **P_4** levels were usually determined by a direct ^{3}H-RIA used for human (*Csernus*, 1982) and equine (*Nagy* et al., 1998) samples, validated for assaying bovine and ovine plasma without modification (*cortisol:* sensitivity: 0.26 nmol/l, intra- and interassay CV: ≤5.6 and ≤9.5%; *P_4*: sensitivity: 0.20 nmol/l, intra- and interassay CV: ≤4.5 and ≤8.9%). Details are described elsewhere (P_4: *Nagy* et al., 1998; cortisol: *Nikolic* et al., 1998; *Jánosi* et al., 2003).

The **P_4** content of previously defatted bovine **milk samples** was determined with a microplate ELISA of *Nagy* et al. (1998) developed for equine plasma, and modified for assaying bovine skim milk (sensitivity: 0.18 nmol/l; depending on the actual concentrations, the inter- and intra-assay coefficients varied between 10.3 and 12.3 %, and between 5.2 and 11.6%, respectively; *Huszenicza* et al. 1998; *Taponen* et al. 2002).

For assaying **P_4-met**, steroid content was extracted from feces according to a slight modification of method described by *Palme* et al. (1996 and 1999). Briefly, 0.50 g of feces was dispersed in 0.50 ml double distilled water in thick-wall glass tubes suitable for centrifugation. After adding 4 ml methanol (80 %) samples were shaken for 20 min. with a multitube vortex. Thereafter 3 ml petroleum ether (bp. 40-70 °C) was added to remove lipids. Than samples were mixed again at high speed for 10 sec. by hand-vortex. After centrifugation

[17] Biogenesis, Poole, UK

(3600 g, 30 min., + 4 °C) samples were taken at -70 °C for 25-30 minutes to separate the three phases of petroleum ether (above), methanol (in middle) and the frozen water with the extracted feces (below). 0.5-1 ml of the methanol phase was pipetted into clean tubes, than 1:10, 1:20 or 1:50 working solutions were diluted with PBS buffer (pH: 7.4) when low or high P_4-met levels were expected, respectively (if values were out of the expected range, the sample was re-assayed). P_4-met concentrations were measured in triplicate 20 µl aliquots of fecal extracts with a micro-plate ELISA (*Nagy* et al., 1998), based on the use of an anti-P_4 monoclonal antibody (5D4) cross-reacting with wide range of gestagen metabolites (*Siklódi* et al., 1995)[18].

In all of these assay systems the binding pattern of serially diluted bovine, ovine plasma and fecal extract samples was parallel to that of the standard curves. The recovery rates of added known quantity of hormones were the following: (i) standards of tested steroids[19] to bovine and ovine plasma samples (n=3): between 89 and 108 %; (ii) P_4 and 5β-pregnan-3,20-dion (125, 250 and 500 nmol/g of each) to methanolic extract of standard ovine fecal samples (n=3): between 93 and 102 %; (iii) 5β-pregnan-3,20-dion (125, 250 and 500 nmol/g) to standard ovine fecal samples (n=3): between 78 and 87 %.

4.4. Statistics

For presentation of results the group **means** and their **standard errors** (SEM) were expressed. The results were usually subjected to chi-square (χ^2) test (distributions), Student's t test (pair-wise comparison of group means), or a single trait analysis of variance (ANOVA; for comparison of 3 or more group means in a particular stage). If ANOVA proved significant difference, the least significant differences were calculated at 5% ($LSD_{P<0.05}$) for further comparison. In *Exp. 4a* Tukey-Kramer test was conducted to analyze the between-group differences. Temporal patterns were usually tested by repeated measures ANOVA (*Exp. 1c*, *1d*, *1e*, *2*, *5a* and *7c*), or by one-way ANOVA with a post hoc testing by Neuman-Keuls test (*Exp. 7a* and *7b*). Interrelations were estimated by using Pearson correlation, linear and quad-

[18] 5β-pregnan-3,20-dion: 100 %; 11α-OH-progesterone: 20 %; 5α-progesterone -3,20-dion: 15,6 %; 17α-OH-progesterone: 3,6 %; pregnenolon: 1.8 %; 11β-OH-progesterone: 1.6 %

[19] All from Steraloids Inc., Newport, USA

ratic regression, or mixed procedure of SAS (2000)[20] (only in *Exp. 4a*). These methods were employed and conducted, as suggested by *Juvancz and Paksy* (1982), *Snedecor and Cochran* (1982) and *Petrie* and *Watson* (1999). Analyses were done using the Windows-XP Excel program and the statistical package S-PLUS (2000)[21] or SAS (2000)[19]. Further details of statistical methods are also given at the description of studies.

5. Studies

5.1. Biological validation of the leptin assay (*Exp. 1a, 1b, 1c, 1d* and *1e*)

After the development of our leptin assay the first responsibility was to check and improve the biological validity of findings with this laboratory procedure. For this purpose, five experiments were conducted. Within the framework of these studies we wished to reproduce some tendencies known from the literature, such as the effects of

(i) 24 h feed deprivation (in mid- to late-lactation dairy cows: *Exp. 1a*),

(ii) reproductive status and lactation (late pregnancy vs. early and mid lactation in dairy cows: *Exp. 1b*),

(iii) gender, furthermore ovariectomy / estrogen administration, and castration / testosterone replacement (in sheep: *Exp.1c* and *Exp. 1d*), as well as

(iv) presence or absence of cycle-related changes (in non-lactating ewes: *Exp. 1d*; in dairy cows, about 9-10 weeks after calving: *Exp. 1e*) and diurnal variability (in lactating cows: *Exp. 1e*).

Exp. 1a

Animals. Sampling

In a large scale dairy farm non-pregnant lactating Holstein Friesian cows (n=6; BW: 545-625 kg; BCS: 2.75-3.50; parity: 3 to 5) were involved in this study. The cows calved 137-191 days earlier, and yielded 7.8-23.0 kg/day milk. All of them had been culled due to chronic recurrent mastitis, and produced high somatic cell count milk (>500 000/ml in individual bulk milk samples), but were clinically symptomless at the time of sampling. After morning

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[20] Statistical Analysis System (SAS Institute Inc., Cary, NC, USA); by A. Meikle (Faculty of Veterinary Medicine, Montevideo, Uruguay; *Exp. 4a*), T. Kokkonen and T. Lehtolainen (Faculty of Veterinary Medicine, Helsinki, Finland; *Exp. 6*, *7a*, *7b* and *7c*).

[21] MathSoft Inc. (Seattle, Washington, USA); by J. Reiczigel and A. Gáspárdy).

milking, they were separated from the others in two boxes (3 in each) with no edible bedding for 24 h, during which no feed was provided at all, but free access to drinking water was available. As usually, the cows were milked in the late afternoon, and again next morning. Blood samples were taken before the feed deprivation, and 24 h later (after the morning milking) to assay the plasma leptin. After the second sampling all cows went back to their production group.

Results

Before fasting the plasma leptin levels varied between 0.347 and 0.527 nmol/l, and were in mild positive correlation with BCS (r=0.376), but not with the BW. After 24 h complete feed deprivation significantly lower plasma leptin contents were detected, which did not correlate with BCS and BW (*Fig. 5.1.1.*).

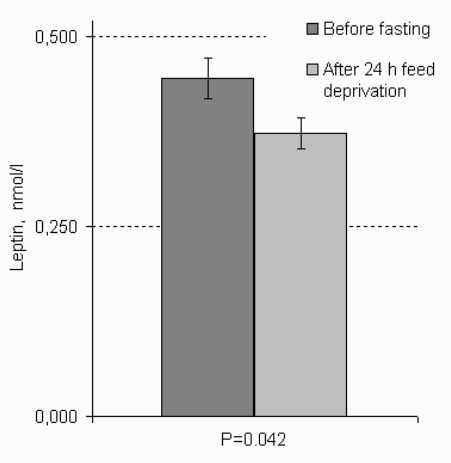

Fig. 5.1.1.: Plasma leptin levels of non-pregnant mid- to late-lactation dairy cows (n=6; BCS: 2.75-3.50), before fasting and after 24 h feed deprivation (mean ± SEM; *Exp. 1a*)

Exp. 1b

Animals. Sampling

In a Holstein Friesian herd three consecutive blood samples were taken from 16 cows (parity: 2 to 5; milk yield in previous lactation: 7218 - 9744 kg FCM), 4-5 weeks before the expected time of calving (during the late pregnancy), furthermore 2-5 days and 12 weeks after calving. However, during the early lactation 5 of them were affected by puerperal metritis and/or severe forms of mastitis, and were excluded. So at the end the plasma leptin content of 11 cows was determined. The housing and feeding conditions were the same as usual.

Results

During the dry period the plasma leptin content was high (varying between 0.465 and 0.679 nmol/l; *Fig. 5.1.2.*), and showed moderate positive correlation with the BCS (r= 0.468). On day 2-5 after calving markedly lower leptin levels were measured, with no correlation with BCS and/or BW. A tendency of further reduction was detected in samples taken 12 weeks later, and the correlation between leptin and BCS remained almost negligible (r= +0.282).

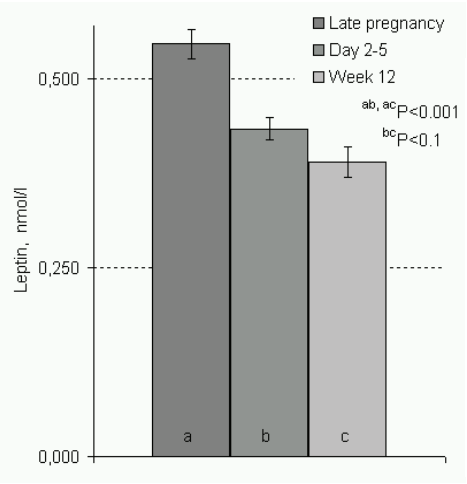

Fig. 5.1.2.: Plasma leptin content of dairy cows (n=11) during the late pregnancy, furthermore 2-5 days and 12 weeks after calving (mean ± SEM; *Exp. 1b*)

	BCS range	BW, kg range
Late pregnancy	3.25 - 4.00	---
Day 2-5 (week 1)	3.00 - 3.75	575 - 645
Week 12	2.50 - 3.25	---

Exp. 1c and ***Exp. 1d***

Animals. Sampling

This study was conducted in healthy Merino ewes (n=7) and rams (n=7) in moderate to good body condition (age of both sexes: 3-5 years), in October - November. Precedingly all of them were bred regularly for years, and were culled before this study due to economic reason. Before the experiment they were kept on pastureland, under usual flock conditions (but despite the season rams were separated from ewes). The ovarian cyclicity of ewes was synchronized with a 14-day-long gestagen[22] administration combined with 500 IU eCG[23] at the time of vaginal sponge removal. Simultaneously, the ewes and rams were moved into two separated paddocks (were free access to grass hay and drinking water was provided continuously), and were sampled regularly thereafter.

In *Exp. 1c*, after once-a-day sampling for 3 days, all **rams** were fasted for 24 h. Then a fourth sample was collected, and all rams were castrated. After a two-week-long postopera-

22 Vaginal sponge of 40 mg Cronolone (Chrono-gest™, Intervet International B.V., Angers, France)

23 Folligon inj.™ (Intervet International B.V., Angers, France; i.m.)

tive recovery period the once-a-day sampling procedure was continued. After taking the third post-castration sample, a combination of various testosterone esters[24] (250 mg; providing a rapid and powerful androgenic activity, which persists for about one month following the administration) was injected. Then further 6 samples were collected 24 h apart.

In *Exp. 1d*, after gestagen removal the **ewes** were sampled for 22 days, 24 h apart. Then hay was deprived for 24 h, a blood sample was taken, and all ewes were ovariectomized. When animals were over a two-week-long postoperative recovery period, the once-a-day sampling procedure was continued for additional 5 days. After taking the third post-ovariectomy sample 1 mg of estradiol benzoate[25] was injected.

Gonads were removed with standard surgical methods, following a 24-h-long preoperative feed deprivation, and after general and local anesthesia. In order to avoid measuring the combined effects of inflammatory cytokines and the postoperative depression of feed intake, no samples were collected for endocrine determinations in the first two weeks after the surgical interventions. Animals were slaughtered on day 55 of the study (e.g. following a 5-week withholding period after the testosterone supplementation).

All blood samples were assayed for testosterone and leptin (*Exp. 1c*), and for P4 and leptin (*Exp. 1d*).

Results

At the beginning of the study the plasma leptin content was higher in the ewes than in the rams (*Fig. 5.1.3.*), although both sexes were in moderate to good body condition, and the rams were heavier (BW, ewes: 49-57 kg; rams: 59-67 kg). During the 24-h-long preoperative feed deprivation the plasma leptin content declined in both sexes (compared to those found before fasting, differences were $P<0.05$ and $P<0.1$ in ewes and rams, respectively), but remained still in higher range in females than in males (*Fig. 5.1.3.*).

Before castration the testosterone levels were within the physiological range seen in rams usually during the breeding season. Two weeks after castration very low levels of testosterone were detected, and compared to those measured at the beginning of the study, higher concentrations of leptin were found (*Fig. 5.1.4*.), whereas neither the BW (measured), nor the

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[24] Durasteron inj.™ (Intervet International B.V., Angers, France; i.m.). Ingredients per ml: Testosterone propionate: 6 mg, Testosterone phenylpropionate: 12 mg; Testosterone isocaproate: 12 mg; Testosterone decanoete: 20 mg. Dose and indication for ram: 250 mg, in case of aging and debility.

[25] Oestradion benzoate inj.™ (Intervet International B.V., Angers, France; i.m.); ingredients per ml: 0.2 mg estradiol.

body condition (estimated) changed significantly (data not shown). Following supplementation, a sharp increase in testosterone was seen, forming a plateau lasting till the end of the sampling process. Simultaneously – after two days decrease – the plasma leptin content stabilized in a lower range, which was almost equal to the pre-castration levels (*Fig. 5.1.4.*).

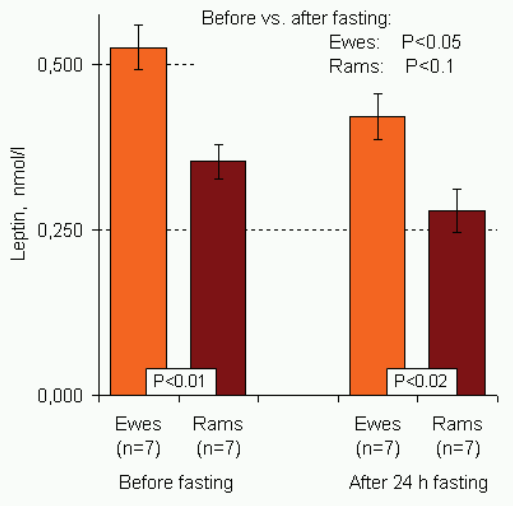


Fig. 5.1.3.: Plasma leptin content of non-lactating ewes (n=7) and rams (n=7) before the preoperative feed deprivation (mean of 3 samples of each animal), and after 24 h fasting (mean ± SEM; *Exp. 1c* and *1d*).

Fig. 5.1.4.: 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*).

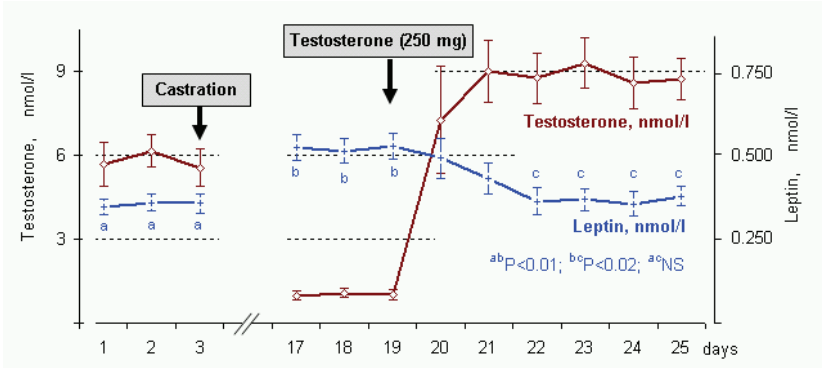
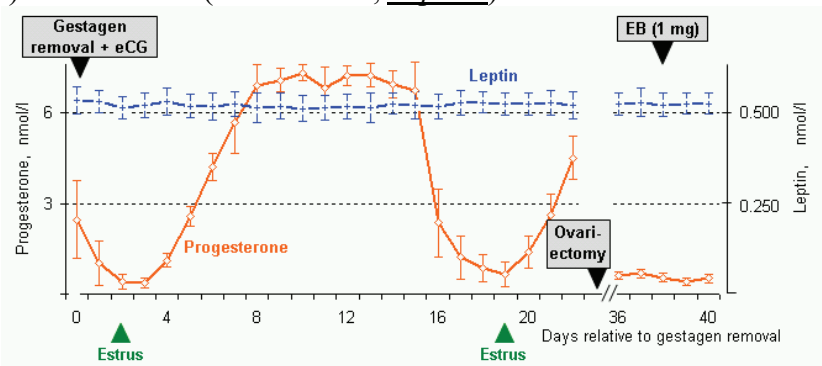


Fig. 5.1.5.: Time-related changes in plasma progesterone (P_4) and leptin contents of ewes (n=7), after a gestagen-based cycle synchronization, during the subsequent cycle, two weeks after ovariectomy, and after a single-dose estradiol benzoate (EB) administration (mean ± SEM; *Exp. 1d*).



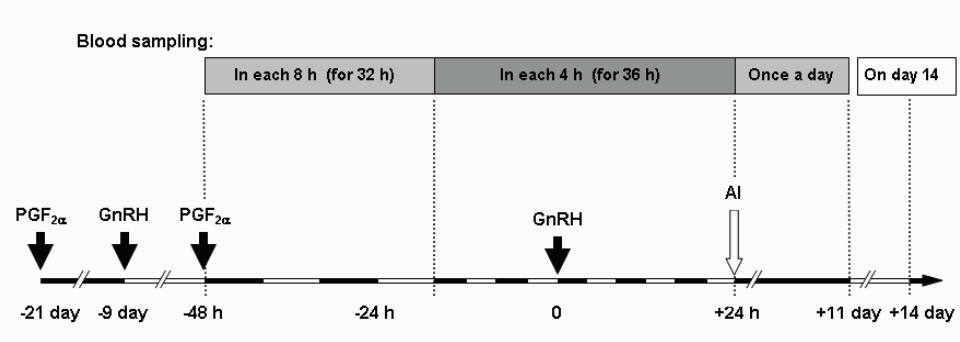
In all ewes the time-related changes of P_4 proved a regular response to the gestagen-based cycle synchronization technique, with ovulation and cyclic ovarian function subsequently. All ewes were ovariectomized in the early metoestral phase of the second cycle. Two weeks after ovariectomy very low P_4 content was found. However, the plasma leptin content remained unchanged during the synchronized ovarian cycle, after ovariectomy, and/or following the postoperative single-dose estradiol benzoate administration (evaluated by repeated measures ANOVA; *Fig. 5.1.5*.).

Exp. 1e

Animals. Sampling

This experiment was conducted as a part of a large trial in a TMR-fed large-scale dairy herd. For this study two blocks of healthy Holstein Friesian cows (4 in each; parity: 2 to 5; actual milk production: 29 to 44 kg/day; BCS: 2.50 to 3.00) were selected. All of them were kept, housed and fed in the same group of animals since calving. Starting on day 49 to 63 postpartum, a combined administration of $PGF_{2\alpha}$[26] and GnRH[27] (e.g. $PGF_{2\alpha}$, 12 d later: GnRH, 7 d later: $PGF_{2\alpha}$, 48 h later: GnRH, completed with fixed-time AI 17-24 h later; Pre-Synch protocol, *Moreira* et al., 2001) was used for synchronization of follicle growth and ovulation. After the second $PGF_{2\alpha}$ treatment blood samples were taken in each 8 h (for 32 h), thereafter in each 4 h (until the fixed-time AI), later once a day for 10 days, and again on day 14 (*Fig. 5.1.6*.). Hormones rflecting

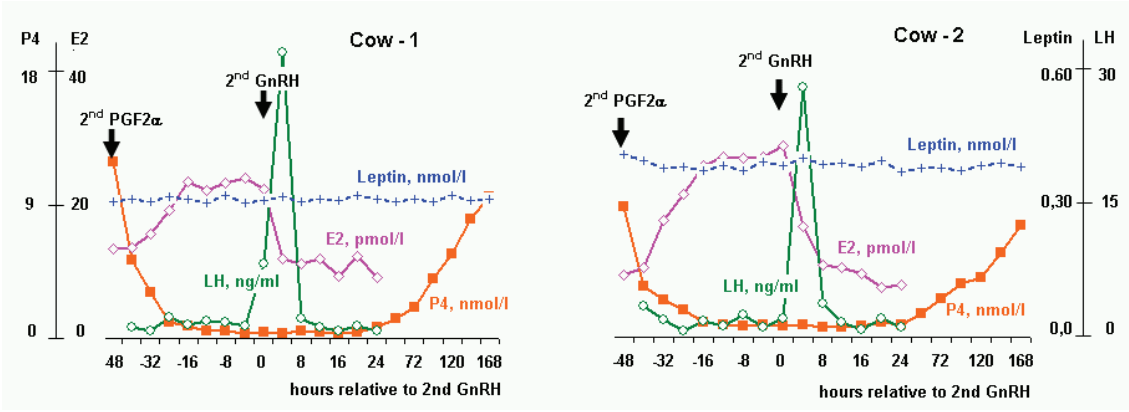
Fig. 5.1.6.: The treatment and sampling schedule in *Exp. 1e* (all procedures were started on day 50 to 64 after calving; time scale relative to the second GnRH treatment of Pre-Synch; *Moreira* et al., 2001).



[26] 0.021 mg Buserelin acetate (5 ml of Receptal™ inj., Intervet AG, Angers, France).

[27] 15 mg of Luprostiol (2 ml of Prosolvin™ inj., Intervet AG, Angers).

Fig. 5.1.7.: Endocrine changes after the second $PGF_{2\alpha}$ treatment of the Pre-Synch protocol (illustrated by individual data of two cows; *Exp. 1e*).



the ovarian cyclicity and CL function (P_4) and follicle growth (E_2), as well as LH and leptin were determined in each sample (P_4, leptin), or in samples collected until the fixed-time AI (E_2, LH).

Results

As illustrated on *Fig. 5.1.7.*, at the time and after the second $PGF_{2\alpha}$ treatment of the Pre-Synch protocol all cows had growing or dominant follicles producing E_2 for about 16 to 32 h. The second GnRH administration was followed by a temporary (=8 h) preovulatory-like LH release, ovulation, and luteinization (indicated by post-ovulatory P_4 rise). Peak levels of LH were measured 4 h after the GnRH challenge. The individual means of plasma leptin content varied between 0. 156 and 0.421 nmol/l (e.g. <0.250 nmol/l, between 0.250 and 0.330, and >0.330 in 4, 2 and 2 cows, respectively), with an intra-individual variability of 9 to 16 %. Similar means of leptin were measured during the E_2 dominance (e.g. 32 h before the GnRH-induced LH peak), during the first 72 h after the LH peak (when neither the E_2 nor the P4 contents were elevated), and thereafter (during the P_4 dominance): the repeated measures ANOVA could not reveal any cycle-related or diurnal changes in plasma leptin content (details are not given).

Conclusion of *Exp. 1a - 1e*

Finding of *Exp 1a* to *Exp. 1e* showed that the 24-h feed deprivation reduced the plasma leptin content. Significantly lower leptin levels are seen during the early lactation than in dry cows 4-5 weeks before calving. There are obvious gender-related differences also in ruminants, with lower leptin levels in plasma of males (although the total body fat mass of animals

was not determined). These sex-dependent differences are mediated by the suppressive effect of testosterone: two weeks after castration the leptin levels increase, whereas in wethers the high-dose slow-release testosterone administration depresses the plasma leptin again. However, neither cycle-related, nor diurnal changes seem to occur in plasma leptin contents of ruminants: in cyclic ewes and cows we could not detect any changes in plasma leptin, and also the ovariectomy, and the E_2 treatment administered two weeks later failed to alter the circulating leptin (tested only in ewes). These findings fully agreed with the data of literature (discussed in Chapter 3.3.), improved the reliability of our leptin assay system, and – regarding the gender dichotomy and the lack of cycle-related changes in plasma leptin of ruminants – represented also novelty value.

5.2. Influence of pregnancy stage and number of fetuses on maternal plasma leptin in ewes (*Exp. 2*)

As it was discussed in Chapter 3.6., 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.

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 P_4 and fecal P_4 metabolite (P_4-met) contents.

Materials and methods

The study was conducted on Prolific Merino ewes (n=61; age: 2 to 5 years; body weight: 33 - 43 kg) of mild to moderate body condition at the experimental farm of University of Debrecen at the end of the breeding season in January. During the first 72 days the group of animals was kept in a pen, and fed *ad libitum* with grass hay, whereas after the beginning of the grazing season in April they were moved to a pasture land on alkaline soil, providing abundant grass until lambing in June. Free access to drinking water was available throughout the

experiment. In January 2002 the ovarian activity of ewes was induced / synchronized with a 14-day-long gestagen[28] administration, combined with 500 IU eCG[29] at the time of vaginal sponge removal. Sixty hours later the ewes were inseminated (AI) once with fresh diluted semen, which was deposited with Salamon's method (*Evans* and *Maxwell*, 1987) by laparoscopy, after 24-hour feed and water deprivation. Pregnancy rate and number of fetuses were calculated upon the data of lambing in June.

At the beginning of the feed deprivation preceding the AI (d 0) and again 41, 81 and 101 d later blood samples (10 ml) were taken assaying for P_4, insulin and leptin. Simultaneously also feces (5-10 g) was collected for P_4-met. Body weight was measured simultaneously with the first sampling.

Pregnancy-related changes in plasma levels of these hormones were evaluated by repeated measures ANOVA (S-PLUS) using time (d 41, d 81, and d 101) as the within subject factor, the number of fetuses (1, 2, and ≥3) as a between subject factor and the initial (d 0) measurement as a covariate.

Results

Data of 56 ewes were evaluated, whereas the others were excluded due to incomplete sampling (n=4), or conception later than the AI in late January (n=1). Twenty-four of the 56 animals (43 %) conceived at the AI studied, and delivered one (n=12), two (n=6) or 3 to 5 lambs (n=6) after a regular gestation in June.

With the exception of 4, all the other ewes had low plasma P_4 and fecal P_4-met concentrations (<1.50 nmol/l and <250 nmol/g, respectively) at the time of AI, representing the range known as physiological in the follicular phase of cyclic small ruminants (*Schwarzenberger* et al., 1996; *Pereira* et al., 2006). None of these 4 individuals with elevated plasma P_4 (1.57 to 4.54 nmol/l) and fecal P_4-met (268 to 424 nmol/g) contents conceived at that time. During pregnancy the P_4 and P_4-met levels rose continuously, peaking in the last samples taken on d 101 of gestation (*Fig. 5.2.1.*). The plasma P_4 and fecal P_4-met were in significant positive correlation in all stages of the study (r= 0.526, 0.612, 0.810 and 0.881 on d 0, 41, 81 and 101, respectively; P<0.001). The P_4 and P_4-met contents of those not conceived at the AI remained low (details are not given), proving the lack of cyclic ovarian function in this season.

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[28] Vaginal sponge of 40 mg Cronolone (Chrono-gest™, Intervet International B.V., Angers, France)

Fig. 5.2.1. The plasma progesterone (P_4) and fecal progesterone metabolite (P_4-met) levels of pregnant ewes compared to those not conceived at the artificial insemination (day 0), and became acyclic throughout the spring-time sampling period (mean ± SEM; time-related changes in P_4 and P_4-met of pregnants: P<0.001, with single trait ANOVA; *Exp. 2*)

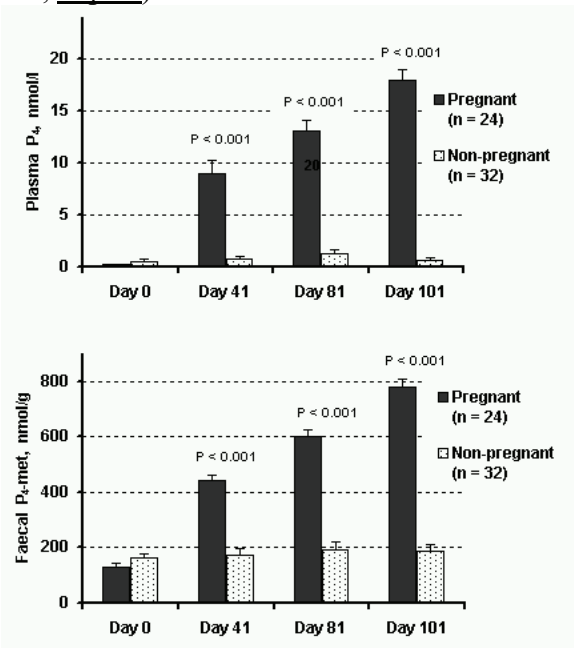
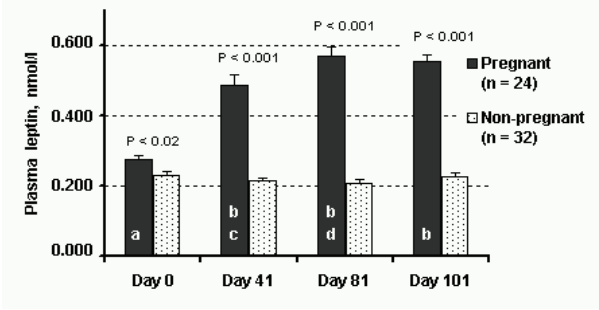


Fig. 5.2.2. The plasma leptin levels of pregnant ewes compared to of those not conceived at the artificial insemination (day 0), and became acyclic throughout the spring-time sampling period (mean ± SEM; time-related changes of pregnants: bars signed with diverse letters represent significantly different values: $^{a\text{-}b}P<0.001$; $^{c\text{-}d}P<0.05$, with single trait ANOVA; *Exp. 2*)



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 interrelationship remained existing throughout the study in non-pregnants only (n=32; r= +0.438; P<0.001). No correlation was found, however, between leptin and body weight. The d 0 level of leptin was higher in ewes became pregnant (n=24) than in those remained non-pregnant (n=32) (*Fig. 5.2.2.*), but the mean of body

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[29] Folligon inj.™ (Intervet International B.V., Angers, France; i.m.)

weight in these two groups did not differ (data not shown). By d 41 the plasma leptin of pregnants had doubled, it showed a moderate further increase on d 81, and decreased slightly thereafter (*Fig. 5.2.2.*). The plasma insulin levels did not show significant gestation-related changes, and during pregnancy significant correlation was not found between these two hormones (details are not given).

Fig. 5.2.3. The plasma progesterone (P_4) and fecal progesterone metabolite (P_4-met) levels of pregnant ewes bearing single vs. twin and multiple (3-5) fetuses (mean ± SEM; pregnancy-related changes in plasma P_4 and P_4-met: P=0.044 and 0.059, respectively; with repeated measures ANOVA; *Exp. 2*)

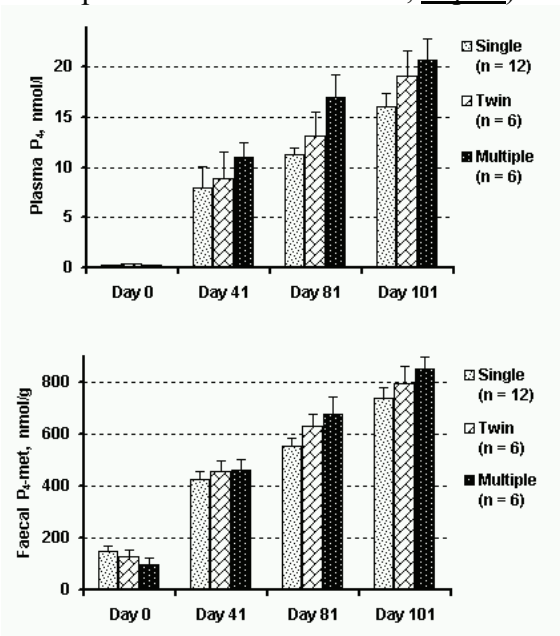
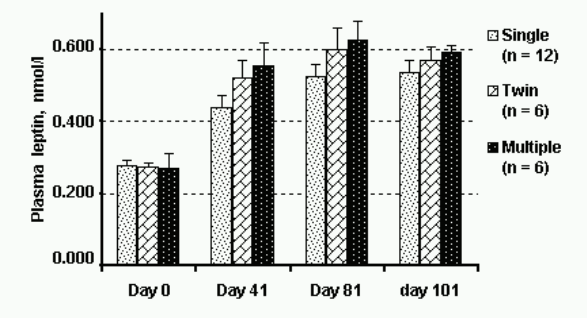


Fig. 5.2.4. The plasma leptin levels of pregnant ewes bearing single vs. twin and multiple (3-5) fetuses (mean ± SEM; pregnancy-related changes: P=0.042; with repeated measures ANOVA; *Exp. 2*)



The mean levels of P_4, P_4-met and leptin were slightly lower in ewes bearing single fetus than those with 2 or 3-5 fetuses (*Fig. 5.2.3.* and *Fig. 5.2.4.*). According to the number of fetuses the repeated measures ANOVA proved significant differences in P_4 and leptin, but not in P_4-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 P_4 ($r = +0.353$, $+0.389$ and $+0.329$, respectively) and P_4-met ($r= +0.297$, $+0.305$ and $+0.303$, respectively).

Discussion

Pregnancy provides a long-lasting exposure to various hormones for the dam. In ewes the corpus luteum (CL) is the exclusive source of P_4 during the first 50 d. Placental enzymes (Δ^{4-5}isomerase and 3β-ol-dehydrogenase) converting pregnenolone to P_4 activate on d 50 to 60, and thereafter this hormone comes mainly from the placenta. In general the greater the amount of placental tissues (as seen in twin or multiple pregnancies), the more P_4 is produced. However, this correlation is not close, and plasma P_4 in ewes bearing single, twin and multiple fetuses may overlap. Placental estrogen production starts to increase around d 70-80 after conception, but really high levels are determined only in the last 3-4 weeks of gestation (*Edqvist* and *Forsberg*, 1997; *Senger*, 2003). During the ovine pregnancy distinct alteration is seen also in function of adipo-insular axis. As usual in non-lactating ruminants, the concentration of insulin is relatively high (*Prior* and *Christenson*, 1978; *Harmon*, 1992; *Petterson* et al., 1993), while insulin resistance develops, which is reflected by decreased ability of adipocytes and skeletal muscle to utilize glucose (*Ehrhardt* et al., 2001). Also considerable hiperleptinaemia typical of pregnancy occurs in this species (*Ehrhardt* et al., 2001; *Thomas* et al., 2001; see also in Chapter 3.6.); mainly due to a 2.3 fold increase in leptin mRNA content of adipocytes. Its degree is unrelated to the energy balance of the dam (*Ehrhardt* et al., 2001). During the pregnancy-associated hyperleptinaemia a decrease in hypothalamic leptin sensitivity was confirmed in lab rodents (*Waddell* and *Smith*, 2003). These alterations in levels of and sensitivity to insulin and leptin are involved in the maternal metabolic adaptation to pregnancy, controlling the partition and expenditure of energy sources and body reserves (*Ehrhardt* et al., 2001).

According to the experiences in lab rodents and Primates (*Ashworth* et al., 2000; *Christou* et al., 2003; *Henson* and *Castracane*, 2003; *Islami* and *Bischof*, 2003; *Waddell* and *Smith*, 2003; *Yoon* et al., 2005; *Zieba* et al., 2005), this metabolic effect of maternal leptin is completed by the local intrauterine activity of leptin - LR system, which may be an important regulator of implantation, as well as of the placental and fetal growth and development. In human fetuses adiposity is an important factor influencing plasma leptin concentration. Plasma leptin remains static until week 33 of gestation, and then it increases rapidly parallel

with the rate of fat deposition. Plasma leptin is already present in the plasma of ovine fetuses by 40 day, but in contrast to humans it increases at a very slow rate during the fetal life (*Ehrhardt* et al., 2002). In fetal sheep leptin mRNA abundance in perirenal adipose tissue increases with gestational age toward term (*Yuen* et al., 1999; *Thomas* et al., 2001; *Forehead* et al., 2002; *Yuen* et al., 2004). These differences may be attributed to the much lower body fat content of fetal sheep, particularly near term. Absence of adipose depots in fetal sheep before 70 day of pregnancy implies that plasma leptin originates from other tissues (fetal brain and liver) during early-mid pregnancy. The ontogenic changes in plasma leptin and tissue mRNA levels coincide with the rise in plasma cortisol concentration seen in the fetus near term. On d 138 the fetal (but not the maternal) plasma leptin concentrations are inversely related to the uterine blood flow and fetal and placental weight, proving the involvement of this hormone in an adaptive response to intrauterine growth restriction (*Buchbinder* et al., 2001). These data suggest that the ovine fetus cannot contribute meaningful amounts of leptin to the maternal circulation.

Our samples taken during pregnancy in this study represented the last days of CL-dependent stage (d 41), and the early and middle period of placenta-dependency (d 81 and 101). On d 81 and 101 also the estrogen concentrations may be elevated, but not very high until late pregnancy. Glucocorticoids, which can also stimulate leptin synthesis (*Chilliard* et al., 2005; see also Chapter 3.4.), are not increased in the ovine plasma until just before term. Similarly, increase in the plasma level of prolactin and placental lactogen does not occur until late pregnancy (*Edqvist* and *Forsberg*, 1997; *Senger*, 2003). So only the P_4, P_4-met and insulin were determined in the current experiment.

In accordance with our expectation the P_4 and P_4-met levels were continuously elevated throughout the pregnancy, and they related positively to the number of fetuses. The relevance of this tendency was reflected by its interrelation with plasma leptin. The production rate of gestagens relates positively both to plasma P_4 and fecal P_4-met contents in ruminants (*Rabiee* et al., 2002). However, the plasma P_4 showed closer correlation with leptin than fecal P_4-met in this study, perhaps due to the variability in dry matter content of fecal samples. As discussed in Chapter 5.1, in lab rodents and Primates the testosterone suppresses, whereas E_2, P_4 or their combination can stimulate the leptin production. However, the plasma levels of E_2 and P_4 are more elevated in *Primates* than in ruminants. Our results suggest that the pregnancy-

associated long-term increase of gestagens may be one of the factors enhancing the leptin production in pregnant ewes.

The plasma insulin levels were relatively high in the d 0 samples, and comparing to the initial values no further changes were seen throughout the gestation. The insulin and leptin concentrations correlated only before AI, and in non-pregnant animals, but not during pregnancy, which confirms the finding of *Ehrhardt* et al. (2001) that in ewes the gestation-challenged increase in leptin production is not related to changes in insulin or whole-body glucose response to insulin.

Body condition relates well with plasma leptin in ewes (r=+0.68; *Delavaud* et al., 2000). However, lack of significant correlation was observed between the body weight and plasma leptin of dams in this study, obviously due to the pregnancy-associated leptin resistance, and the age-related variability in size of our animals. Confirming this idea the animals conceived at AI had higher plasma leptin than those not conceived, which suggests that ewes in better condition can become pregnant more easily at the end or out of the breeding season. We think that the body weight was not enough sensitive parameter to detect this tendency: future studies using more precise parameters of body fat content should be required to prove this hypothesis.

As a summary of these results we think that although the degree of pregnancy-associated hyperleptinaemia is affected by number of fetuses and level of P_4 in ewes, but pregnancy stage is a more important regulator than these additional factors.

5.3. Plasma leptin levels of normo- and hyperketonaemic late-pregnant ewes (*Exp. 3*)

There are several hormonal components in pathogenesis of hyperketonaemia and ketosis in postpartum dairy cattle. Lower rate of insulin to glucagon and GH is proved to act as the main endocrine factor (*Sartin* et al., 1988), which is completed with depressed circulating concentrations of IGF-I, T_4 and T_3, whereas the plasma level of the inactivated thyroid hormone formation (rT_3) increases (*Huszenicza et al., 2006*; see also in Chapter 5.5.). These endocrine alterations reflect the current severity of NEB (*Pethes* et al., 1985; *Ronge* et al., 1988), and have a crucial role in pathomechanism of NEB/ketosis-related ovarian malfunctions in dairy cattle (*Butler*, 2000 and 2003; *Diskin* et al., 2003).

A related metabolic disorder (ovine ketosis; syn. gestational or pregnancy toxaemia) is known to occur 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. Reduced capacity of dry matter intake resulted from the physically compressed rumen, consumption of low energy containing daily ration, and/or poor utilization of the available energy sources may induce the depletion of intrahepatic glycogen store, with unstable blood glucose levels, a rapid mobilization of fat reserves, and a concomitant increase in ketone production (*Pastor et al., 2001*). 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 (*Harmon*, 1990; *Henze* et al., 1998; *Van Saun*, 2000): there have not been available data yet showing how this pregnancy-associated hyperleptinaemia of ewes interacts with hyperketonaemia / gestational toxaemia. Furthermore, the subsequent reproductive performance of survivals has not been studied yet, either.

Leptin acts central role in regulation of energy metabolism (see also Chapter 3.5.2.), so both in cattle and sheep its involvement may be hypothesized, but has not been clearly proved in pathogenesis of ketosis.

The aims of this trial were to study (i) the endocrine alterations – including the supposed changes in plasma leptin level – in gestational toxaemia of spring-lambing Merino ewes kept under intensive large-scale farm conditions, and (ii) the reproductive consequences of this disease when the affected animals receive a gestagen + eCG based treatment regime to induce their ovarian cyclicity soon after weaning, out of the breeding season.

Materials and methods

Farm conditions. Case history

Using the opportunity of a natural incidence of gestational toxemia this trial was carried out in a Merino flock, where – as a regular part of the breeding technology – hormonal procedures were frequently used for synchronization of estruses in breeding season, and for induction of ovarian cyclicity out of the breeding season. In the proceeding summer ewes (parity: 1 to 4; n=121) were kept on a riverside pastureland with abundant grass production. In the second half of August 300 g/day/animal concentrate was also fed. At the end of August the ovar-

ian cyclicity was synchronized with a gestagen + eCG treatment[30] Ewes in estrus were inseminated (AI) once with fresh diluted semen 48 hours after gestagen removal. Thereafter, rams were introduced into the flock to breed the ewes coming into estrus later. After the grazing season the animals were fed *ad libitum* with grass hay. From the day 105 of pregnancy 350 g/day/animal concentrate was given, and this ratio was planned to continue till the end of pregnancy. Unfortunately, however, the further feeding of concentrate was ceased at day 125 of gestation, because of the owner's unexpected temporary financial difficulty. Between days 136 and 139 of pregnancy, three ewes suddenly died after a short faintness. At that time the owner asked for our assistance to diagnose this disease and to prevent the further losses.

Examinations during late pregnancy

At the inspection of the flock on day 140 after AI another cadaver was found. At necropsy its liver showed the signs of excessive fatty infiltration, and using a dip-and-read strip test[31] large quantity of ketone bodies was detected in the urine. In the uterus there were three well-developed, but already dead fetuses. The anamnesis and these observations raised the suspicion of gestational toxaemia. The owner requested us to monitor the ketone status of the whole flock, in order to select and treat the affected individuals. So while the immediate restart of the concentrate feeding was recommended, on the same day (e.g. day 140 of pregnancy) blood samples were taken. Plasma BHB content was determined within 48 hours. All ewes with BHB level ≥1.60 mmol/l were considered to suffer from the subclinical form of ketosis (*Henze* et al., 1998), and on day 143 of pregnancy the animals were started to receive a 3-day-long peroral antiketogenic treatment (20 g Propylene glycol + 16 g Sodium propionate + 1 g Nicotinic acid amide + 8 g Choline chloride per day)[32]. In addition to this, within the framework of our research program later we analyzed the plasma activity of aspartate-aminotransferase (AST), as well as the levels of certain metabolites (glucose, NEFA, and total cholesterol, TCH) and metabolic hormones (insulin, T_4, T_3, cortisol, IGF-I and leptin). At lambing the viability and number of newborn lambs were recorded.

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30 14-day-long gestagen (vaginal sponge of 40 mg Cronolone; Chrono-gest™, Intervet International B.V., Angers, France) plus 500 IU eCG (Folligon inj.™, Intervet International B.V., Angers, France; i.m., simultaneously with the gestagen removal) administration.

31 Ketostix test™ (Ames Company, Division of Miles Laboratories Limited, Stoke Poges, England)

32 Energa Veyxol solution™, Veyx-Pharma GmbH, Schwarzenborn, Germany)

Farming conditions after lambing

After parturition the lambs were allowed to suckle their mothers, and the ewes were milked for 72-75 days. During the lactation period the dams received grass hay *ad libitum*, which was completed with 450-500 g/day/animal concentrate. When the weather allowed it the ewes were let to the pasture again. However, through the evening and morning hours grass hay was given *ad libitum*. The lambs were weaned at 72-75 days of age, and the dams were dried off simultaneously.

Induction of cyclic ovarian function. Checking the metabolism and ovarian response

On day 78-80 postpartum (at the beginning of May) all ewes received a gestagen + eCG treatment[33] to induce their cyclic ovarian function again. They were inseminated at the same time and way as previously in the autumn period, regardless of the presence of visible estrus. At the time of AI (on day 92-94 after lambing), and 10 days later again, blood samples were collected from each ewe. The same metabolites and hormones were determined from the samples taken at AI as previously on day 140 of pregnancy. In order to monitor the ovarian response to the cycle-inducing treatment the P_4 content was also assayed (in both samples). We considered as a proof of ovulation and successful cycle induction if the P_4 increased from the base-line level at AI to =3.20 nmol/l detecting luteal activity 10 days later. Also the data on the next lambing were collected in the subsequent autumn period.

Results

Another pregnant ewe died before starting the antiketogenic treatment. After that, however, no further animal died. Altogether, 116 dams lambed, 102 of them on days 149 to 152 after the AI. Each newborn lamb was healthy. The 14 ewes lambed 14-18 days later than the others, indicating that they had conceived from a natural mating by rams, rather than at the AI, were not considered thereafter. The 102 dams conceiving at the AI delivered single lamb (n=41), twins (n=57) or triplets (n=4). Hereafter, we introduced and evaluated the metabolic and reproductive data of the 98 ewes which conceived at the AI and delivered and suckled one or two lambs.

[33] 14-day-long gestagen (vaginal sponge of 30 mg Cronolone; Chrono-gest™, Intervet International B.V., Angers, France) plus 500 IU eCG (Folligon inj.™, Intervet International B.V., Angers, France; i.m., simultaneously with the gestagen removal) administration.

On day 140 of gestation the plasma BHB level varied between 0.27 and 1.32 mmol/l (single pregnancy) and 0.35-3.65 mmol/l (twin pregnancy). The group mean of BHB was more than twice higher in the twin-bearing ewes than in those with single pregnancy (1.62±1.37 mmol/l vs. 0.78±0.36 mmol/l; P<0.001). According to the suggested threshold (BHB: ≥1.60 mmol/l; *Henze* et al., 1998), almost half of the twin-bearing ewes (n=27= 47 %), but none of those with single pregnancy were considered to have hyperketonaemia-related elevation in plasma BHB. Slightly more than half of hyperketonaemic animals (n=15) were also hypoglycaemic (2.37-2.99 mmol/l) simultaneously. In the others the plasma glucose content was within the normal range (3.00-3.5 mmol/l; n=8), or increased at a negligible degree (3.51-3.77 mmol/l; n=4).

Table 5.3.1. The metabolic and endocrine characteristics of ewes with single and twin pregnancies on day 140 of gestation (*Exp. 3*).

	Ewes with single pregnancy (n=41)	Ewes with twin pregnancy	
		BHB on day 140 of gestation	
		<1.60 mmol/l (n=30)	≥1.60 mmol/l (n=27)
	mean ± SEM		
On day 140 of pregnancy:			
Glucose, mmol/l	3.38 ± 0.10	3.19 ± 0.14	2.88 ± 0.31
NEFA, mmol/l	0.27 ± 0.02 [a]	0.31 ± 0.03 [a]	0.44 ± 0.04 [b]
AST, IU/l	87 ± 4 [a]	95 ± 6 [a]	131 ± 14 [b]
TCH, mmol/l	2.18 ± 0.07 [a]	2.21 ± 0.12 [a]	1.78 ± 0.14 [b]
Insulin, pmol/l	112 ± 9 [a]	91 ± 12 [a]	49 ± 8 [b]
T_4, nmol/l	75.1 ± 4.5	69.3 ± 5.7	61.1 ± 6.0
T_3, nmol/l	1.14 ± 0.06 [a]	1.07 ± 0.07 [a]	0.87 ± 0.08 [b]
Cortisol, nmol/l	35.1 ± 3.98 [a]	37.3 ± 4.93 [a]	59.4 ± 7.54 [b]
IGF-I, nmol/l	2.26 ± 0.09 [a]	2.01 ± 0.10 [a]	1.18 ± 0.18 [b]

[a - b] P<0.05

There were no essential differences in the metabolic and endocrine characteristics between the single-lambing and twin-lambing normoketonaemic ewes (BHB: <1.60 mmol/l; n=30), while their twin-bearing hyperketonaemic flock mates had a remarkably higher AST activity, as well as higher NEFA and cortisol levels, and significantly lower insulin, T_3 and IGF-I concentrations (*Table 5.3.1.*). 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 (*Fig. 5.3.1.*), 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, T_3 and BHB (r = +0.355, +0.327, +0.305 and -0.336, respectively; P<0.05 to 0.01).

These ketosis-related metabolic differences had fully disappeared by the time of the induced estrus and AI on day 92-94 after lambing (data not shown). At that time 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 (*Fig. 5.3.1.*). 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).

Fig. 5.3.1. The plasma leptin levels in ewes with single pregnancy and in their twin-bearing flock mates with normoketonaemia (BHB: <1.60 mmol/l) or hyperketonaemia (BHB: ≥1.60 mmol/l) (mean ± SEM; *Exp. 3*).)

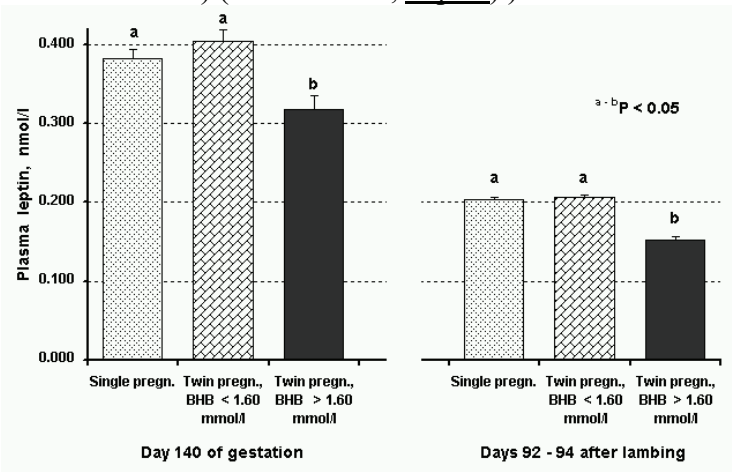
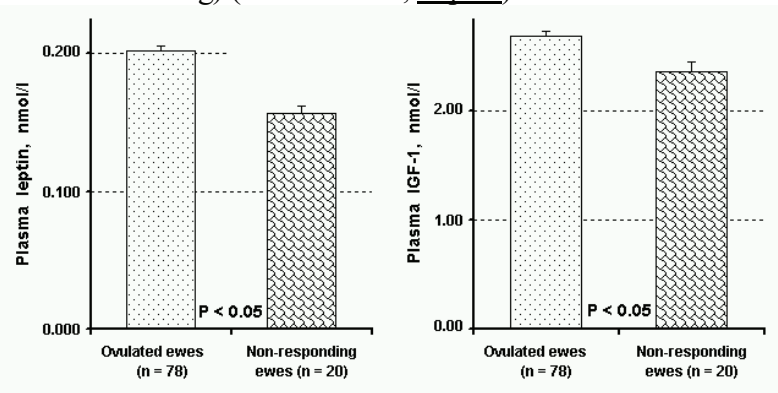


Table 5.3.2. The ovarian response and reproductive performance of ewes following a standard Chronolon + eCG cycle inducing protocol started on day 78-80 after lambing (*Exp. 3*).

	Ewes with single pregnancy (n=41)	Ewes with twin pregnancy		P<
		BHB on day 140 of gestation:		
		<1.60 mmol/l (n=30)	≥1.60 mmol/l (n=27)	
	n (%)			
Following the induction of cyclicity:				
Ewes ovulated	90.2 % (n=37)	83.3 % (n=25)	59.3 % (n=16)	0.01
Ewes re-conceived	85.4% (n=35)	80.0 % (n=24)	55.6 % (n=15)	0.05
of them: twin pregnancies	60.0 % of pregnancies (n=21)	41.7 % of pregnancies (n=10)	26.7 % of pregnancies (n=4)	(0.1)

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 (*Table 5.3.2.*). The leptin and IGF-I levels of non-responders (n=20) were lower than of those ovulated (n=78) (*Fig. 5.3.2.*).

Fig. 5.3.2. The plasma leptin and IGF-I levels in ewes ovulated after a cycle-inducing Chronolone + eCG administration, and in those not responding to this treatment (day 92-94 after lambing) (mean ± SEM; *Exp. 3*).



Discussion

Within the framework of this trial the endocrine characteristics and suspected reproductive consequences of ovine ketosis were studied in a commercial mutton producing flock with intensive breeding technology. Concerning the plasma glucose, BHB, TCH, insulin and cortisol levels, as well as the AST activity found in late pregnant normo- and hyperketonaemic ewes our results are comparable to the data of *Henze* et al. (1998), *Van Saun* (2000) and *Pastor* et al. (2001). To the best of our knowledge, however, neither the ketosis-related alterations in IGF-I, thyroid hormone and leptin levels, nor the late ovarian consequences of this disease have been examined in sheep previously. No data were found in our experiment suggesting why hyperketonaemia developed in one part of twin-pregnant dams, but not in the others.

The metabolic and endocrine properties of normoketonaemic twin-bearing animals were close to those of dams with single pregnancy, but obviously differed from those found in hyperketonaemic individuals. In our hyperketonaemic ewes the plasma levels of constituents related to the energy metabolism (e.g. BHB, NEFA), as well as of the most of hormones regulating the energy homeostasis (e.g. insulin, IGF-I, T_4 and T_3) were similar to those determined

in feed-restricted (*Agenäs* et al., 2003; *Chelikani* et al., 2004) or high-producing (*Wensing* et al., 1997) postpartum dairy cows, and reflected NEB-related tendencies (*Ronge* et al., 1988; *Chilliard* et al., 1998; *Veerkamp* et al., 2003). In accordance with the experiences of *Henze* et al. (1998) these findings reinforce the supposed role of lower than normal secretory capacity of pancreatic β-cells and/or the low level of insulin in plasma, as primary endocrine factors in the pathogenesis of pregnancy toxaemia. As observed in cattle (*Ronge* et al., 1988; *Diskin* et al., 2003), the decreased concentration of IGF-I may reflect the NEB also in late pregnant hyperketonaemic ewes. In response to feed deprivation low insulin and IGF-I levels were seen also in pregnant beef cows (*Lents* et al., 2005) and postpartum dairy cows (*Agenäs* et al., 2003; *Chelikani* et al., 2004). The low plasma levels of thyroid hormones can be explained by the increased degree of their inactivation (inner-ring deiodation) in peripheral tissues, and/or alternatively by the decreased capacity of T_4 activation (namely the outer-ring deiodation of T_4), diminishing the transformation of T_4 to T_3 (*Pethes* et al. 1985; *Ronge* et al., 1988; *Huszenicza* et al., 2006; *Lents* et al., 2005). As reviewed earlier (*Huszenicza* et al., 2002), these changes represent important mechanisms in adaptation to NEB.

The temporary hyperleptinaemia resulted from a gestation-induced 2.3 fold increase in leptin mRNA content of adipocytes is one of the species-based characteristics of ovine pregnancy (*Ehrhardt* et al., 2001; *Thomas* et al., 2001; see also in Chapters 3.5.3. and 5.2.). Its actual degree is unrelated to the energy balance of the dam (*Ehrhardt* et al., 2001), but it is associated with the stage of gestation (*Ehrhardt* et al., 2001; *Thomas* et al., 2001), and – as seen in Chapter 5.2. – also with the number of fetuses. In dairy cows the peri-parturient leptin levels were the highest during the late pregnancy and declined by about 50 % after calving. The plasma leptin concentration remained depressed during early lactation despite a gradual improvement in energy balance. This postpartum reduction in circulating leptin was the obvious consequence of NEB, because leptin remained high in cows not milked at that time. Plasma leptin was positively correlated with concentrations of insulin and glucose, while related negatively to GH and NEFA (*Block et al., 2001*). Similar peri-parturient decrease was not seen in ewes (*McFadin* et al., 2002), but sampling was started relatively late at that study, only one week before the expected time of lambing. *Ehrhardt* et al. (2001) could detect almost the same concentration of plasma leptin on 15-22 days after lambing, as 20 to 40 days before breeding, and this level was significantly lower than those found in mid and late pregnancy. In

accordance with the earlier observations (*Ehrhardt* et al., 2001; own data in Chapter 5.2.), in the current study the level of leptin was much higher on d 140 of gestation then that detected on d 92-94 after lambing. However, the mean of leptin content was lower in the twin-bearing hyperketonaemic dams than in the others, demonstrating the combined effects of NEB and gestation on leptin production. Certain undefined gestation-related factors are known to increase the abundance of leptin mRNA in adipocytes of pregnant ewes (*Ehrhardt* et al., 2001). These factors are supposed to override the effect of NEB on circulating level of leptin in ketotic dams for a while. The situation seems to be different in late pregnant dairy cows in positive energy balance, where feeding level clearly interacts with pregnancy stage on plasma leptin level (*Holtenius* et al., 2003). The actual feeding level (rather than body fat content or stage of gestation) was the primary factor determining the plasma leptin concentration also in feed-restricted pregnant beef cows (*Lents* et al., 2005). All the ketosis-induced metabolic differences completely disappeared during lactation. The significantly lower plasma leptin level of formerly hyperketonaemic ewes was the only exception existing at the time of the induced estrus and AI on day 92-94 after lambing, perhaps due to a longer and more intensive loss of whole-body lipid reserves presumed in these animals. Unfitness of the feeding system to the intensive breeding technology could also be responsible for these prolonged differences.

In this study fewer formerly hyperketonaemic than normoketonaemic ewes responded with ovulation to a standard cycle-inducing procedure, and those ovulated after this treatment were characterized by somewhat higher leptin and IGF-I levels than the non-responding individuals. The same tendency with the advantage of normoketonaemic dams was observed also in the rate of re-conceived animals. Of course the direct effects of lower leptin and IGF-I levels on hypothalamic-anterior pituitary GnRH/LH secretion and/or on follicular growth and maturation (*Spicer* et al., 1993; *Spicer* and *Stewart*, 1996; *Zieba* et al., 2005) can not be completely excluded. However, it could be hypothesized that these tendencies in leptin and IGF-I reflect only the smaller cell size and triglyceride content of adipocytes, and the poorer body condition, rather than they are involved as direct causative agents in pathophysiology of the poorer reproductive performance. In other respects, a hyperketonaemia-related impairment of follicular development is supposed to be a more likely reason of the poor ovarian response. The time needed for development of primordial follicular structures into pre-ovulatory follicles is longer in sheep than in cattle, and it takes approximately 180 days (*Cahill* and

Mauleon, 1980; *Monniaux* et al., 1997a and 1997b). So in the current study the dominant follicles expected to yield fully competent oocytes following the induction of cyclicity on day 92-94 after lambing started to develop before delivery. Therefore in the affected dams the developing follicles were exposed to all the hyperketonaemia-related metabolic and endocrine changes. The long-lasting effects of NEB and its decompensation (ketosis, fatty liver) on intrafollicular steroidogenic capacity and oocyte quality are well-documented in high-producing dairy cows (*Wensing* et al., 1997; *Kruip* et al., 1999; *O'Callaghan* and *Boland*, 1999; *Gwazdauskas* et al., 2000; *Walters* et al., 2002; *Vanholder* et al., 2005 and 2006). So, the poor reproductive performance of our formerly hyperketonaemic ewes could be based mainly on an analogue follicular malfunction. Further studies are needed, however, to prove this hypothesis. These reproductive consequences of pregnancy toxaemia could be recognized in this study, because our ewes lambed in winter time, and their ovarian cyclicity was attempted to be induced just at the end of lactation, in animals out of the breeding season. By the time of the traditional breeding season in autumn these impairments would have been eliminated obviously. Of course it can not be stated that the gestational toxaemia in sheep acts a nearly similar role in the possible reasons of reproductive disorders as the NEB and its consequences in postpartum dairy cows. However, occasionally we have to count with this form of diminished reproductive performance also in ewes targeted by intensive breeding technology. It might have particular importance in dairy flocks.

As concluding remarks of this study the subclinical form of ovine ketosis is characterized by complex endocrine alterations, reflecting NEB. However, in late pregnant ketotic ewes the leptin level seems to be influenced also by leptin resistance, and certain further NEB-unrelated factors, which remained undefined in this trial. If some weeks after lambing the ovarian cyclicity is induced out of the breeding season, the plasma leptin level, ovarian response and fertility of formerly ketotic ewes may be depressed.

5.4. Plasma leptin and ovarian function in postpartum dairy cows (*Exp. 4a*, *Exp. 4b*)

Genetic selection for milk production during the last decades has been associated with decreased reproductive efficiency in dairy cows (*Lucy*, 2001). However, reduced fertility is not a direct consequence of the high genetic merit (*Veerkamp* et al., 2003; *Wathes* et al.,

2003 and 2007): also a high-producing dairy cow can re-conceive at a reasonable time (day 50-60 to 100-120) after calving, if – not regarding the andrological aspects currently – (i) the dam ovulates from one of the first 3-4 waves of follicle growth, her ovarian function becomes cyclic, and she shows estrus thereafter, (ii) her uterine involution is finished with a complete histological regeneration of the endometrium, (iii) she is capable of ovulating a fully competent oocyte, furthermore (iv) after AI and fertilization the endocrine and metabolic conditions provide a proper intratubal/intrauterine environment for the developing embryo (recently reviewed by *Huszenicza*, 2003; *Huszenicza* et al., 2003).

The first postpartum ovulation and resumption of ovarian cyclicity is a milestone on the way to re-conception, which is closely related to the NEB in this period: the time to the beginning of the recovery of energy balance is positively correlated with the time to first ovulation (*Butler* et al. 1981; *Beam* and *Butler*, 1999). *Butler* and *Smith* (1989) found that cows that lost less than 0.5 units of body condition score (BCS) during the first 5 weeks postpartum had higher conception rates at the first service than cows that lost more than 0.5 BCS. 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 GH - IGF-I system, insulin, thyroid hormones and leptin (*Blache* et al., 2007). *Spicer* et al. (1990) were the first in proposing that the effect of NEB on the time of first ovulation may be mediated by the IGF-I. Although GH concentrations are usually high in early-lactating ruminants, the intrahepatic production of its mediator IGF-I is diminished (*Chilliard*, 1999). Circulating concentrations of IGF-I in the peri-partum period were good indicators of the capacity of energy-restricted cows to resume cyclicity after parturition (*Roberts* et al. 1997). Cows with ovulatory estrogen-active (E_2-producing) follicles had higher circulating IGF-I concentrations during the first 2 weeks than cows with anovulatory follicles (*Beam* and *Butler* 1997, 1998). Both the insulin and IGF-I are known to stimulate the *in vitro* steroidogenesis and proliferation of bovine thecal and granulosal cell cultures (*Spicer* et al. 1993; *Spicer* and *Stewart* 1996). Likewise, cows that ovulated within 35 days postpartum presented higher IGF-I concentrations as well as higher glucose and insulin and lower NEFA and BHB concentrations (*Huszenicza* et al., 2001). Cows in postpartum NEB have also low concentrations of thyroid hormones (*Pethes* et al. 1985; *Capuco* et al. 2001). A role of these hormones in

regulating steroidogenesis has been reported (*Spicer* 2001), but data regarding their effect on ovarian function *in vivo* is limited and controversial (*Huszenicza* et al. 2002).

As it was discussed earlier (Chapters 3.3. and 3.5.), leptin acts as an energy reserve signal for the hypothalamic regions that control feeding behavior, metabolism and endocrine function, so as to maintain energy homeostasis, and its plasma concentrations vary with changes in body weight and percentage of body fat (*Delavaud* et al. 2002). Dairy cows often lose over 60 % of their body fat during early lactation (*Tamminga* et al. 1997; *Chilliard* 1999), and it was shown that leptin concentration declined shortly before parturition (*Kadokawa* et al. 2000; *Block* et al. 2001; *Liefers* et al. 2003). Less agreement is on leptin concentrations after parturition: increasing (*Kadokawa* et al. 2000), unchanged (*Huszenicza* et al. 2001), diminished (*Block* et al. 2001; *Holtenius* et al. 2003), or a transient increase (*Liefers* et al. 2003) have been reported. The postpartum leptin reduction is likely to be due in part to the NEB because plasma leptin remained high in cows not milked after parturition (*Block* et al. 2001). There are only few reports on the relation between leptin concentrations and resumption of cyclic ovarian activity during the postpartum period (*Kadokawa* et al. 2000; *Huszenicza* et al. 2001; *Liefers* et al. 2003; *Kadokawa* et al. 2006). The interval from parturition to first ovulation correlated significantly with the interval from parturition to leptin nadir, but was not correlated with the prepartum, pre- and/or postovulatory leptin values (*Kadokawa et al. 2000*). *Liefers* et al. (2003) found that although there was lack of relationship between leptin and first postpartum luteal activity, higher leptin concentrations were associated with shorter intervals to first observed oestrus. However, the supposed altering effects of inflammatory diseases with intensive endotoxin / cytokine release (*puerperal metritis, mastitis*; see also in Chapters 3.8. and 5.7.) were not taken into consideration in these early field studies.

The objective of *Exp. 4a* was (i) to characterize the insulin, IGF-I, thyroid hormones and leptin concentrations along with BHB and NEFA profiles in healthy dairy cows. In addition, (ii) the effect of parity and BCS at parturition on endocrine and metabolite patterns and reproductive parameters were determined. Furthermore, in *Exp. 4b* we wished to compare the plasma levels of these metabolic hormones and metabolites in cows with already *cyclic* vs. still *acyclic* ovarian function at the desirable time of the first postpartum AI.

Materials and methods

Exp. 4a

Holstein Friesian cows with 2 to 5 parturitions (parity: ≥2, multiparous cows; n=23) and cows without previous parturitions (parity: 1, primiparous cows; n=24) (average 305 day milk yield: 6000 and 4800 kg respectively) calving in the autumn period of the southern hemisphere (April) were selected from a dairy herd (experimental farm of the Agronomy College; Paysandú, Uruguay). Animals were grazing on improved pastures (mixture of grasses and legumes) and three weeks before parturition they were offered a diet that consisted in a mixture of 12 kg of corn silage and 4 kg of a commercial concentrate (14 % CP, 7.11 MJ NEl/kg) which were given once a day, and bales of *Setaria Itálica*, *ad libitum*. After parturition cows had access to a daily strip of pasture (mixture of grasses and legumes), 15 kg (fresh basis) of corn silage (33 % DM, 6.8 % CP) and 6 kg DM of a commercial concentrate (17 % CP, 1.7 MCal Nel/kg). The pasture sward mass (1650 ± 230 kg DM) was estimated with a comparative yield method adapted from *Haydock* and *Shaw* (1975), and an allowance of 15 to 18 kg of DM per cow per day was offered through weekly adjustments of the daily strip size. The cow had access to the grazing plot between the morning and the afternoon milking. The corn silage was fed in the afternoon (after milking), and the concentrate equally distributed during milking time (twice a day). Cows were milked twice a day and milk production was measured every 15 days. BCS was determined every 15 days by the same person from 2 months before until the third month after parturition. At the same time, body weight (BW) was determined. 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)[34]. Accordingly, terms of LEAN and FAT cows were used thereafter in this study. Estrus was detected twice a day, and animals were inseminated 12 h after heat detection (voluntary waiting period = 50 days). Reproduction was seasonally with a breeding period of 5 months (from May to September). Pregnancy diagnosis was performed by rectal palpation 45 days after artificial insemination. Blood samples were obtained three times a week, from around 30 days prepartum to 60 days postpartum. The reinitiation of ovarian cyclicity was determined by P_4 concentrations in plasma 3 times per week. Reproductive parameters measured were days to first ovulation, intervals from parturition to

first service and to conception. Samples taken every 10 days from one month before to two months after parturition were assayed for metabolites (NEFA, BHB)[35] and metabolic hormones.

In this study the milk production, BCS, metabolites and hormonal concentrations were analyzed by mixed procedure (Statistical Analysis System, SAS Institute Inc., Cary, NC, USA 2000). The statistical model included the effects of parity (categories: primiparous=L1 or multiparous=L2), BCS at parturition (BCS <3.00 or ≥3.00), days including pre and postpartum periods (linear and quadratic functions) and interactions. The covariance structure was autoregressive order 1 and cow within parity*BCS at parturition was set as random effect. Functions were calculated for each dependent variable and differences in the estimates of the curves were analyzed according to parity and BCS at parturition. Postpartum days were categorized in intervals of 10 days during the experimental period (day 0 = day of parturition) and data are presented in graphs as group means ± pooled standard error. To study reproductive parameters a general linear model was used and the fixed effects were parity and BCS at parturition. Tukey-Kramer tests were conducted to analyze differences between groups. The re-initiation of ovarian cyclicity was defined as the day when P_4 increased from basal concentrations in two consecutive samples of >1.60 nmol/l or one sample of >3.20 nmol/l. If no detectable P_4 was observed until day ≈70 postpartum, the last day of sampling was arbitrarily considered as the initiation of ovarian cyclicity for that cow. Correlation coefficients were calculated to study relationships between variables (Proc Corr, SAS). Factors affecting the re-initiation of ovarian cyclicity were evaluated by regression analysis using a backwards elimination procedure (initial inclusion of all independent variables and sequential elimination of the variables with P >0.10 using the regression procedure of SAS) to determine those with P <0.10. The dependent variable was the re-initiation of ovarian cyclicity and the independent variables included were parity, BCS at parturition, BW, actual BCS, milk production, NEFA, BHB, insulin, T_3, T_4, IGF-I and leptin. The last observation before the re-initiation of ovarian cyclicity in each cow was included in this study. Regression analyses were performed to study relationships between leptin and BCS, leptin and NEFA and leptin and IGF-I before and after parturition in cows with low and high BCS at parturition.

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[34] BCS of excluded animals (n=5) are not involved.

Exp. 4b

This trial was carried out in 7 Hungarian large-scale dairy herds, on 1 to 8 parity Holstein Friesian cows calving from late November, 2000 to early January, 2001 (in 5 of these 7 herds), and in February and March, 2002 (in the further 2 herds). Health condition and genitals were examined, and body condition was scored ≤72 h after calving (day 1-3), and again on days 7-10, 28-35 and 49-63. At the last examination also blood samples were collected to determine the circulating levels of some metabolites (NEFA, BHB, TCH) and metabolic hormones (insulin, IGF-I, leptin, T_3 and T_4). From day 5-8 to 49-63 milk samples were taken three times a week for analyzing the P_4 content, and according to their individual P_4 profiles, the cows not ovulating during the first ≈50 days after calving (e.g. acyclic cows) were distinguished from those with already cyclic ovarian function at that time (*Huszenicza* et al., 1987; *Huszenicza*, 2003). Cows showing general signs of puerperal metritis, mastitis (e.g. depressed feed intake, rectal temperature: >39.5 °C), and/or any other diseases (including lameness) were not involved, or were excluded.

Results

***Exp. 4a*:** *Clinical data. Milk production. Body weight and body condition score*

Data of 3 primiparous and 2 multiparous cows were excluded, due to dystocia, retained placenta and puerperal metritis (one first-parity young cow), or mastitis causing also severe general signs (depressed feed intake, rectal temperature: >39.5 °C; n=4). During the sampling period the others remained healthy. So altogether 21 - 21 cows were evaluated in both age groups. Primiparous cows produced less milk than multiparous cows during the experimental period (P<0.001, *Fig. 5.4.1*.). Milk production was affected by days postpartum (*Table 5.4.1*.). Milk production was negatively correlated with body condition and with NEFA (r=-0.35, n=165, P<0.05 and r=-0.24, n=165, P<0.05, respectively). The average 305-day milk yield was also higher in the multiparous than primiparous cows (6013±350 and 4822±213 kg, respectively; P<0.001).

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[35] Also the total protein, albumin, urea, TCH, calcium, phosphorus and magnesium contents, and AST activities were determined and evaluated. These data, however, are not shown in this dissertation (for details, see *Cavestany* et al., 2005).

Variable	n	P	BCS-C	DPP	DPP^2	P*BCS-C	DPP*P	DPP*BCS-C	DPP^2*P	DPP^2*BCS-C
BCS	328	0.07	***	***	***	0.11	***	0.11		0.08
BW	292	***	*	***	***		*	0.10		
Milk	165			***	***					
NEFA	446	0.11		***	***		***		**	
BHB	441			*	*		0.07		0.08	
Insulin	446			*	***				0.09	
T_4	446	0.10		***	***					
T_3	446	*		***	***					
IGF-I	446	***	*	***	***		**	**	***	0.09
Leptin	446	0.13	**	***	***		0.07	0.07		

Table 5.4.1. F-tests of fixed effects included in the model for measured parameters in cows under grazing conditions [fixed effects are the effects of parity (P), body condition score at parturition (BCS-C), days postpartum (linear – DPP- and quadratic functions – DPP^2) and interactions between them; *P<0.05, **P<0.01, ***P<0.001; *Exp. 4a*].

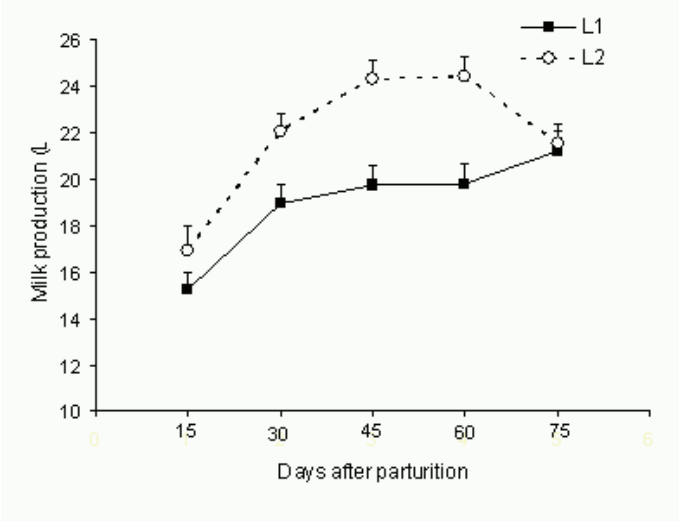


Fig. 5.4.1. Milk production of primiparous (Lactation 1, L1, n=21) and multiparous (Lactation ≥2, L2, n=21) cows under grazing conditions (mean±SEM; *Exp. 4a*).

Fig. 5.4.2. Evolution of body condition score in LEAN (L1-BCS<3 n=12) and FAT (L1-BCS≥ 3 n=9) primiparous cows, furthermore in LEAN (L2-BCS<3 n=8) and FAT (L2-BCS≥ 3 n=13) multiparous cows (mean±SEM; *Exp. 4a*).

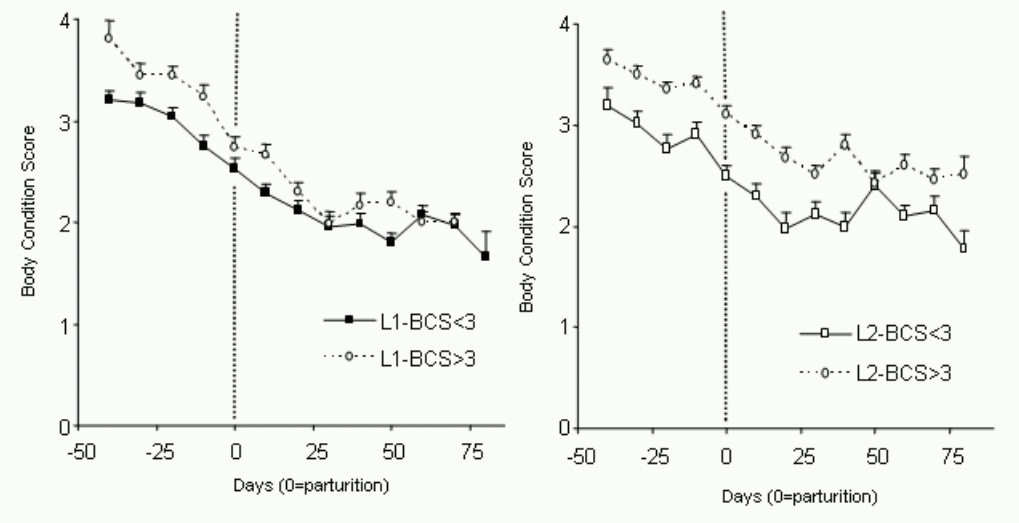


Table 5.4.2. Estimates of the functions in primiparous and multiparous cows. Only variables with significant differences according to parity are presented (*Exp. 4a*).

Variable (unit)	Intercept	DPP	DPP^2
Primiparous cows			
BCS (scale 1 – 5)	2.61^a	- 0.01861^a	0.00013^a
BW (kg)	521^a	-1.6933^a	0.009153
NEFA (mmol/l)	0.42	0.0039^a	-0.00010^a
IGF-I (nmol/l)	3.81^a	- 0.039^a	0.000884^a
Leptin (nmol/l)	0.40^a	-0.00312^x	0.0000479
Multiparous cows			
BCS (scale 1 – 5)	2.75^b	-0.01284^b	0.00008^b
BW (kg)	583^b	-1.3286^b	0.005954
NEFA (mmol/l)	0.39	0.0017^b	-0.00006^b
IGF-I (nmol/l)	4.52^b	-0.0247^b	0.000559^b
Leptin (nmol/l)	0.431^b	-0.0025^y	0.0000398

DPP: days postpartum (linear – DPP- and quadratic functions – DPP^2)

^{ab} Values with different superscripts within estimates (intercept, DPP and DPP^2) differ according to parity P<0.05.

^{xy} x vs. y differ P=0.066

The loss of BCS is shown in *Fig. 5.4.2*. LEAN cows (BCS at parturition <3.00) had less BCS during the experimental period, while FAT cows (≥3.00) tend to loose more BCS. BCS was affected by parity and days postpartum, with an interaction between both effects (*Table 5.4.1*.). Primiparous cows had a steeper decline in BCS than multiparous cows, but they recuperated faster (*Table 5.4.2*.). BCS was negatively corre-lated with NEFA and BHB (r=-0.35, n=298, P<0.05 and r=-0.17, n=295, P<0.05 respectively). Multiparous cows had more body weight than primiparous cows (576±7 vs. 510±7 kg, P=0.0001). Parity and BCS

at parturition affected BW changes during the experimental period. Statistical differences were found in the drop of BW (-1.7 vs. -1.3 kg/day in primi- vs. multiparous cows, respectively) but not on its recuperation (*Table 5.4.2*.). A close correlation between BW and BCS was found for primiparous cows (r=0.76, n=146, P<0.0001) and multiparous cows (r=0.74, n=145, P<0.0001).

Metabolites, metabolic hormones

NEFA concentrations started to increase before parturition, reached peak concentrations at day ≈14 and ≈20 after calving (in multiparous vs. primiparous cows, respectively), and began to decline thereafter (*Fig. 5.4.3*.). Surprisingly, the increase observed in NEFA level was more intensive for primiparous cows and levels remained high for a longer period (*Table 5.4.2*.). **BHB** concentrations were low at parturition, rose sharply up to day ≈10, with a slow decrease thereafter (*Fig. 5.4.3*.). NEFA and BHB levels were highly correlated (r=0.53, n=441, P < 0.001). When BHB values of >1.00 mmol/l (e.g. cows with supraphysiological levels; *Whitaker* et al., 1999) were considered, primiparous cows had more samples with these levels (P <0.05). However, BHB profiles related to subclinical ketosis (BHB >1.60 nmol/l, in at least two subsequent samples) were not observed.

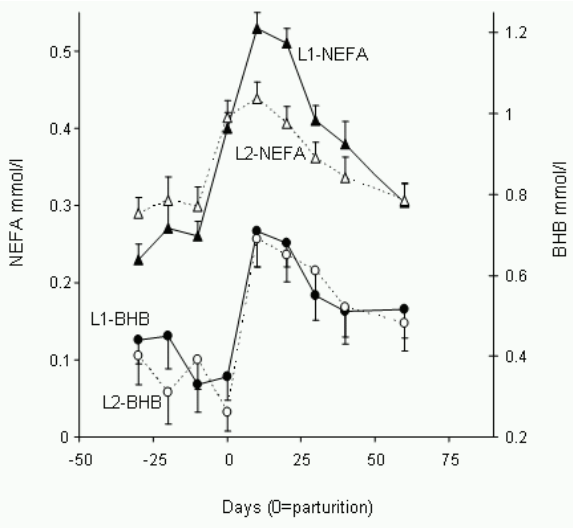
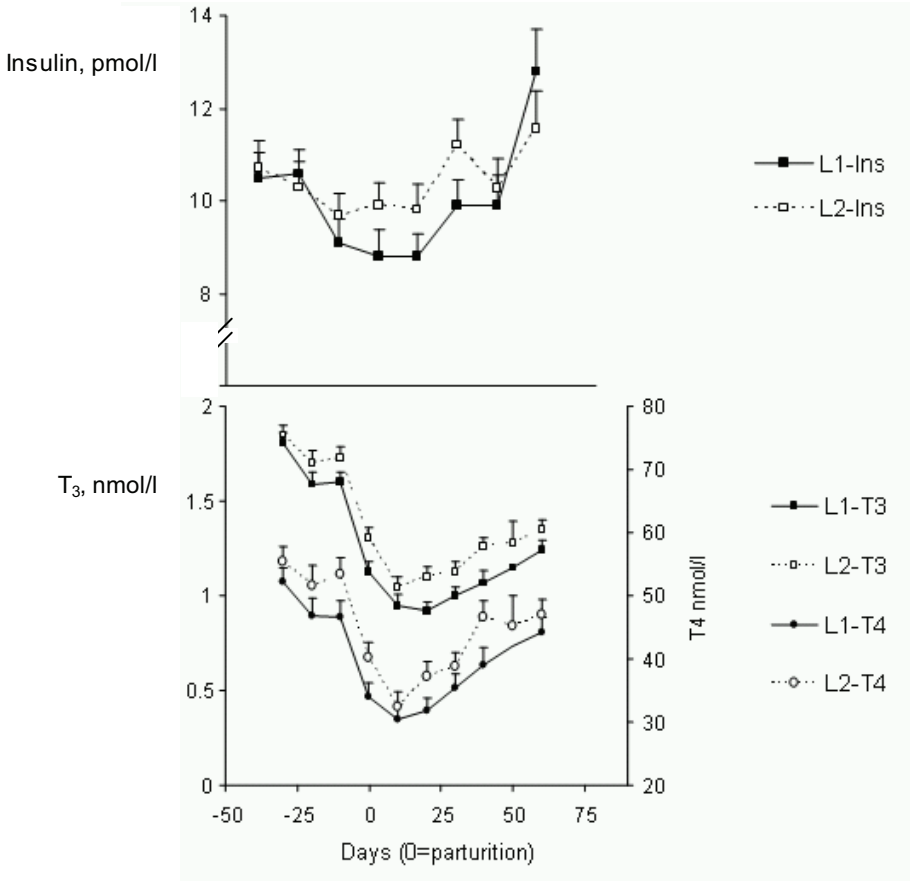


Fig. 5.4.3. Levels of non-esterified fatty acids (NEFA) and ßOH-butyrate (BHB) in primiparous (L1, n=21) and multiparous (L2, n=21) cows under grazing conditions (mean ± SEM; *Exp. 4a*).

Insulin concentrations differed according to days postpartum: levels started to decrease before parturition, minimum concentrations were found around parturition but levels were fully recovered at Day 30 postpartum (*Fig. 5.4.4*.). There was no effect of parity or BCS at parturition on insulin concentrations (*Table 5.4.1*.). Both **thyroid hormones** were affected by days postpartum (*Table 5.4.1*.), but no other effect was found and parallel curves could be ob-

served in both categories (*Fig. 5.4.4*.). Concentrations started to decrease before parturition, and minimum levels were found soon after parturition. Thyroid hormones did not recover prepartum concentrations until the end of the study. Multiparous cows had higher T_3 concentrations than primiparous cows (1.36±0.03 vs. 1.23±0.03 nmol/l), and T_4 concentrations tended to be different (43.0±1.8 vs. 39.4±1.7 nmol/l, $P<0.1$).

Fig. 5.4.4. Concentrations of insulin (Ins), thyroxine (T_4) and 3,3',5 triiodothyronin (T_3) in primiparous (L1, n=21) and multiparous (L2, n=21) cows under grazing conditions (mean±SEM; *Exp. 4a*).

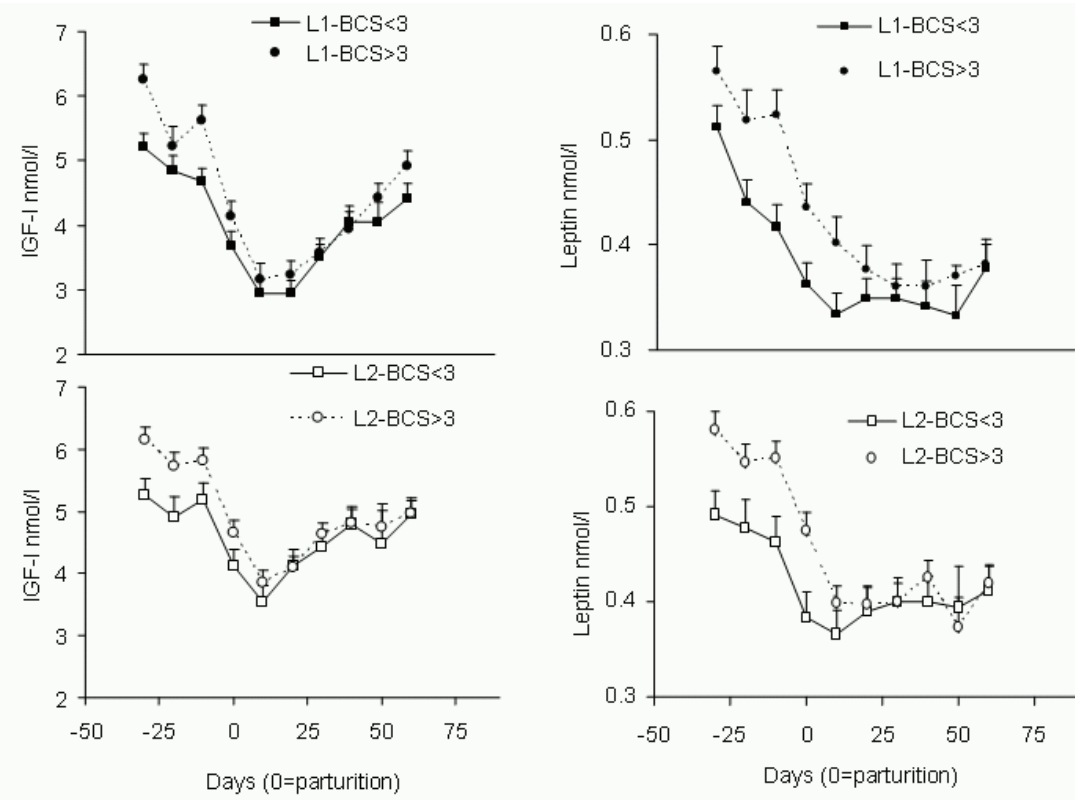


Effects of parity and BCS at parturition were found in **IGF-I** concentrations: primiparous cows and LEAN cows had lower concentrations of IGF-I (*Fig. 5.4.5*.). Concentrations of IGF-I started to decrease 20 days before parturition in all cows to reach half of the prepartum values after parturition. The curves of IGF-I concentration differ according to category (*Table 5.4.2*.). Primiparous cows had a steeper decrease than multiparous cows, remained lower for more days but they tended to recover IGF-I concentrations faster (*Fig. 5.4.5*.). There was also an interaction between BCS at parturition and days postpartum: IGF-I (nmol/l)

in FAT cows fall more sharply than in LEAN cows ($4.36^{a} - 0.039^{a}$ x + 0.00078^{y} x^2 vs. 3.96^{b} – 0.026^{b}x + 0.00065^{z} x^2, P< 0.05, a vs. b P<0.05; y vs. z, P=0.094).

Leptin levels decreased sharply before parturition and – contrasting with the pattern of the other hormones – concentrations remained low during the sampling period (*Fig. 5.4.5*.). BCS at parturition affected leptin concentrations (*Table 5.4.1.*). Leptin concentrations (nmol/l) during late pregnancy and first two weeks of lactation were higher in cows with higher body condition score. Leptin concentrations of primiparous cows tended to present a steeper peri-partum decay than multiparous cows (P=0.066; *Fig. 5.4.5*. and *Table 5.4.2*.), and reached a lower level postpartum (P <0.01). FAT cows presented a steeper peripartum decay when compared to LEAN cows (0.444^{a} – 0.00312^{y} x + 0.00005 x^2 vs. 0.387^{b} – 0.0025^{z}x + 0.000044 x^2, P< 0.05, a vs. b P<0.05; y vs. z, P=0.073). In LEAN cows leptin nadir was reached 10 days before FAT cows. Furthermore, plasma leptin at 10 days of lactation was much lower in LEAN than FAT heifers (*Fig. 5.4.5*.).

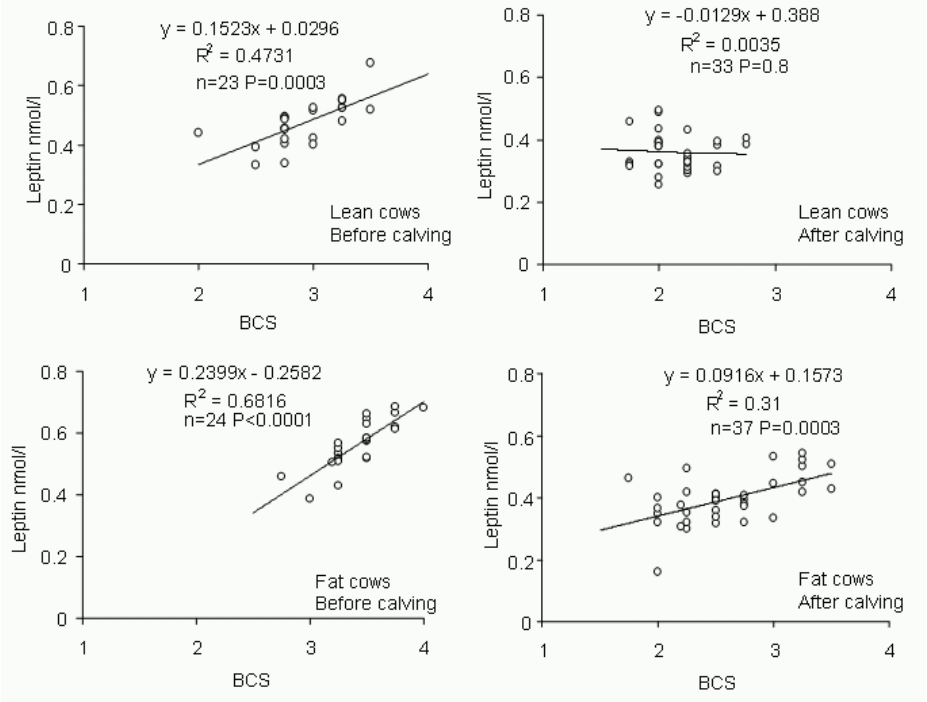
Fig. 5.4.5. Concentrations of insulin-like growth factor I (IGF-I) and leptin in LEAN (L1-BCS<3 n=12) and FAT (L1-BCS≥ 3 n=9) primiparous cows and LEAN (L2-BCS<3 n=8) and FAT (L2-BCS≥ 3 n=13) multiparous cows (mean±SEM; *Exp. 4a*).



Correlations among variables. Regression analyses

T_4, T_3, IGF-I and leptin were correlated with BCS; the latter presented the highest correlation coefficient (r=0.51, r=0.68, r=0.61 and r=0.80, respectively; n=292, P<0.001 for all). Milk production was associated with T_3 and with a higher significance to IGF-I (r=0.22, n=152, P<0.05 and r=0.26, n=152, P<0.01, respectively). All metabolic hormones were negatively correlated with NEFA and BHB and high correlation coefficients for NEFA vs. T_3 (r=-0.67, n=446, P<0.001) and NEFA vs. IGF-I (r=-0.70, n=446, P<0.001) were found. All hormone concentrations were positively correlated and T_3 vs. T_4 (r=0.83, n=446, P<0.001), T_3 vs. IGF-I (r=0.78, n=446, P<0.001) and IGF-I vs. leptin (r=0.70, n=446, P<0.001) were the ones most closely related.

Fig. 5.4.6. Relationships between plasma leptin and body condition score (BCS) in LEAN or FAT dairy cows (i.e. with BCS at parturition of BCS<3 or BCS=3 respectively) measured either one month before or two months after parturition (*Exp. 4a*).



The regression analyses between plasma leptin and BCS before and after parturition in LEAN and FAT cows are shown in *Fig. 5.4.6*. While leptin concentrations were consistently related to BCS during the experimental period in FAT cows, this was present only before parturition in LEAN cows. Plasma leptin concentration (nmol/l) was less related to NEFA concentration in FAT than in LEAN cows, either before or after parturition (FAT cows: y=

0.669 - 0.431x n=91 R^2= 0.14 or y= 0.444 -0.125x n=156 R^2= 0.05; LEAN cows: y= 0.612 - 0.538x n=70 R^2= 0.32 or y= 0.444 - 0.194x n=129 R^2= 0.21 respectively P<0.005 for all). Similar observations occurred when analyzing leptin vs. IGF-I concentration. The functions for FAT cows were y= 0.325 + 0.005x n=91 R^2= 0.19 before parturition and y= 0.25 + 0.0038x n=156 R^2= 0.2 after parturition; LEAN cows y= 0.125 + 0.0088x n=70 R^2= 0.51 and y= 0.238 + 0.0044x n=129 R^2= 0.33, P<0.0001 for all).

Table 5.4.3. Intervals from calving to first ovulation, first service and re-conception in primiparous (L 1) or multiparous (L 2) cows with BCS at parturition of <3 or ≥3 (*Exp. 4a*).

Parity / BCS at parturition	Intervals from parturition to		
	1st ovulation	1st service	Re-conception
	mean (± SEM) of days		
L 1 / BCS < 3 (n=12)	52.8^{a} ± 4.8 (n=12)	139.0^{d} ± 11.8 (n=10)	143.0^{gh} ± 13.0 (n=8)
L 1 / BCS ≥ 3 (n= 9)	37.4^{b} ± 5.6 (n= 9)	122.2^{de} ± 12.8 (n= 9)	149.4^{g} ±12.2 (n=8)
L 2 / BCS < 3 (n= 8)	19.0^{c} ± 6.3 (n= 8)	99.9^{ef} ± 13.7 (n= 8)	114.1^{hi} ± 13.0 (n=8)
L 2 / BCS ≥ 3 (n=13)	23.0^{c} ± 4.5 (n=13)	93.4^{f} ± 10.0 (n=11)	104.0^{i} ± 10.0 (n=11)

Values with different letters within the same column differ P<0.05 except e vs. f (P =0.085) and g vs. h (P=0.058)

Reproduction

There was an effect of parity on the initiation of ovarian cyclicity, parturition to first service and parturition to conception intervals. The interaction effect of BCS at parturition and parity was significant only for the re-initiation of ovarian cyclicity (*Table 5.4.3*.). Postpartum **acyclicity** was longer in primiparous than in multiparous cows: 45 vs. 21 days (P<0.0001). Primiparous LEAN cows presented a longer interval from parturition to first ovulation than primiparous FAT cows, but this was not observed for multiparous cows.

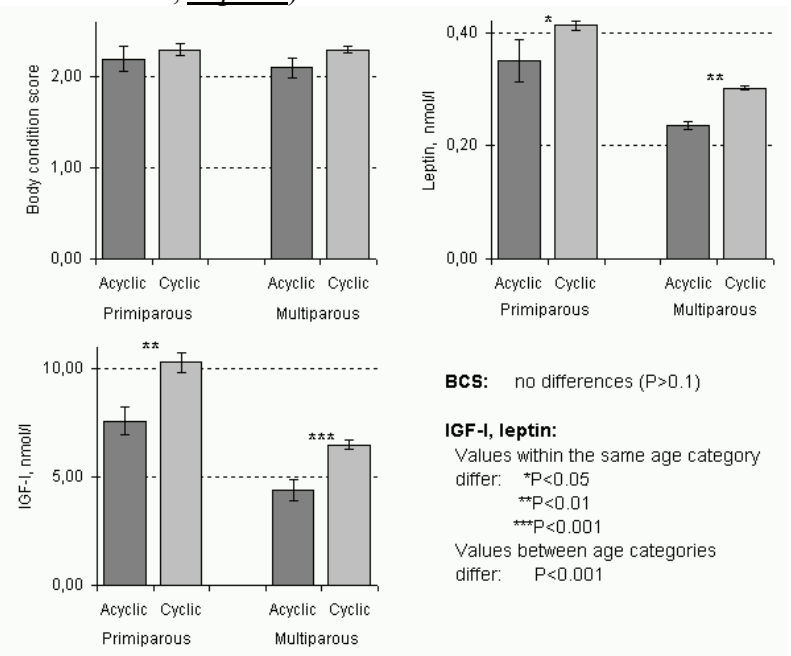
Overall, the interval from parturition to **first service** was 131 and 97 days for primiparous and multiparous cows respectively (P<0.01). The interval from parturition to **re-conception** was 146 and 109 days for primiparous and multiparous cows (P<0.01). An interesting finding was that multiparous LEAN cows re-initiate ovarian cyclicity earlier than the primiparous FAT cows. This was reflected in the parturition to conception interval (*Table 5.4.3*.).

Body condition score at parturition, BCS, insulin, leptin and BHB (all with P<0.05), furthermore the T_3 (P<0.08) were associated with the re-initiation of ovarian cyclicity, as determined by regression analysis (r= 0.78, P=0.0001).

Exp. 4b

In the 7 herds 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±0.7 vs. 30.5±0.4 kg/day; mean±SEM, P<0.001). The BHB was <1.00 mmol/l in all animals, and also the plasma concentrations of NEFA, TCH, insulin, IGF-I and thyroid hormones were within the physiological range of lactating dairy cows, with minimal between-herd variability (data are not shown). 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 (*Fig. 5.4.7.*; all these party-associated differences: P<0.001).

Fig. 5.4.7. Body condition score and concentrations of insulin-like growth factor I (IGF-I) and leptin in primiparous (L1) and multiparous (L2) cows at day 49-63 of lactation: acyclic vs. cyclic animals (n: L1-acyclic: 37, L1-cyclic: 95, L2-acyclic: 46, L2-cyclic: 205; means ± SEM; *Exp. 4b*).



Most of the cows ovulated during the first 7 weeks of lactation: the ovarian activity remained still acyclic only in 83 animals (22 %). The rate of acyclic cows varied between 16 and 25 %, but the between-herd differences were not significant. However, by day 50 more primiparous than multiparous cows did not ovulate yet (37/132 = 28% vs. 46/251 = 18 %, respectively; P<0.001). Although their BCS was similar, in both age categories the acyclic animals were characterized by lower levels of leptin and IGF-I than the cyclic cows (*Fig. 5.4.7.*). If, however, data of primi- and multiparous animals were pooled, due to the age-dependent variability differences between acyclic and cyclic cows were not significant. There were no further differences in plasma levels of NEFA, BHB, TCH, insulin and thyroid hormones, either (details are not given).

Discussion

In *Exp. 4a* we intended to collect basic data on peri-parturient changes of leptin. Therefore we wanted to avoid the suspected influence of endotoxin / cytokine induced hormonal interactions discussed in Chapters 3.8. and 5.7, which may be intensive first of all during the earliest 2 to 4 weeks of lactation (for further details, see also our related papers: *Jánosi* et al., 1998 and 2003; *Huszenicza* et al., 2004 and 2005; *Földi* et al., 2006). So the *Exp. 4a* was conducted in grazing animals, in a small experimental herd, where – due to the better hygienic conditions – the incidence of *mastitis* caused by environmental pathogens, as well as of *bacterial complications of uterine involution* (such as puerperal metritis) is usually low (*Bruun* et al., 2002; *Sheldon* and *Dobson* 2004). However, in pasture-fed cows the peri-parturient changes in body condition, as well as in plasma levels of certain metabolites and metabolic hormones may be different from those seen in animals fed total mixed ration (TMR) in large-scale daily herds. Although the physiological mechanisms that the dairy cow undergoes to adapt to lactation requirements is basically similar among different production systems, the energy demands due to regular physical exercise and grazing might modify the transformations that take place during this period (*Nagy* et al., 1984, *Roche* et al., 2005; *Chagas* et al., 2006). Moreover, dry matter intake in this production system is usually lower than in confined systems, and may be insufficient to sustain the high milk production that can be achieved with the genetic potential (*Kolver* and *Muller*, 1998).

Data of *Exp. 4a* demonstrated that parity affects NEFA, T3, T4 and IGF-I concentrations as well as reproductive parameters in dairy cows while BCS at parturition has an effect

on IGF-I and leptin concentrations and reproductive parameters. Body condition decreased from 30 days before parturition, and this trend was steeper during the first 4 weeks after parturition. NEFA and BHB levels increased around parturition, reflecting the NEB of animals (*Invgarsten* and *Andersen*, 2000). In this study the primiparous cows had lower BCS than multiparous cows and this is consistent with the higher NEFA levels and the greater number of BHB samples >1.00 mmol/l in this category (supraphysiological levels, according to *Whitaker* et al. 1999). This probably is related to increased needs for growth in primiparous cows occurring simultaneously with the demands of lactation, and their lower feed intake capacity (*Rémond* et al. 1991). In large-scale TMR-fed dairy herds in Hungary (*Huszenicza* et al., 1988) the peri-parturient metabolic and hormonal changes of primiparous animals are usually more intensive than those seen in multiparous cows (*Wathes* et al., 2007), which are in agreement with these experiences. The only exception is that the keton (BHB) production is usually more elevated in multiparous than in young primiparous cows (*Huszenicza* et al., 1988; *Wathes* et al., 2007). Clear age-related variability of leptin and IGF-I was found also in the *Exp. 4b*.

Studies investigating the potential metabolic signals for the reproductive axis have been focused primarily on blood metabolites and metabolic hormones known to fluctuate during altered states of energy metabolism. Decreased insulin concentrations around parturition were found in agreement with previous reports (*Holtenius* et al. 2003). Insulin plays a central role in homeostatic control of energy metabolism and its concentration is positively correlated with energy intake (*Chilliard* et al. 1998). The diminished concentration is consistent with the reduction in dry matter intake that characterizes this period (*Bertics* et al. 1992). Both insulin and IGF-I are known to stimulate follicular growth (*Spicer* et al. 1993), however in this study insulin did not seem to be related to reinitiation of ovarian cyclicity: insulin concentrations were fully recovered 30 days postpartum and while multiparous cows ovulated before that period, primiparous cows ovulated afterwards.

The peripheral tissues try to fit their current local energy metabolism to the postpartum catabolic condition, for example by interfering in the path of thyroid hormones resulting in diminished circulation as observed here as well by others (*Kunz* and *Blum*, 1985; *Pethes* et al. 1985; *Heyden* et al. 1993; *Tiirats*, 1997). Lower concentrations of T_3 were found in primiparous cows, which is in disagreement with data of *Cisse* et al. (1991) who suggested that higher

T_3 concentrations in first lactation could be due to a lower milk yield since thyroid hormones are excreted by the mammary gland. On the other hand, a lower level of T_3 was reported to be associated with lower energy balance (*Blum* et al. 1983), which seems to be the case in our study taking into account that primiparous cows presented comparative greater body condition loss and an overall misbalanced metabolic condition. This could explain the apparent discrepancy with the study of *Cisse* et al (1991), in which heifers and cows had the same energy balances. Low thyroid hormone concentrations have been suggested to be associated with low reproductive performance in the postpartum cow (*Huszenicza* et al. 2002; *Huszenicza*, 2003).

In *Exp. 4a*, parity affected IGF-I concentrations, as primiparous cows had lower values of this hormone. This contrasts with findings of *Wathes* et al. (2003, 2007), and also the results of *Exp. 4b*, where IGF-I concentrations were higher in the younger animals. Since it has been suggested that low insulin and IGF-I are the metabolic signals that delay ovulation (*Beam* and *Butler*, 1999) and IGF-I concentrations were high in primiparous cows, *Taylor* et al. (2003) suggested that in this category – which is still growing – insulin concentrations may be limiting, whereas in older cows a closer association is observed between IGF-I and fertility parameters. The results of *Exp. 4a* do not support this hypothesis, since primiparous cows presented lower IGF-I but similar insulin concentrations, and presented a delayed ovulation when compared to multiparous cows. Peri-parturient metabolic differences of pastured and TMR-fed dairy cows may also explain these contradictions. IGF-I concentrations were good indicators of the capacity to resume cyclicity after parturition in agreement with *Roberts* et al. (1997): FAT cows and multiparous cows had higher IGF-I concentrations and better reproductive performance.

As previously reported in adult dry non-pregnant ruminants (*Delavaud* et al. 2000, 2002), plasma leptin content in this study was a good indicator of body fatness (BCS) in peripartum dairy cows, especially when measured 3 weeks before calving, i.e. at a stage where leptin is highly expressed. On the other hand, *Holtenius* et al. (2003) reported no relation between leptin concentrations and BCS after parturition. Differences among studies could be due to the different production system, energy intake, energy balance, range of BCS among cows, genetic background and/or frequency of sampling. In the *Exp. 4a* leptin concentrations started to decrease by 20 days before parturition in agreement with other studies (*Kadokawa*

et al. 2000; *Block* et al. 2001; *Liefers* et al. 2003), and then remained low until 2 months after parturition. This tendency is at variance with the reported increase around day 10 postpartum (*Kadokawa* et al. 2000), or the progressive increase from week 1 to week 12 (*Reist* et al. 2003), the unchanged concentrations up to week 5 (*Huszenicza* et al. 2001), or with the transient increase at week 2 (*Liefers* et al. 2003), but consistent with the lower concentrations found by *Block* et al. (2001) and *Holtenius* et al. (2003).

In the *Exp. 4a* baseline leptin levels were reached later postpartum in FAT cows consistent with a later maximal loss in BCS in these animals, suggesting that the decrease in leptin concentrations due to NEB may be masked in FAT cows by the amount secreted by the adipose tissue reserves, that may be still high during the first month of lactation. This hypothesis is supported by the regression analyses of plasma leptin during pre- and postpartum period in LEAN and FAT cows: while no association was found between leptin levels and BCS after parturition in LEAN cows, this was maintained in FAT cows during the same period. Overall, our results suggest interactions between the effects of BCS, parity and lactation stage on peripartum leptin regulation.

The reinitiation of ovarian cyclicity was delayed in primiparous cows and in LEAN animals and this was consistent with longer intervals from parturition to first service and to conception in these animals. The anestrous duration was associated with BCS loss and was longer in primiparous cows as shown previously (*Butler* and *Smith* 1989). These authors and others (*Huszenicza* et al. 1987, 1988) demonstrated that the sooner the cows restore the energy balance, the sooner they will start cycling and will become pregnant. It is interesting to point out that multiparous LEAN cows resumed cyclic activity sooner than primiparous FAT cows and this could be due to the patterns of the endocrine signals or to NEB due to lower intake, ascendant lactation curve and/or growth requirements in heifers. All these parameters could indeed be related to the high body lipid mobilization (*Verite* and *Chilliard*, 1992), BCS loss (*Rémond* et al. 1991) and higher plasma NEFA (*Cisse* et al. 1991), that were more intensive in primiparous cows vs. multiparous cows in *Exp. 4a*, despite the fact the formers yielded less milk. In this study parity was a more important effect for reproductive performance than BCS at parturition; it should be taken in account that these animals were under grazing conditions, where an effect such as dominance for food availability is present (*Grant* and *Albright*, 2001).

Cows with better reproductive performance had higher IGF-I and leptin concentrations. Similarly, a negative relationship between IGF-I concentrations after parturition and the interval to the resumption of ovarian cyclicity was reported (*Butler* 2000). Data regarding leptin and reproductive performance is conflicting. Cows with decreased leptin have been linked to delayed onset of cyclicity or longer intervals to first oestrus during the postpartum period (*Kadokawa* et al. 2000; *Liefers* et al. 2003). Similarly, a slightly increasing tendency of leptin pattern was seen in cows resuming their ovarian cyclicity within 35 days (*Huszenicza* et al. 2001). In two most recent field trials (*Mann* et al., 2005; *Wathes* et al., 2007) using milk P_4 profiles to monitor the ovarian function, delayed onset of cyclicity and other cycle abnormalities were associated with increased milk yield, elevated plasma BHB and reduced plasma leptin and IGF-I concentrations, as well as with lower BW and BCS of the affected cows. Latest model studies in dairy cattle illustrated a close link between plasma leptin concentrations and LH pulsatility during the postpartum period (correlations of leptin with LH pulse frequency, and with amplitude of the LH pulses were r=+0.73 and r=+0.53, respectively; *Kadokawa* et al. 2006), although a triggering role for leptin in the termination of anoestrus was not always supported (*Chagas* et al. 2006).

The data presented here does not support the theory that the restoration in leptin concentrations activate the hypothalamic-pituitary-ovarian axis, since unlike the other hormones, it remained low until 60 days postpartum. On the other hand, not only the hormone concentration itself and the tissue sensitivity to it (receptors) are important for the reproductive axis, but also the hormonal dynamics, (e.g., leptin decay – as for IGF-I – was more precipitous in primiparous cows) could be read by the endocrine system as a different signal. As previously reported (*Bocquier* et al. 1998; *Clarke* and *Henry* et al. 1999) our results suggest that leptin may play a permissive role when increasing above a critical threshold in the activation of the hypothalamus-pituitary axis and consequent reinitation of ovarian activity in the postpartum cow. This would allow cows, which are in good condition at parturition and thus have a higher leptinaemia during early lactation, to have a facilitated reproductive activity. The most recent observations (*Mann* et al., 2005; *Chagas* et al. 2006; *Kadokawa* et al. 2006; *Wathes* et al., 2007) are in full agreement with our findings in *Exp. 4a* and *4b*. Of course these data were not yet available for us when our experiments were conducted and published. Currently the theory

on permissive role of leptin in regulation of resumption of ovarian cyclicity is fully accepted (*Zieba* et al., 2005; see also in Chapter 3.5.3.)

In *Exp. 4b* data of primiparous and multiparous cows ovulating during the first 7 weeks of lactation were compared to those remained still acyclic in this period. At the time of sampling (day 49-63) the cyclic cows had higher IGF-I and leptin levels than their acyclic herd mates. However, none of their other parameters differed. The lack of more obvious metabolic differences may be due to the fact that cows showing the pathognostic clinical symptoms of metabolic disorders (such as ketosis) had been excluded from the study, furthermore most of the cows must have been over the nadir of NEB, e.g. the phase of lactation characterized by the highest NEFA and ketone levels. The first postpartum ovulation of our still acyclic cows must have been postponed by this earlier metabolic condition: at sampling the long-lasting acyclicity and the poor BCS were the only coinciding clinical consequences. The metabolic impairment of these cows was proven, however, by their low IGF-I and leptin.

In summary, the age-related differences must be considered when the peri-parturient metabolic and endocrine changes are monitored. Under grazing conditions primiparous cows showed a metabolic/endocrine profile more disbalanced when compared to multiparous cows, reflecting that they are recovering from the negative energy balance period with more difficulty. The endocrine signals that most likely could inform the reproductive axis regarding the NEB and/or the level of body reserves that may explain the reproductive performance found in this study were IGF-I and leptin.

5.5. Plasma leptin levels in normo- and hyperketonaemic dairy cows during the peri-parturient period (*Exp. 5a* and *5b*)

Ketosis (accompanied usually with remarkable lipid accumulation in the liver) is known, as a consequence of decompensated NEB in high-producing postpartum dairy cows (*Chilliard* et al., 1998; *Duffield* et al., 1998; *Whitaker* et al., 1999). Several endocrine aspects of this metabolic disorder have already been reported or supposed to occur: the lower than normal rate of insulin to glucagon and GH was proved as the main endocrine factor of its pathogenesis (*Sartin* et al., 1988), and also the role of thyroid and adrenocortical (mal)functions was hypothesized long time ago (*Hill* et al., 1950; *Robertson* et al., 1957; *Durdevic* et al., 1980). Since then the involvement of thyroid hormones in postpartum adaptation of energy

metabolism has become fully accepted (*Kunz* and *Blum*, 1985; *Pethes* et al., 1985; *Tiirats*, 1997), and glucocorticoids have been widely used in therapy of bovine ketosis for many years (*Baird*, 1980). Low concentrations of IGF-I (*Ronge* et al., 1988) and leptin (*Block* et al. 2001) were proved to reflect the degree of energy imbalance of cows in early period of lactation (*Ronge* et al., 1988; *Chelikani* et al., 2004). However, only limited data are available on plasma leptin of hyperketonaemic individuals (*Reist* et al., 2003). In one of our former trials we studied extensively the endocrine characteristics coinciding with hyperketonaemia in postpartum Holstein Friesian cows showing various forms of BHB pattern[36] (*Huszenicza* et al., 2006). Simultaneously with the hyperketonaemic stage increased NEFA, depressed TCh and glucose, furthermore decreased insulin, IGF-I, T_4 and T_3 concentrations were detected in almost all the cases. Overt endocrine alterations were found, however, only in the long-lasting forms of hyperketonaemia. The TRH-stimulated T_4 and T_3 responses remained almost unaffected proving intact thyroid function in most of the cows; depressed thyroid response was detected only in the most severe cases of long-lasting hyperketonaemia. The cows characterized by lower than normal (<mean - SD of normoketonaemic cows) ACTH-stimulated cortisol response on days 1-3 after calving showed poorer chance for spontaneous recovery. The involvement of leptin in ketosis-related endocrine cascade, however, was not studied in that experiment.

As it was discussed earlier (Chapters 3.3., 3.6. and 5.4.), 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 NEB 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.

[36] According to *Whitaker* et al. (1999), plasma BHB of >1.00 mmol/l was considered as a supraphysisological level, and was called as hyperketonaemia in this study.

Materials and methods

Farm conditions. Animals. Sampling

Exp. 5a

This study was conducted in a large-scale dairy herd, on group-fed multiparous (parity: 3 to 5) Holstein Friesian cows producing at least 6000 kg of milk/305-d in the previous lactation. On day 259-265 of gestation the cows expected to calve in February and March were scored for BCS. All healthy individuals meeting these inclusion criteria (parity, minimum milk yield, expected calving date) were selected for this study: they were separated from the others in a different paddock (for 14-16 days), and later in boxes of the maternity unit (3-4 cows in each; in the latest days of pregnancy, at calving and thereafter for 7 days). The group of late-pregnant cows received the same diet (consisting of 3.5 kg commercial concentrate mixed into 15 kg of maize silage, with free access to grass hay available *ad libitum*) for 14-16 days. Thereafter (when the cows were placed in boxes of 3-4 animals) growing quantity of concentrate and corn silage was given: the quantity of concentrate increased continuously (with about 1.0 kg in every alternate days till the end of the sampling period, with the maximum of 9.0 kg on day 7 after calving), whereas increased quantity (25 kg) of maize silage was given only after calving. This ration was administered twice a day, in equal portions. Grass hay was available *ad libitum* also in this period. The cows were milked twice a day. All cows left the maternity unit 7 days after calving, when they were examined for puerperal metritis[37], and their BCS was recorded again. Blood samples were taken 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.

Exp. 5b

Cows (parity ≥2; milk yield in previous lactation: at least 4500 kg) calving within a 4-5 week-long study period[38] in 8 large-scale dairy herds were involved in the experiment. Animals were kept in loose-housing systems, and were milked twice daily. Cows were fed a ration of maize silage, alfalfa, grass hay and concentrate (including vitamins and minerals) in accordance with NRC (2001) recommendations. Once between day 4-12 postpartum all cows were ex-

[37] Presence of malodorous, reddish-brown, watery vaginal discharge.

[38] Between February and June.

amined for the presence of clinical mastitis and puerperal metritis, and simultaneously they were sampled for assaying metabolites and hormones.

Analysis of samples. Evaluation

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 mastitis[39] and/or retained fetal membrane resulting in puerperal metritis[40] 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.

Results

In *Exp. 5a* and *Exp. 5b* data of 22 and 253 cows not showing overt clinical signs of any diseases were evaluated, respectively.

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 week positive correlation existed between these two parameters ($r = +0.271$; $P<0.048$). The plasma BHB started to increase some days before calving, with wide individual variation (*Fig. 5.5.1*.). Three forms of peri-parturient BHB pattern were observed. 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 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

[39] Inappetance, rectal temperature >40.5 °C

[40] Presence of malodorous, reddish-brown vaginal discharge.

Fig. 5.5.1. Peri-parturient changes of plasma βOH-butyrate (BHB) content (i) in *normoketonaemic* individuals (BHB: not elevated, e.g. <1.00 mmol/l throughout the study; n=9), (ii) in those with *transient ketone increase* (BHB: >1.00 mmol/l only at calving and/or on the next day; n=7), and (iii) in cows showing *continuous* form of hyperketonaemia (BHB: >1.00 mmol/l in at least 5 consecutive days; n=6) (means ± SEM; *Exp. 5a*).

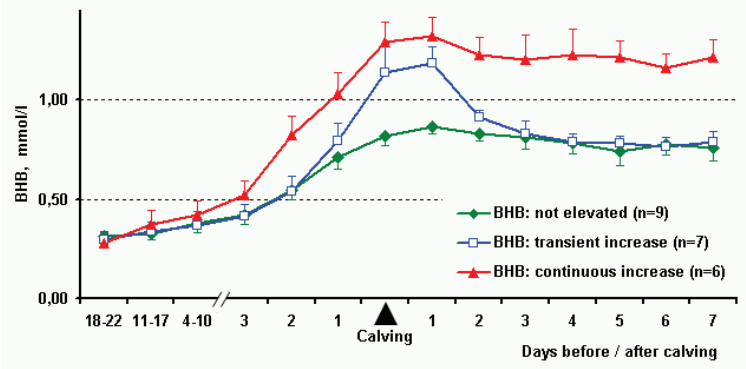


Fig. 5.5.2. Peri-parturient changes of plasma insulin and leptin (i) in *normoketonaemic* individuals, (ii) in those with *transient ketone increase*, and (iii) in cows showing *continuous* form of hyperketonaemia (means ± SEM; *Exp. 5a*).

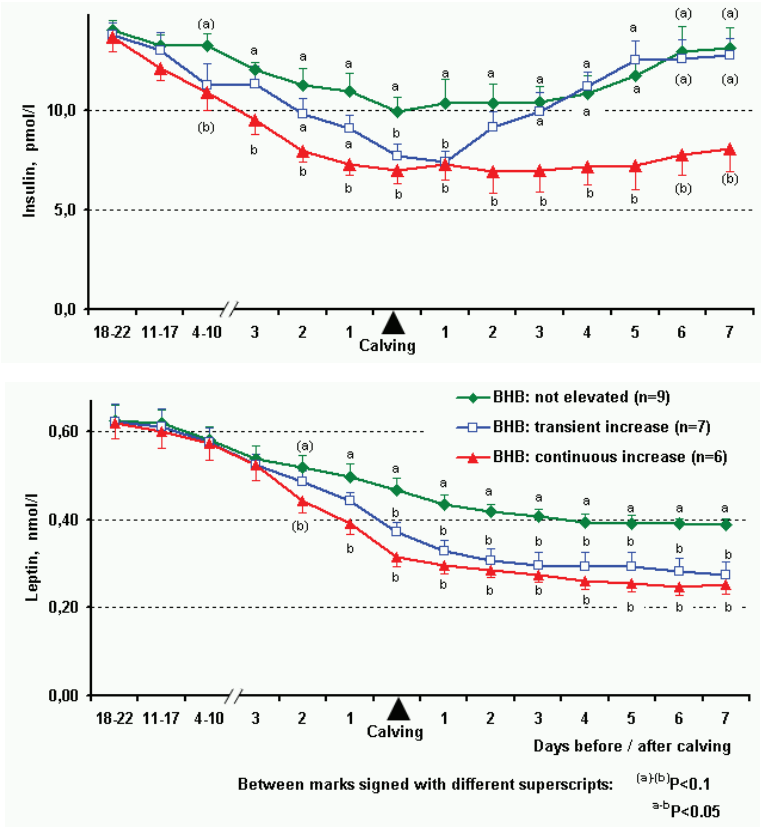


Table 5.5.1. Correlation between plasma levels of BHB, NEFA, insulin and leptin 18-22 days before calving, furthermore 1 and 7 days postpartum (r values are given, if P <0.05; n = 22; *Exp. 5b*)

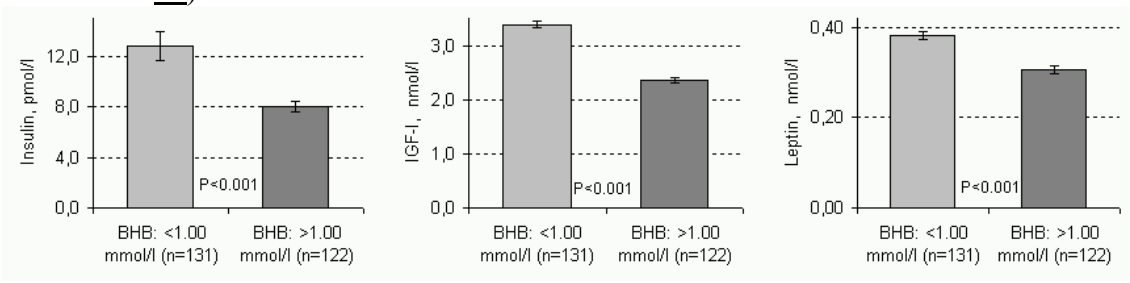
	18-22 days before	1 day after calving	7 days after
BHB - NEFA	+0.76	+0.43	+0.44
BHB - Insulin		-0.38	-0.53
BHB - Leptin		-0.53	-0.52
NEFA - Insulin		-0.54	-0.65
NEFA - Leptin		-0.40	
Insulin - Leptin	+0.40	+0.50	+0.31

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 (details are not given). 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 with continuous hyperketonaemia. The prepartum decrease of insulin begun some days later, and remained less obvious in normoketonaemic than in hyperketonaemic individuals (*Fig. 5.5.2*., upper panel). 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 (*Fig. 5.5.2*., lower panel). There were significant positive correlations between BHB and NEFA, and insulin and leptin at any time of sampling. However, negative correlations of BHB and NEFA with insulin and leptin became remarkable only around the time of parturition and thereafter (only data on days -22 to -18, +1 and +7 are given; *Table 5.5.1*.).

The cows in *Exp. 5b* were sampled on day 4-12 after calving. Almost half of them had elevated BHB levels (BHB: >1.00 mmol/l, with the maximum of 5.18 mmol/l; 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 (details are not given). Plasma levels of BHB (0.65±0.26 to 1.98±0.32 mmol/l) and insulin (11.20±1.65 to 25.56±3.88 pmol/l) differed significantly between herds, and there was a tendency (P=0.095) for different plasma

levels of IGF-I (2.68±0.10 to 3.16±0.19 nmol/l), as well. Plasma insulin levels were different by production level within herds (P= 0.06). 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 (*Fig. 5.5.3*.). 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).

Fig. 5.5.3. Plasma insulin, IGF-I and leptin concentrations in Holstein Friesian cows showing *normal* (<1.00 mmol/l) or *elevated* (>1.00 mmol/l) βOH-butyrate (BHB) concentrations on day 4-12 after calving (3 to 5 parity cows; means ± SEM; *Exp. 5b*).



Discussion

In full agreement with the experiences of *Exp. 5.4a*, on day 18-22 before calving, the metabolic and endocrine condition of the late pregnant, non-lactating cows was stabile, and characterized with high plasma levels of insulin, IGF-I and leptin, and low concentrations of NEFA and BHB also in the current study. Although the individual variation was quite wide, the production and plasma levels of ketone bodies (such as BHB, furthermore acetone and acetoacetic acid, not determined in this trial) usually started to increase some days before calving, with a coinciding decline in plasma insulin, and elevation in circulating NEFA content. Although development of clinical ketosis is expected when BHB is above >1.60 mmol/l for several days (*Bruss*, 1997; *Whitaker* et al., 1999), also the consequences of less obvious, subclinical forms of hyperketonaemia (which is usually detected, as ketonuria, in the practice; *Markusfeld*, 1985; *Kégl*, 1990; *Kégl and Gaál*, 1992) may have clinical relevance simultaneously with, or

soon after calving. Due to the BHB elevation and decreased IGF-I content, in these cows the capacity of intrauterine and intramammary anti-microbial self-defense mechanisms (first of all the migration and killing activity of neutrophil granulocytes) is impaired, predisposing the affected animal for puerperal metritis and mastitis (*Hussain*, 1989; *Cai* et al., 1994; *Wensing* et al., 1997; *Suriyasathaporn* et al., 1999 and 2000; *Zerbe* et al., 2000; *Jánosi* et al., 2003; *Vangroenweghe* et al., 2005). Furthermore, also the postpartum resumption of cyclic ovarian function may be postponed, and at the desirable time of first insemination the quality of oocyte may be impaired (*Wensing* et al., 1997; *Veerkamp* et al., 2003; *Huszenicza*, 2003; *Huszenicza* et al., 2006).

In accordance with some other reports (*Kadokawa* et al., 2000; *Block* et al., 2001; *Liefers* et al., 2003; *Chagas* et al., 2006; *Kadokawa* et al., 2006), as well as with our earlier findings (*Exp. 4a*), in late-pregnant non-lactating cows the plasma leptin was elevated, was in positive correlation with the BCS, and declined towards parturition also in the current study. Like the observation of *Holtenius* et al. (2003), low leptin concentration with mild correlation with BCS was found about one week after calving. As it was discussed also in Chapters 3.3. and 3.6., this decrease in plasma leptin of late pregnant cows may be due to a decline in dry matter intake together with an increased energy demand of the fetus (*Hayirli* et al., 2002), furthermore due to changes in production and plasma level of insulin and GH initiated by this pre-calving NEB. In dairy cattle the onset of NEB was associated with decreased plasma insulin and increased plasma GH (*Block* et al., 2001 and 2003; *Leury* et al., 2003). Administration of insulin increased circulating leptin in both late pregnant and early lactating cows, whereas GH treatment did not reduce plasma leptin (*Block* et al., 2003; *Leury* et al., 2003), suggesting that insulin rather than GH is the main endocrine regulator of leptin concentrations during the periparturient period. The experiences of our current studies are in agreement with these observations. In *Exp. 5a* the cows with continuous hyperketonaemia showed higher BHB and lower insulin levels than their normoketonaemic mates from day 4-10 before calving, and these tendencies existed throughout the study. Also the plasma leptin started to decrease at that time, however, the first significant differences between the normo- and hyperketonaemic cows were observed some days later, only on day 2 before calving. The BHB and insulin patterns of those with transient ketone increase around parturition represented an intermediate position. It was interesting to see that in cows, which had shown elevated BHB levels for a

while, the postpartum leptin levels were low subsequently in the first week of lactation, regardless of the presence or absence of coinciding BHB elevation. This tendency proved distinct BHB-related changes in function of adipo-insular axis in hyperketonaemic cows, resulting in long-time depression in circulating leptin level. Impairments in endocrine regulation of energy homeostasis were reflected also in data of cows in *Exp. 5b*, sampled only once between days 4 and 12 after calving.

As a conclusion, these data demonstrate obvious differences between circulating leptin levels of normo- and hyperketonaemic dairy cows during the peri-parturient period and at the beginning of lactation, with lower leptin content in plasma of those which have had >1.00 mmol/l BHB. It has remained still unknown, whether this hypoleptinaemia may promote the hyperphagia of lactation, and if so, why this promoting effect exists only in certain individuals (e.g. in those with transient ketone increase). However, this low leptin level seems to be a consequence, rather than a causative factor in pathogenesis of subclinical ketosis.

5.6. Effects of fat supplementation in postpartum dairy cows (*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. Energy density of fat is high and long chain fatty acids are used efficiently for milk fat synthesis. Large amounts of fat may, however, have a negative impact on microbial fiber digestion in the rumen and reduce feed intake. Feeding inert fats with melting point lower than the normal temperature of the ruminal juice or calcium salts of fatty acids alleviates this problem (*Coppock* and *Wilks*, 1991). Another potential drawback of fat supplementation can be, paradoxically, increased lipid mobilization from adipose tissue, which is possibly facilitated by decreased blood insulin or merely a consequence of increased milk yield (*Chilliard*, 1993, *Staples* et al., 1998). In monogastric models the high-fat diet feeding reduced the diurnal variation of plasma leptin concentration (in rats; *Cha* et al., 2000), a one-week fat supplementation (2 % conjugated linoleic acid, provided as a safflower oil enriched diet) reduced the serum leptin levels (in rats; *Yamasaki* et al., 2000), but a similar diet elevated the circulating leptin level in rabbits (*Corino* et al., 2002). The intravenous administration of an aqueous emulsion of 20 % soybean oil did not affect plasma leptin, insulin and IGF-I concentrations in early-lactation dairy cows, whereas stimulated the leptin secretion (but not the insulin and IGF-I) in late-lactation animals (*Chelikani* et

al., 2003b). In 2002-2003 fat supplementation was a widely used technology in dairy herds, but up to our knowledge no data had been published yet on its influence on circulating leptin levels. So in cooperation with Finnish nutritionists we studied the effects of a commercially available inert fat source on insulin and leptin levels in postpartum dairy cows.

Material and methods

In a randomized complete block design 24 high-producing Ayrshire cows were divided to groups with 0 (**Group NF**; n=8), 3.5 (**Group MF**; n=8) or 7% (**Group HF**; n=8) of calcium salts of palm fatty acids (Ca-PFA) in concentrate[41,42]. For the first 8 weeks of lactation, cows were kept and fed individually; grass silage was provided *ad libitum*, and concentrate allowance was increased to 15 kg/d within 20 days. Metabolizable energy content of the silage was 11.0 MJ/kg dry matter, whereas the metabolizable energy contents of the concentrates were 12.6, 13.1 and 13.6 MJ/kg DM for NF, MF and HF, respectively[43].

Milk yield and feed intake were recorded daily. Milk samples for composition analyses were taken at 1, 2, 4, 6 and 8 wk after parturition. Body weights were recorded daily during the week of calving, and at 2, 4, 6 and 8 wk after calving. At the day of calving, furthermore 4 and 8 weeks later the body fat reserves (represented by subcutaneous fat depths) were measured with a real-time B-mode ultrasonographic scanning method[44] of *Kokkonen* et al (2005) at 3 locations, as follows: (i) on the left transversal process of the fourth lumbar vertebra, 2 to 3 cm medially from the lateral end, (ii) in the middle of the line connecting the ventral corner of the left tuber coxae and the dorsal tuberosity of the left tuber ischiadicum, and (iii) on the same line, 8 cm cranially from the dorsal tuberosity of the tuber ischiadicum. Fat depth at a particular time was given as a mean of values determined at these 3 locations. Blood samples were taken from the *superficial epigastric* (mammary) vein at 1, 3, 5, 7, 14, 21, 28, and 56 d after calving and from the *coccygeal* (tail) vessel 5 and 28 d after calving.

The data for milk yield, composition, and feed intake from weeks 1 to 4 and weeks 5 to 8, as well as blood composition data from 1 to 28 d, were analyzed as repeated measures.

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41 Raisio Feed Ltd, Raisio, Finland

42 Also a digestibility experiment was conducted with 12 cows, using acid-insoluble ash, as an internal marker. These data, however, are out of the subject of the current dissertation, and are not presented and discussed in details thereafter.

43 According to the national standard in Finland, the metabolizable energy content of constituents were calculated and given in this study.

44 Aloka SSD-210DXII, equipped with a 7.5-MHz transducer (Aloka Co. Ltd., Tokyo, Japan)

The statistical model included effects of treatment, block, time, and interactions of time and treatment, and time and block. The changes of body weight and fat depth, digestibility of diet, average metabolizable energy balance within the two periods and plasma NEFA at 56 d after calving were analyzed with the model including the fixed effect of treatment and random effect of block.

Results

Although silage and total dry matter intakes were not significantly different between treatments (*Table 5.6.1*.), the increase of total dry matter intake in group MF tended to be slower (time x treatment P<0.10) than in the other groups. Apparent (orga-

Table 5.6.1.: Feed intake, milk yield and diet digestibility (*Exp. 6*).

					Significance			
	NF	MF	HF	SEM	Lin.	Quadr	Time	Time x treatm.
Weeks 1 to 4								
Silage, kg DM/d	9.7	9.2	10.0	0.33			***	
Total DMI, kg/d	19.9	19.4	20.5	0.46			***	+
ME, MJ/d	239	236	256	5.5	º		***	+
Milk yield, kg/d	40.9	39.9	40.7	1.39			***	
Milk fat content, g/kg	44.2	47.8	49.2	1.33	*		***	
Weeks 5 to 8								
Silage, kg DM/d	9.5	9.3	9.3	0.49			º	
Total DMI, kg/d	23.0	23.0	23.0	0.51			*	
ME, MJ/d	275	279	290	5.8			º	
Milk yield, kg/d	46.3	48.2	48.4	1.33			**	+
Milk fat content, g/kg	40.9	41.0	41.6	1.55				

+P<0.10 * P<0.05 ** P<0.01 *** P<0.001
DM: dry matter; DMI: dry matter intake; ME: metabolizable energy

ganic matter) digestibility of diet did not differ between treatments (no further details are given). Ca-PFA had no significant effect on milk yield, but the decline of milk yield during lactation weeks 5 to 8 tended to be smaller in MF and HF groups than in NF group (time x treatment P<0.10). Ca-PFA increased milk fat content (linear effect, P<0.05) during lactation weeks 1 to 4. Group MF had greater body weight loss (quadratic effect, P<0.05) (*Table 5.6.2*.) MF had greater body weight loss (quadratic effect, P<0.05) (*Table 5.6.2*.) during lactation weeks 1 to 4 and tended to have greater decrease of fat depth during lactation weeks 5 to 8 than the other groups (quadratic effect, P<0.10). Plasma NEFA increased linearly

(P<0.01) with Ca-PFA 8 weeks after calving, whereas the leptin, insulin, T_3, T_4 and IGF-I contents were not significantly affected by treatment (*Table 5.6.3*.).

Table 5.6.2.: Energy balance, live weight and fat depth changes and plasma NEFA (*Exp. 6*).

					Significance	
	NF	MF	HF	SEM	Lin.	Quadr.
Weeks 1 to 4						
ME balance, MJ/d	-43.8	-54.7	-40.2	12.62		
Body weight change, kg	-9	-38	-16	9.5		*
Fat depth change, mm	-1.8	-2.3	-2.2	0.62		
NEFA (tail vein), mmol/l [1]	0.42	0.53	0.51	0.063		
NEFA (mammary vein), mmol/l [1]	0.39	0.52	0.48	0.042		
Weeks 5 to 8						
ME balance, MJ/d	-20.3	-30.9	-22.3	8.25		
Body weight change, kg	-9	-8	-2	6.4		
Fat depth change, mm	-0.9	-2.4	-0.5	0.68		+
NEFA (mammary vein, 56 d), mmol/l	0.15	0.26	0.28	0.026	**	

[1] Significant time effect (P<0.001), no significant (P>0.10) time x treatment interaction
+P<0.10 * P<0.05 ** P<0.01
ME: metabolizable energy

Table 5.6.3.: Concentrations of plasma hormones during lactation weeks 1 to 4 (*Exp. 6*).

					Significance			
	NF	MF	HF	SEM	Lin.	Quadr.	Time	Time x treatm.
Leptin, nmol/l	0.297	0.282	0.272	0.016			***	
Insulin, pmol/l	11.47	10.72	10.33	1.07			***	
T_3, nmol/l	2.04	1.71	1.83	0.126			***	
T_4, nmol/l	36.37	31.80	34.56	2.558			***	
IGF-I, nmol/l	3.78	3.03	3.22	0.323			***	

*** P<0.001

Discussion

A review by *Allen* (2000) shows that supplementation of dairy cow diets with Ca-PFA has a linear negative effect on dry matter intake, which may be partly a problem of acceptability. In the present trial, no acceptability problems were observed. However, the effect of Ca-PFA on feed intake was not uniform. Feed intake tended to decrease in group MF, but not in group HF. Our findings in this study could not explain this contradiction.

Supplemental dietary fat increases concentration of NEFA in plasma (*Chilliard*, 1993; *Staples* et al., 1998). Therefore, plasma NEFA is not a very good indicator of lipid mobilization in fat supplementation studies. Nevertheless, contrary to expected linear increase, plasma

NEFA (in mammary vein) tended to be highest in MF group (P=0.11, quadratic effect) during lactation weeks 1 to 4. Along with greater body weight and fat depth loss, this gives evidence for increased tissue mobilization in group MF. Reviews by *Chilliard* (1993) and *Staples* et al. (1998) suggested that dietary fat supplementation with saturated fatty acid sources may depress plasma insulin and enhance lipolysis in adipose tissue. The present study cannot fully confirm this theory, as there were no significant differences in plasma concentrations of insulin, IGF-I, T_4, T_3 and leptin. In a recent study of *Kay* et al. (2006) 600 g/day supplementation with conjugated linoleic acid increased the milk yield, but failed to affect plasma glucose, insulin, leptin and NEFA in transition dairy cows grazing fresh pasture in New Zealand. However, due to the differences between pastured and concentrate-fed animals, the metabolic consequences of polyunsaturated fatty acid supplementation (including the supposed changes of leptin) should be studied also in TMR-fed animals.

As a conclusion, the consumption of the studied diet – enriched with calcium salts of palm fatty acids (Ca-PFA) in concentrate for the first 8 weeks of lactation – did not influence the plasma leptin, insulin, T_3, T_4 and IGF-I levels. However, this diet elevated the milk fat content during lactation weeks 1 to 4, and tended to increase the persistency of peak yield during lactation weeks 5 to 8. Although a tendency towards increased lipid mobilization was observed with medium Ca-PFA, no consistent shift towards increased catabolism was seen with Ca-PFA.

5.7. Effects of inflammatory diseases with intensive endotoxin / cytokine release: postpartum mastitis (*Exp. 7a*, *Exp. 7b*) and puerperal metritis (*Exp. 7c*)

In high-producing dairy cows perhaps the postpartum *mastitis* (mainly the cases resulted from GN infections) 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). The further aspects of pathogenesis are summarized in our recent reviews (*Huszenicza* et al., 2004; *Földi* et al., 2006), but are not detailed here, due to the size limit of this dissertation. In our current work we wished to determine, whether there are any changes in circulating leptin levels related to these diseases in postpartum dairy cows. Three experiments were conducted: a model study with experimental intramammary endotoxin challenge (*Exp. 7a*; where also responses shown during the early vs. late lactation were compared), furthermore two field studies, with natural cases of postpartum mastitis (*Exp. 7b*) and puerperal metritis (*Exp. 7c*).

Materials and methods

Exp. 7a

In this model study 9 clinically healthy Finnish Ayrshire cows participated: two of them were primiparous and seven multiparous (2-4 parity)[45] . Cows were fed good quality grass silage and hay freely, and concentrate was given twice daily. They were milked twice a day at 6 am and 5 pm, and their milk yield was 17 to 38 kg per day in early lactation, and 5 to 19 kg per day in late lactation. All the cows were free from mastitis pathogens[46], and had low SCC in their milk (<150 000 cells /ml). A cross-over design was used: each cow (serving as its own control) was challenged twice, in the *early lactation period* (EL; 6 to 15 days of lactation), and in the *late lactation period* (LL; 137 to 77 days before the next parturition). Cows were randomly allocated into two groups; one group was challenged in the EL, and the other group in the LL for the first time. After the morning milking cows were infused into one hind quarter with 100 µg of endotoxin (*E. coli* 0111:B4 lipopolysaccharide B)[47] diluted into 5 ml of sterile physiological saline (0.9 % w/v NaCl). The same quarter was used at both challenges. Systemic and local signs of mastitis were recorded throughout the experiment. Systemic signs included general disposition, heart rate, rectal temperature, and appetite; local signs included swelling, temperature, and pain in the udder. Milk's appearance in the challenged quarter was also recorded. Systemic and local signs and milk appearance were scored from 1 to 3, using also half numbers. In this scoring system 1 resembled normal and 3 severe reaction, meaning

45 One more cow was involved, but it had to be excluded, as it did not re-conceive following the experimental endotoxin challenge in the early lactation period.

46 Proven by repeated sampling for bacteriology previously.

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

systemically rectal temperature >40.5 °C, anorexia and depression, locally severe swelling and pain into udder, and milk appearance changed to watery or pus-like (*Pyörälä* et al. 1994)[48]. Severity of the response was assessed: <2 was classified as mild, ≥2 but <3 as moderate, and 3 as severe response.

Blood (plasma) samples were taken from each cow at the time of endotoxin challenge (0 h), furthermore 2, 4, 8, 12, 24, 32, 48, and 72 h thereafter, and concentrations of NEFA, BHB, cortisol, IGF-I, insulin, leptin, T_4 and T_3 were determined[49].

Exp. 7b

This field trial was carried out in a Hungarian large-scale dairy herd with ≈1800 Holstein Friesian cows kept under free housing conditions. The farm, which was free of *Str. agalactiae,* had a continuous mastitis control program, and produced low (<400 000 / ml) SCC milk for many years. All identified cases of *S. aureus* mastitis were immediately separated, and these cows were culled soon. The cows calved in maternity boxes in groups of 3-4 animals, and calves were weaned when their dams left the unit at the end of the colostral period. Separated groups were formed for fresh-milking primi- and multiparous animals. During early weeks of lactation all cows were milked[50] 3 times a day ≈8 h apart (at ≈8.00-9.30 h, 15.30-17.00 h and 23.00-0.30 h), and were checked for mastitis[51] at the beginning of each milking. As a regular practice of the udder health care, clinical mastitis was diagnosed when the macroscopic appearance of milk was changed, with or without any other local and/or generally signs.

Within the framework of the current experiment we followed the supposed endocrine alterations in multiparous (≥2 parity) cows affected by *natural outbreak of mastitis* on days 1-14 after calving. From early April to late June 1998, all new cases of postpartum mastitis diagnosed in the herd at morning milking (e.g. at ≈8.00-9.30 h) were involved, if also systemic signs including anorexia were observed. As *controls*, healthy herd mates (almost identical in parity, days of lactation and current milk yield) were selected. In order to avoid the interfer-

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48 The response-parameter was formed by calculating the average of these scores. This parameter is handled as continuous parameter and it fits well to the normal distribution curve.

49 In addition also (i) some haematological parameters (packed cell volume, hemoglobin, total and differential leukocyte count) were determined, (ii) at each time point quarter-based milk samples were collected for somatic cell counting and measuring of N-acetyl-β-D-glucose-amininidase activity, furthermore (iii) the daily milk yield was recorded. These data, however, were published elsewhere (*Lehtolainen* et al., 2003), and are not included in the current dissertation.

50 In a milking house; milking parlors were furnished with BouMatic technology.

51 With macroscopic appearance of milk in the first few jets milked out manually.

ence with other complications, however, only cow produced low SCC milk in the previous lactation were assigned, furthermore those needed manual assistance within the birth canal at calving (dystocia), were affected by retained fetal membrane (RFM), and/or showed signs of puerperal metritis, were not involved (dystocia, RFM), or were excluded from the evaluation (puerperal metritis).

All the selected cows were removed, and separated from the others in the sanitary unit of the farm until their complete recovery (mastitic cows), or until the end of the sampling process (controls). This unit was a stanchion barn, where all animals were kept tied down, and were fed as in the maternity unit (immediate postpartum cows) or in their producing group (fresh milkers). In the sanitary unit the affected udder quarters were sampled for bacteriology, and (after a complete milking-out) were treated with intracysternal administration of 250 mg cephoperasone[52]. Cows showing severe systemic signs (rectal temperature: >40.5 C^o and/or serious anorexia and depression, or recumbency) received also 1 mg/kg BW of ceftiofur[53], repeated 24 h apart for 3-5 days. (The healthy controls were not sampled for bacteriology, and of course they were not treated.) All the mastitis-affected and control cows were regularly sampled for NEFA, BHB, cortisol, IGF-I, insulin, leptin, T_4 and T_3. For this purpose blood samples were taken first time at ≈14.00 h (on day of assignment, which represented the h ≈7 to 14 of clinical course), and again five times 6 h apart (e.g. at 20.00 h, 02.00 h, 8.00 h, 14.00 h and 20.00 h) thereafter. From the aseptic milk samples the mastitis pathogens were isolated and the pure cultures were identified based on the recommendations of *Quinn* et al. (1994) and *Sandholm* et al. (1995)[54]. If more than one mastitis pathogen was isolated, the data of animals were not evaluated. For final evaluation the mastitis-affected cows were blocked into one of 3 sub-groups depending on the category of pathogen isolated, e.g. GP or GN bacteria, or cases with no detected pathogens (NDP), and their data were compared to each others, as well as to those of the healthy controls. Due to the limited number of cases, and their similar clinical appearance and endocrine consequences, however, data from cows with GN and NDP mastitis were evaluated together, as endotoxin mastitis.

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52 Pathozone™ intramammary inf. (Pfizer AH, Exton, PA, USA)

53 Excenel™ sterile powder pro inj. (Pharmacia AH, Puurs, Belgium; currently: Pfizer AH, Exton, PA, USA), 50 mg in aqua dest., as intramuscular inj.

54 By Szilárd Jánosi (Department of Bacteriology, Central Veterinary Institute, Budapest, Hungary)

Exp. 7c

In a large-scale Hungarian 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 ≥2nd parity cows predisposed for this disease (RFM: n=10; dystocia: n=31; calving date: January to May 1997). 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. General condition of cows, furthermore quality of cervical discharge, size and tonicity of the uterus, and possible adhesions were recorded. 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 an intrauterine antibiotic therapy: an oxytetracycline-neomycin combination[55] (before d ≈15), and either a cephapirine[56] or an oxytetracycline-furazolidone-clioquinol[57] containing preparation (between d ≈15 and ≈45 after calving). This treatment was repeated 1 to 6 times, and was combined with daily administration of ergot alkaloids[58]. 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[59], 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, T_4, T_3 and TNFα levels and some acute phase proteins, such as haptoglobin (HP) and α_1-acid glycoprotein (α_1-AG). On d 9 after calving blood samples were taken in every 4 h for 24 h to analyze the diurnal leptin and TNFα patterns. Also uterine swab samples were taken for bacteriology at the beginning of the sampling period (e.g. before antibiotics are administered at the first time), 3 and 10 d later, and again at the 4 to 5 wk after calving. The uterine pathogens were isolated and identified[60], as described

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55 Exuter intrauterine tabl.™ (Biogal, Debrecen, Hungary)

56 Metricure intrauterine inf.™ (Intervet International B.V.; Boxmeer, The Netherlands)

57 Metrijet intrauterine inf.™ (Intervet International B.V.; Boxmeer, The Netherlands; not available since 1998)

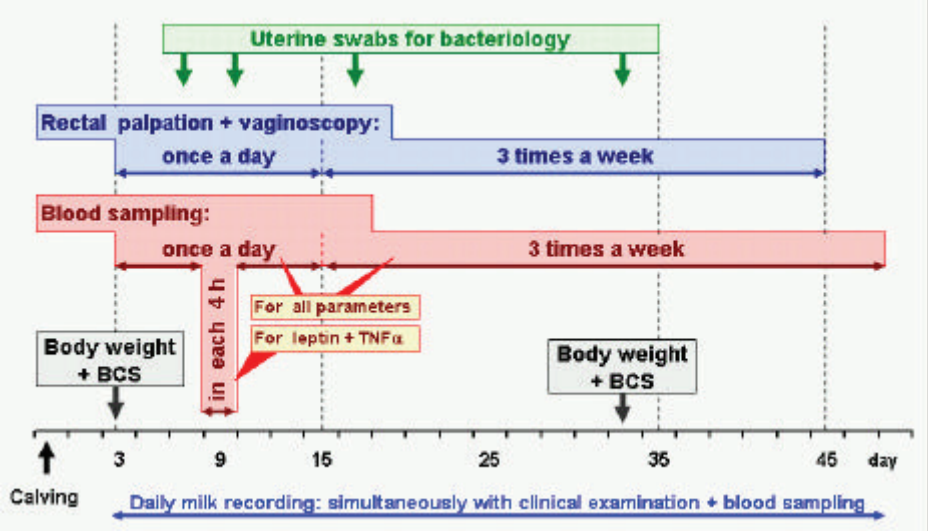
58 10 ml of Ergotin-N inj.™ (Alvetra GmBH, Neumünster, Germany; not available since 1998)

59 Engemycin inj.™ (Intervet International B.V.; Boxmeer, The Netherlands)

60 By Mihaly Fodor and Maria Vamos (Unit for Bacteriology, Central Laboratory, Jósa Andras Hospital, Nyiregyháza)

in our earlier studies (*Dohmen* et al., 1995; *Huszenicza* et al., 1999). The schedule of clinical examinations and sampling procedures are summarized on *Fig. 5.7.1*.

Fig. 5.7.1.: The schedule of clinical examinations and sampling procedures in *Exp. 7c*.



Results

Exp. 7a

After the experimental endotoxin administration clinical signs of mastitis were seen in all cows at both times of challenge, although none of the cows had a severe response. In EL, one cow showed mild and 8 cows moderate reaction, whereas in LL 8 cows showed mild and one cow moderate response. The cow with mild response in EL and the cow with moderate response in LL showed similar responses also at the other challenge time. The difference in means between the cow's response in EL and LL was significant ($P < 0.01$). The peak in systemic signs – including elevated rectal temperature and heart rate, loss of appetite, and discomfort – occurred either at 4 or 8 h post challenge (PC), varying inter-individually and temporally. After 8 h PC, systemic signs started to decrease, and were almost at baseline levels at 24 h PC in both EL and LL. Local signs were the most prominent in all cows at 4 h PC; the udder was swollen, hard, and tender, and some cows leaked milk. After 48 h, the udder returned to normal. The first visible changes in milk of the challenged quarter appeared on average at 8 h PC in EL and at 4 h PC in LL. In some cows, clots continued to be seen in the milk until the end of follow-up. Only mild changes in milk appearance occurred in the control quarters. Milk yield decreased in most cows on the first day after challenge, in average 18% in EL and 35% in LL. The milk yield returned back to baseline levels on the second day in EL and on the third day in LL.

Pre-challenge values of circulating hormones were significantly ($P < 0.05$ - 0.01; IGF-I, insulin and leptin) or tendentiously higher ($P<0.1$; T_4 and T_3), and the plasma contents of NEFA and BHB were tendentiously lower ($P<0.1$) in the LL period than during the EL, whereas the cortisol concentrations did not differ. The plasma levels of IGF-I, leptin ant thyroid hormones remained higher in LL then in EL cows throughout the 72 h-long sampling period. The endotoxin-induced 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, T_4 and T_3. Cortisol responses were similar, regardless of the stage of lactation. However, the other endocrine (insulin, IGF-I, T_4 and T_3) and NEFA changes were pronounced, and showed significant time-related tendencies 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 almost unchanged (*Fig. 5.7.2.*).

Exp. 7b

Data of 13 and 11 cows with endotoxin and GP mastitis were compared to those of healthy controls (n=13) in this study. At the beginning all of our cows with *endotoxin mastitis* 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 in *Exp. 7a*: compared to the data of their healthy counterparts, in endotoxin 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, T_4 and T_3 were on the same level in the first samples and decreased continuously afterwards (*Fig. 5.7.3.*). These endocrine changes were dramatic in the fatal cases. Our cows affected by *GP mastitis* in this study 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 unchanged or showed a mild declining tendency in all the mastitic cows, regardless of the identified pathogens and/or of the clinical course.

Fig. 5.7.2.: Endocrine and metabolic changes in cows with experimental endotoxin mastitis induced in the early vs. late stage of lactation (mean±SEM; *Exp 7a*).

Notes: between-group differences: *P<0.05 **P<0.01 ***P<0.001;
open symbols: compared to the pre-challenge level: P<0.05 (analyzed by one-way ANOVA with a post hoc testing by Neuman-Keuls test);
LPS: 100 µg of *E. coli* 0111:B4 endotoxin infused intramammarily

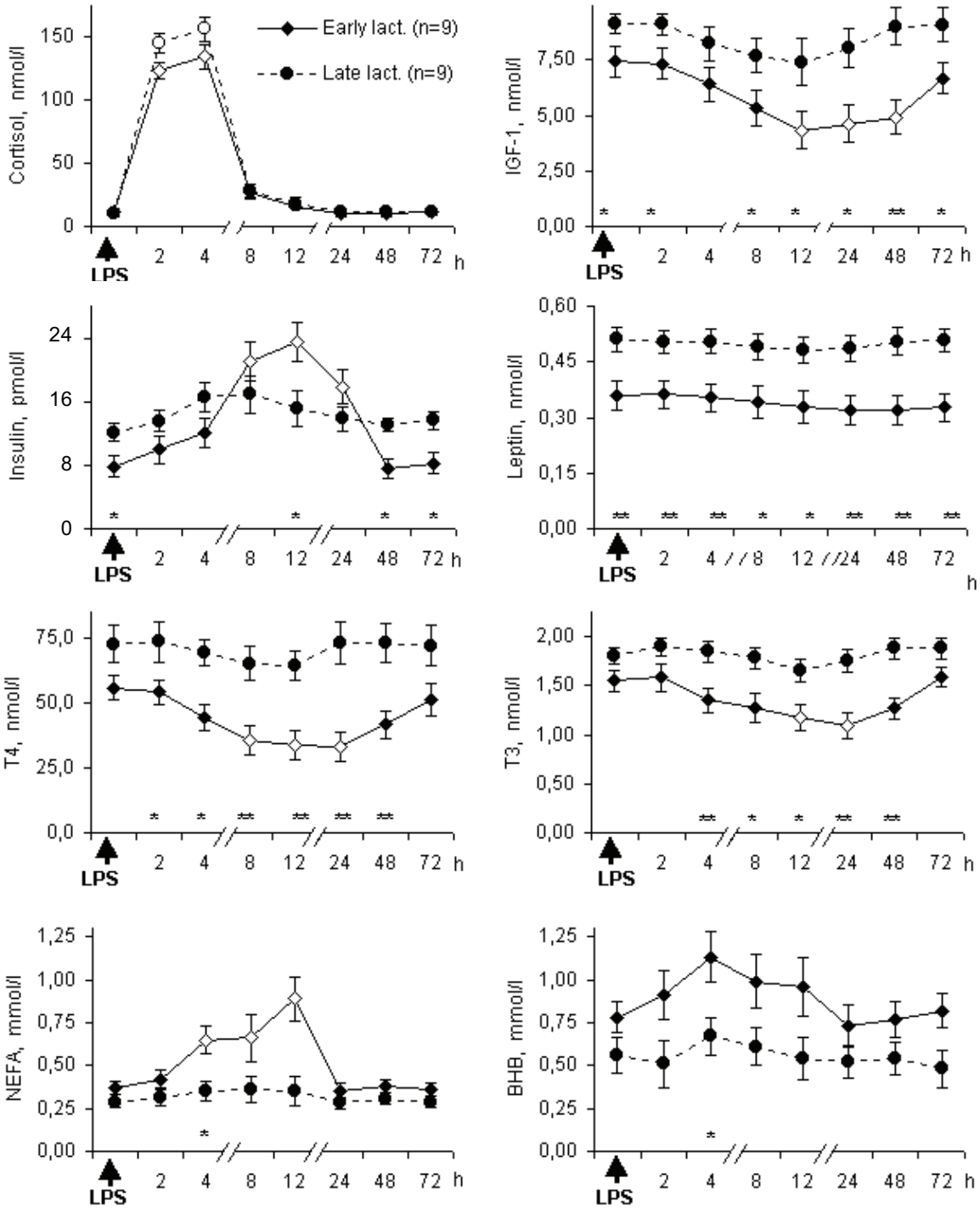
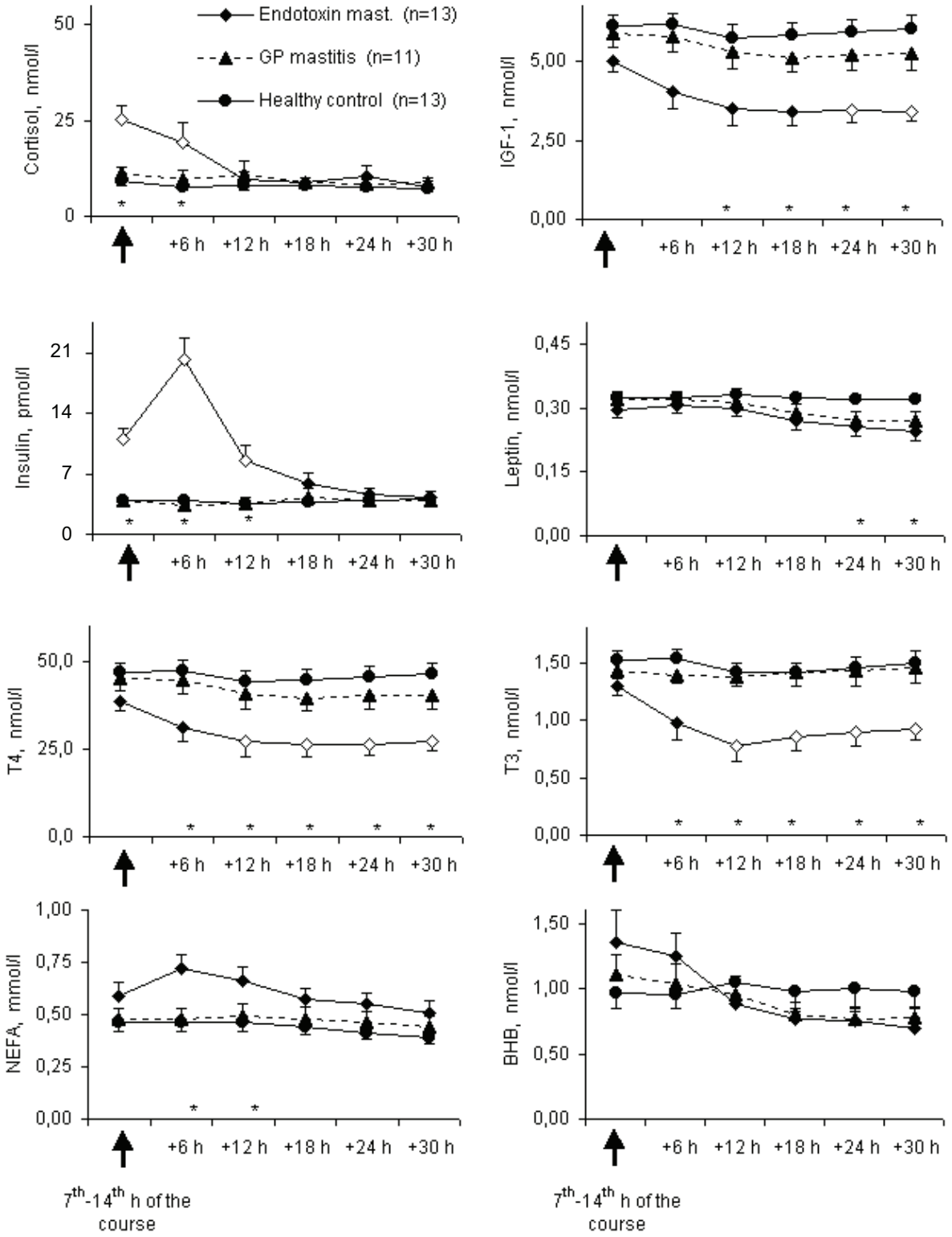


Fig. 5.7.3.: Contemporary endocrine and metabolic changes in cows with natural form of endotoxin mastitis, in cows with Gram-positive (GP) mastitis and in healthy controls (mean±SEM; *Exp 7b*).

Notes: Data of cows with endotoxin mastitis, compared to those with Gram-positive (GP) mastitis as well as to the healthy controls: *P<0.05 **P<0.01 ***P<0.001;
Time-related changes between open and filled symbols: P<0.05 (analyzed by one-way ANOVA with a post hoc testing by Neuman-Keuls test);
Outbreak of mastitis: on days 1-14 after calving;
Regular sampling was started 7-14 h after the clinical outbreak



Exp. 7c

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 (*Fig. 5.7.4*.).

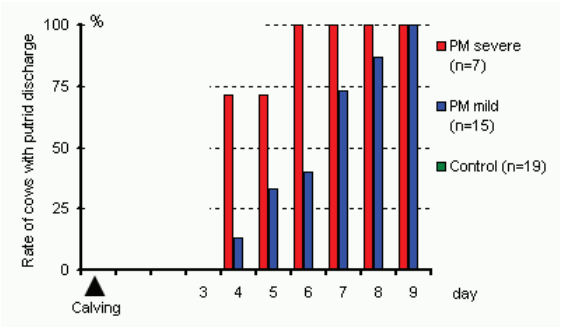
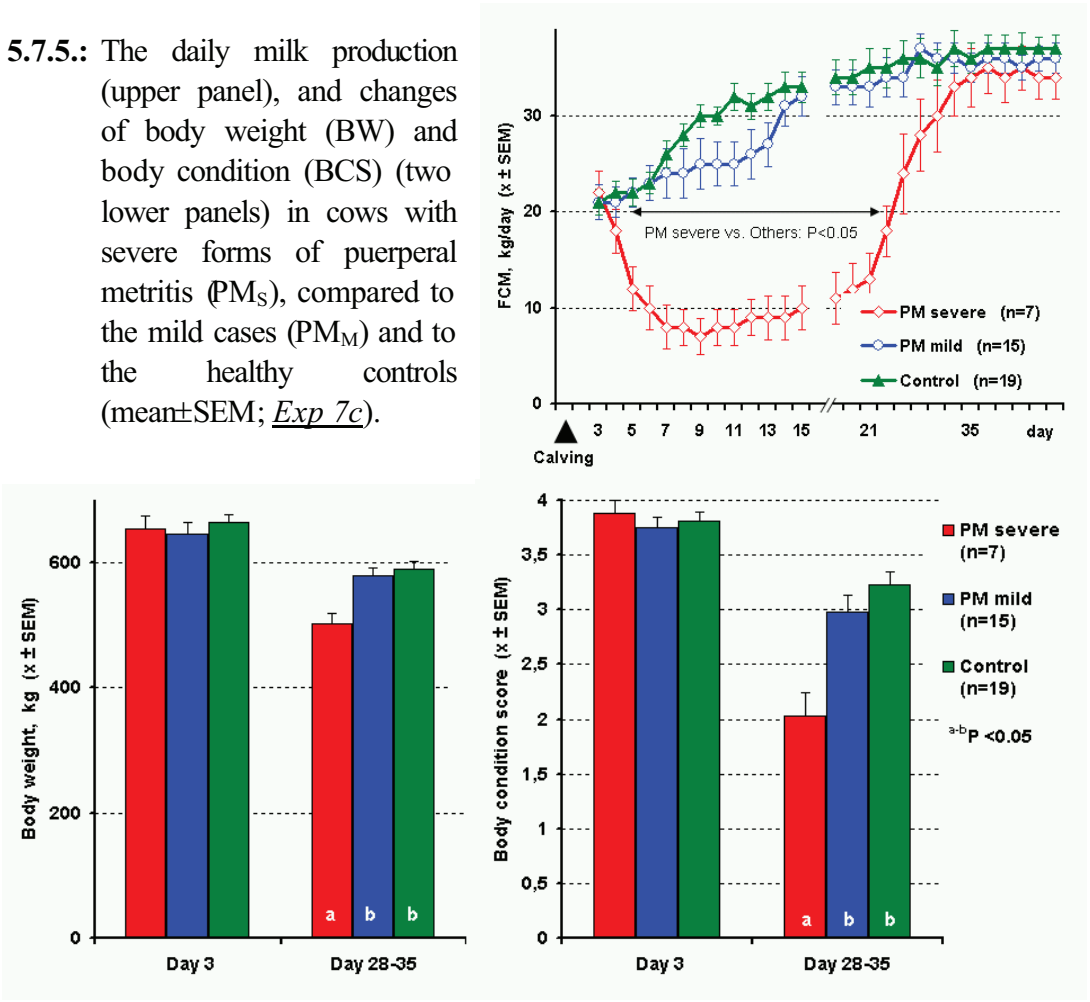


Fig. 5.7.4.: The first appearance of fetid (foul-smelling, reddish, watery) vaginal discharge in cows with severe forms of puerperal metritis, compared to the mild cases (*Exp 7c*).

Compared to those found on d 3 after calving, during the first four weeks of lactation all cows lost body weight (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 (*Fig. 5.7.5*.).

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 (rate of cows with BHB >1.00 mmol/l: *Fig. 5.7.6*., lower panel). At the beginning the NEFA, insulin, IGF-I, leptin, T_4 and T_3 concentra-tions of PM_S, PM_M and healthy cows did not differ. The insulin, IGF-I, T_4 and T_3 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 (IGF-I

Fig. 5.7.5.: The daily milk production (upper panel), and changes of body weight (BW) and body condition (BCS) (two lower panels) in cows with severe forms of puerperal metritis (PM_S), compared to the mild cases (PM_M) and to the healthy controls (mean±SEM; *Exp 7c*).



and leptin: *Fig. 5.7.6.*, upper panel). The α_1-AG content increased at least moderately in all the metritis-affected animals. However, fluctuating elevations in plasma TNFα and HP were detected only in PM_S cows. These tendencies related to the presence and severity of uterine complications, but were not altered by the uterine culture. Neither diurnal pattern of leptin, nor clear tendencies other than irregular fluctuation of TNFα were found in samples taken 4 h apart for 24 h on d 9 after calving (details are not given). Significant delay in resumption of cyclicity and depressed re-conception rate were detected only in PM_S cows (*Fig. 5.7.7.*). The further details on interrelation of uterine bacteriology, clinical condition, changes of acute phase proteins (HP and α_1-AG) and ovarian cyclicity and fertility were published earlier (*Hirvonen* et al., 1999a; *Huszenicza* et al., 1999; Huszenicza, 2003).

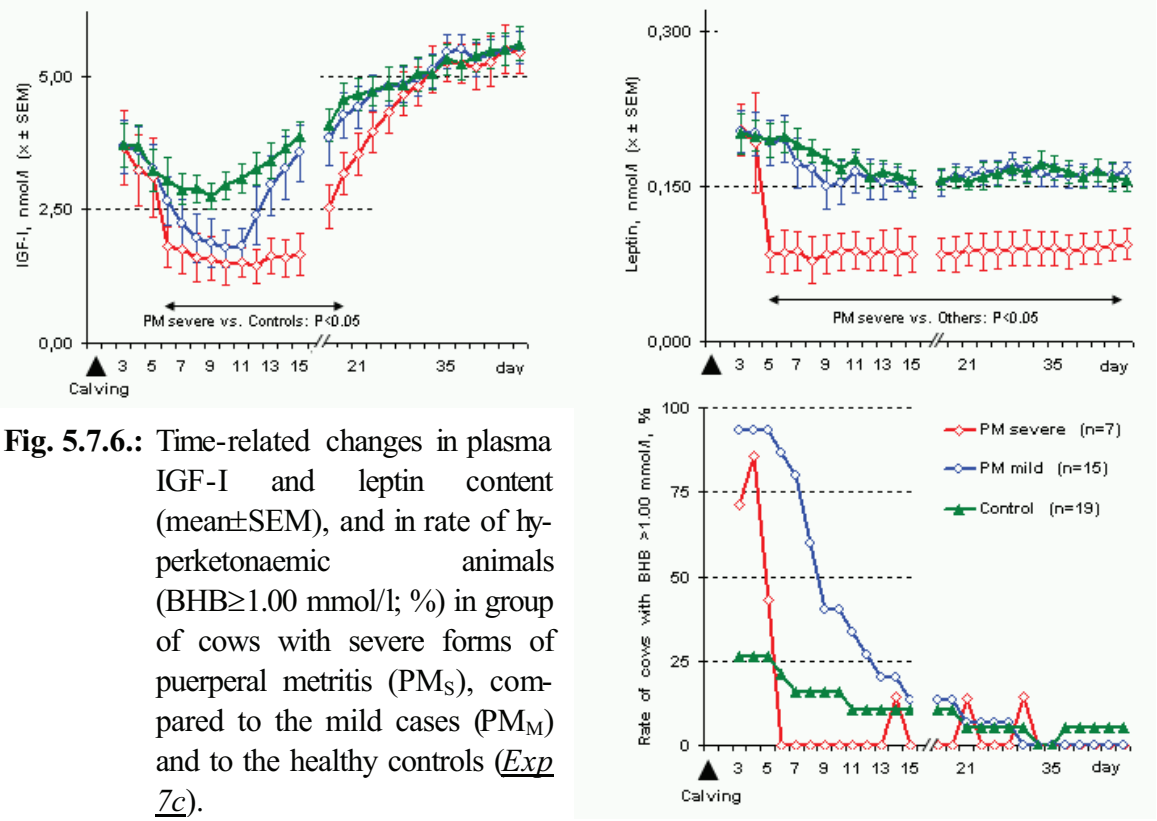


Fig. 5.7.6.: Time-related changes in plasma IGF-I and leptin content (mean±SEM), and in rate of hyperketonaemic animals (BHB≥1.00 mmol/l; %) in group of cows with severe forms of puerperal metritis (PM_S), compared to the mild cases (PM_M) and to the healthy controls (*Exp 7c*).

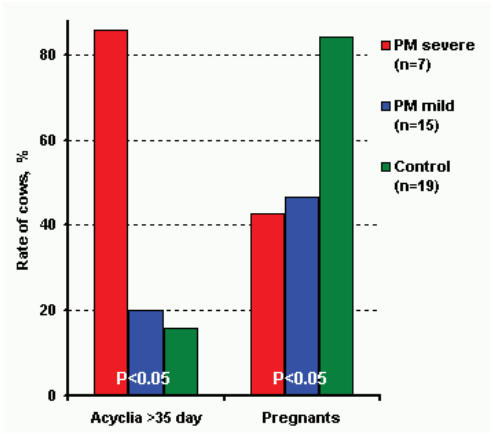


Fig. 5.7.7.: Rate of animals not ovulating in the first 5 wk (e.g. acyclia >35 d), and of those re-conceived until d 150 after calving in the three clinical groups (*Exp 7c*).

Discussion

The most severe cases of *puerperal metritis* and *mastitis* are supposed to result in excessive liberation of cytokines, eicosanoids and oxygen free radicals (*Cullor*, 1992; *Salyers* and *Whitt*, 1994; *Sandholm* et al., 1995; *Sordillo* and *Daley*, 1995; *Jánosi* et al., 1998; *Hirvonen* et al., 1999a and 1999b; *Dohmen* et al., 2000; *Mateus* et al., 2003; *Hoeben* et al., 2000a; *Lehtolainen* et al., 2003). Septicaemia and/or inflammatory diseases with intensive endotoxin and cytokine release may induce a complex endocrine cascade, involving the activity of hypothalamus - anterior pituitary system, both parts of the adrenal, the endocrine pan-

creas, furthermore the thyroid hormone and GH / IGF-I production and metabolism (*Elsasser* et al., 1995, 1996 and 2005). As it was discussed earlier (Chapter 3.8.), in lab rodents and Primates also distinct changes have already been demonstrated in leptin gene expression and circulating leptin levels (*Landman* et al., 2003; *McCann* et al., 2003; *Xiao* et al., 2003), suggesting that leptin can be both released in response to inflammatory cytokines, and act to attenuate the responses to these cytokines. Plasma leptin was elevated in human subjects of acute sepsis (*Bornstein* et al., 1998; *Torpy* et al., 1998; *Arnalich* et al., 1999), with 3-fold higher levels in patients who survived the septic episode than in non-survivors (*Bornstein* at al., 1998). However, to the best of our knowledge the involvement of leptin in the inflammatory-induced cytokine-endocrine cascade has not been studied yet in mastitis and puerperal metritis of postpartum dairy cows.

As seen in rodent models (*Kakizaki* et al., 1999; *Gaillard* et al., 2000), the initial cytokine (TNF-α and IL-1β) response increases the hypothalamic release of corticotrophin-releasing hormone that activates the **pituitary-adrenal axis** also in ruminants. Both the intravenous (iv) and intramammary forms of endotoxin challenge are followed by dose-dependent temporary (≤6-8 hr) elevation of plasma cortisol level also in lactating cows (*Hirvonen* et al., 1999b; *Waldron* et al., 2003). Peak levels are seen 2-4 h after challenge (*Soliman* et al., 2002; *Waldron* et al., 2003). The experimental administration of purified recombinant bovine TNF-α ($_{rb}$TNF-α) induces similar but shorter elevation in plasma cortisol level (*Soliman* et al., 2002).

After iv endotoxin administration increasing **prolactin** levels were seen in rats (*Rettori* et al., 1994). In cows obvious increase in prolactin production was observed only after iv but not after intramammary endotoxin challenge, and it was supposed to play role in the immunmodulation but not in the milk production of the udder (*Jackson* et al., 1990). In sows the experimental endotoxin administration was capable of decreasing both the prolactin level and milk production in the first week after farrowing but not later (*Smith* and *Wagner*, 1984 and 1985). In rat models there are a variety of other cytokine-induced changes in the central nervous system, as well, such as alterations of central and peripheral **catecholamine** levels (*Song* et al., 1999; *Wang* and *White*, 1999), and of **neurotransmitter** release in the hypothalamus (*Mohan-Kumar* et al., 1999). These hypothalamic changes are likely involved in the pathomechanism of endotoxin-induced impairments in the basal and preovulatory-like **LH**

secretion (*Rettori* et al., 1994; *McCann* et al., 2003), which were observed to occur and to damage the final maturation and ovulation of antral follicles not only in lab rodents (*McCann* et al., 2003), but also in ruminants (heifers: *Suzuki* et al., 2001; ewes: *Battaglia* et al., 2000; *Daniel* et al., 2002b; for further details on reproduction, please, see 3 of our most recent papers: *Huszenicza* et al., 2004 and 2005; *Földi* et al., 2006).

Experimental endotoxin treatment decreased plasma **GH** levels in rats (*Rettori* et al., 1994), whereas in cattle (*Elsasser* et al., 1995 and 1996) and sheep (*Daniel* et al., 2002b), it induced mild to moderate elevations in circulating GH concentrations, and a slower but marked reduction in plasma levels of **IGF-I**. This endotoxin action on GH secretion is mediated by TNF-α and IL-1β (*Daniel* et al., 2005). The uncoupling of the GH-IGF-I axis demonstrated by *Elsasser* et al. (1995 and 1996) was accompanied by a decrease in production of one of the IGF-I binding proteins (IGFBP-2). A simultaneous decline in IGF-I and a moderate elevation in IGFBP-1 plasma levels contrasted with the increase in GH secretion suggest that endotoxin causes also a state of resistance to GH, which is exacerbated by a simultaneous reduction in IGF-I bioavailability (*Briard* et al., 2000). In a most recent trial (*Waldron* et al., 2003) the iv endotoxin challenge failed to induce any change in GH and IGF-I levels of multiparous lactating dairy cows. However, IGFBP-s influencing the bioavailability of this hormone were not studied there.

The endotoxin-induced endocrine cascade involves also the glucoregulatory **pancreatic** hormones. Simultaneously with the TNF-α and cortisol elevations a significant, but only transient increase in insulin levels (*Steiger* et al., 1999; *Waldron* et al., 2003), and a clearly dose-dependent, more obvious growth in glucagon levels (*Waldron* et al., 2003) were reported to occur. Following temporary hyperglycemia insulin resistance and decreased glucose concentrations were observed 6 and 24 h after the endotoxin challenge (*McMahon* et al., 1998). Similar changes in glucose and insulin levels, as well as in insulin resistance were induced also by iv administration of $_{rb}$TNF-α (*Kushibiki* et al., 2000; *Soliman* et al., 2002*)*. The exact mechanism inducing these endotoxin-related alterations in endocrine pancreatic function has not been fully understood: beside the effect of nitric oxide and TNF-α also the early elevation of plasma cortisol and catecholamine levels or sympathetic neuroendocrine changes may be implicated (*Waldron* et al., 2003).

A number of infectious and inflammatory (mainly endotoxin-mediated) diseases may be associated with subsequent, obvious changes in **thyroid** status. This so-called *euthyroid sick syndrome* is observed during systemic non-thyroidal illness, and consists of decreasing plasma concentration of the active thyroid hormone (T_3) with a simultaneous elevation in its inactive metabolite (rT_3) and, in severe cases, a reduction in T_4 and TSH concentrations. Most of these changes are caused by a lower T_3 production rate mainly due to the diminished extrathyroidal enzymatic activation (e.g. outer-ring deiodination) of T_4 by 5'-deiodinase (5'D), in combination with a decreased rT_3 clearance rate (*Wartofsky* and *Burman*, 1982). TNF-α and IL-1 may inhibit the TSH release from pituitary cells, and decrease the activity of type-I 5'D in thyroid and liver tissues (*Haastaren* et al., 1994; *Rettori* et al., 1994; *Hashimoto* et al., 1995), which reduce the production and circulating level of T_4, and inhibit the transformation of T_4 to T_3 (*Bartalena* et al., 1998; *Bertók* 1998). In endotoxin-treated cows *Kahl* et al. (2000) reported decreased 5'D activity in the liver, which resulted in lower T_3/T_4 ratio and declining plasma concentrations of thyroid hormones.

In lactating cows simultaneously with these endotoxin-induced endocrine alterations marked changes were reported to occur also in plasma levels of certain **energy-related metabolites**: concentrations of glucose tended to increase initially and subsequently declined, there was a tendency for increased NEFA levels, whereas plasma ketones (BHB) decreased linearly by dose after endotoxin infusion (*Waldron* et al., 2003).

In accordance with the literature cited above, the studied elements of endotoxin/cytokine-induced multiple endocrine response reflected the expected tendencies in all of our 3 related experiments: concentrations of insulin and glucose tended to increase initially and subsequently declined (although sampling was frequent enough to detect these some-hours-long temporary changes only in our mastitis studies), there was a tendency for temporary increased NEFA levels, whereas plasma levels of T_4, T_3, IGF-I and leptin decreased. Endocrine changes found after natural outbreak of mastitis (in *Exp. 7b*) reflected the same tendencies as seen in the corresponding stage of the course after intramammary endotoxin challenge in *Exp. 7a*, and clearly related to dose detected in puerperal metritis (in *Exp. 7c*).The same alterations were recorded to occur in plasma levels of energy-related metabolites, hormones and also in endotoxin- or TNFα-treated lactating cows (*Elsasser* et al., 1995 and 1996; *Kushibiki* et al., 2000; *Soliman* et al., 2002; *Waldron* et al., 2003; *Elsasser* et al.,

2005), and in the natural cases of GN mastitis early in the postpartum period (*Nikolic* et al., 2003).

Cows' responses to standard intramammary endotoxin challenge in *Exp. 7a*, defined by local and systemic signs, to intramammarily infused *E. coli* endotoxin were significantly more severe in EL than in LL. The more severe response in EL has also been observed in experimental *E. coli* mastitis models (*Hill* et al., 1979; *Shuster* et al., 1996), which have, however, used different groups of cows over time. Our cross-over study design diminished the interindividual variation (*Pyörälä* et al., 1994). All animals responded rapidly to the endotoxin infusion, consistent with earlier studies (*Hoeben* et al., 2000a; *Mehrzad* et al., 2001). Local signs were clearly seen at 2 h post challenge (PC), and systemic signs at 4 to 8 h PC. Cows had similar local responses both in EL and LL, but systemic responses were more severe in EL. Both local and systemic signs are mediated by TNFa and interleukins, which are produced by macrophages in the mammary gland. Differences in their production and kinetics may cause variation in local and systemic signs (*Hoeben* et al., 2000a and 2000b). NEFA and BHB values were higher, whereas IGF-I, leptin and thyroid hormone levels were much lower in EL than in LL throughout the experiment, which reflects the metabolic state of early lactation, characterized by fat mobilization and possible subclinical ketosis (*Burvenich* et al., 1994; *Jorritsma* et al., 2001; although none of these alterations of LL cows were very overt, perhaps due to the well-defined individual feeding of animals in this model study). This has been suggested to interfere with neutrophil functions (*Hoeben* et al., 1997; *Suriyasathaporn* et al., 1999 and 2000; *Sartorelli* et al., 1999 and 2000; *Zerbe* et al., 2000), and could be one reason for more severe inflammatory response seen in EL cows in this study.

In *Exp. 7b*, when specific pathogens were not isolated (NDP mastitis) the data were pooled with those from cows affected by GN mastitis in the current study. Chronic subclinical *Staphylococcus aureus* (*S. aureus*) infections can also result in false negative findings in mastitis bacteriology (*Sandholm* et al. 1995). However, cows producing milk with continuously elevated SCC were not involved in this study. So in accordance with *Fang* and *Pyörälä* (1996) we think that at the beginning, most of our cases with NDP mastitis were infected with GN bacteria (mainly *E. coli*), but before sampling, the original pathogens were eliminated by the self-defense mechanisms of the udder.

We conclude that in postpartum dairy cows also the inflammatory diseases with intensive cytokine / endotoxin release (such as puerperal metritis and severe forms of clinical mastitis) influence the circulating levels of metabolic hormones including a depression in plasma leptin. The GN mastitis can induce the same endocrine alterations as the experimental (iv or intramammary) endotoxin or $_{rb}$TNFα challenges. However, in natural cases these changes may vary within a wide range, and may be more protracted and robust. Our cows with puerperal metritis showed more obvious catabolic changes than the controls. The leptin level dropped immediately in all cases, as a part of the inflammatory reaction, and remained continuously low thereafter. We think these changes in plasma leptin are the consequences, rather than the causative elements of anorexia associated with infection-induced inflammatory response in postpartum dairy cows.

6. 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, but testosterone is responsible for the gender dichotomy of plasma leptin (*Exp. 1c, 1d, 1e*).
2) The 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 (*Exp. 2*).
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 (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. 3*).
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 NEB and the level of body reserves, include IGF-I and leptin (*Exp. 4a* and *4b*).
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 (*Exp. 5a* and *5b*).

6) Consumption of a diet enriched with calcium salts of palm fatty acids does not influence the plasma leptin (*Exp. 6*).

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. However, these changes in plasma leptin are only consequences, rather than the causative elements of anorexia associated with infection-induced inflammatory response in ruminants (*Exp. 7a*, *7b* and *7c*).

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Suppl. 1

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 postpartum 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 tejtermelésu tehén takarmányozásának, tejtermelésének és szaporodóképességének kapcsolata. Irodalmi áttekintés. 2. A petefészek muködése az ellés utáni idoszakban. *Magy. Áo. Lapja*, 2003. 125 (58). 75-82. (**IF**: 0.089)

2.2. Kátai L, **Kulcsár M.**, Kiss G., Huszenicza Gy.: A nagy tejtermelésu tehén takarmányozásának, tejtermelésének és szaporodóképességének kapcsolata. Irodalmi áttekintés. 3. Az újravemhesülés zavarai. *Magy. Áo. Lapja*, 2003. 125 (58). 143-146.

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2.3. Huszenicza Gy., **Kulcsár M.**, Dankó G., Balogh O., Gaál T.: A nagy tejtermelésu tehén takarmányozásának, tejtermelésének és szaporodóképességének kapcsolata. Irodalmi áttekintés. 4. A ketonanyag-képzodés 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., Árnyasi M., **Kulcsár M.**, Cseh S., Huszenicza Gy.: Az anyajuhok szaporodási teljesítményének növelésével összefüggo 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 eredetu szövodményei 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., Danko, 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, Deutsland), pp. 133-151.

Suppl. 2

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-muködés újraindulása az ellés utáni idoszakban nagy tejtermelésu szarvasmarhában. In: Csernus V., Mess B.: Biológiai órák: ritmikus biológiai folyamatok az élovilágban. Akadémiai Kiadó, Budapest, 2006. pp. 137-150.

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