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Role of insulin in the development of metabolic and reproductive malfunctions of periparturient dairy cows

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List of abbreviations

AI	artificial insemination
ANOVA	analysis of variance
AUC	area under the curve
BCS	body condition score
BHB	beta-hydroxybutyrate
cHK	continuously hyperketonemic
CL	corpus luteum
CLA	conjugated linoleic acid
Co A	coenzyme A
CR	clearance rate
CTL	control
CV	coefficient of variation
DF	dominant follicle
DMI	dry matter intake
E2	17β-estradiol
ELISA	enzyme-linked immunosorbent assay
FSH	follicle stimulating hormone
GH or STH	growth hormone or somatotrophine
GHR	growth hormone receptor
GLUT	glucose transporter
GnRH	gonadotrope releasing hormone
GTT	glucose tolerance test
HF	Holstein-Friesian
HIEC	hyperinsulinemic-euglycemic clamp test
HK	hyperketonemia
HTH	hypothalamus
IGFBPs	IGF-I binding proteins
IGF-I	insulin-like growth factor-I
IL	interleukin
IR	insulin resistance
IRMA	immunoradiometric assay
ISBGR	insulin stimulated blood glucose response
ITT	insulin tolerance test
iv.	intravenous
IU	international unit
LH	luteinizing hormone
LM	lipid mobilization
LPS	lipopolisacharide
mRNA	messenger ribonucleic acid
NEB	negative energy balance
NEFA	non-esterified fatty acids
NE_L	net energy of lactation
NK	normoketonemic
P4	progesterone
PGF2α	prostaglandin F2 alpha
PGL	propylene glycol
PM	puerperal metritis
pp	postpartum
PPAR	peroxisome proliferator-activated receptor
PUFA	polyunsaturated fatty acid
RIA	radio immunoassay
RQUICKI	revised quantitative insulin sensitivity check index
SCFA	short chain fatty acid
$T_{1/2}$	half-life
T_3	3,3',5-triiodothyronine
T_4	thyroxine
TCA	tricarboxylic acid
TG	triglyceride
tHK	transiently hyperketonemic
TMR	total mixed ration
TNF-α	tumor necrosis factor-alpha
TRH	thyrotropin releasing hormone
VLDL	very low densitiy lipoprotein

Summary

In dairy cows selected for high milk production the periparturient insulin resistance (IR) may play a pivotal role both in adaptation to the energy demands of milk synthesis and in the pathogenesis of some metabolic malfunctions and organic diseases related to the negative energy balance (NEB). Insulin is the most important anabolic hormone with significant functions in carbohydrate, lipid and protein metabolism, which acts to maintain body depots and to prevent ketogenesis. Moreover, insulin is one of the key metabolic molecules which mediate the crosstalk between the hypothalamic-ovarian axis and the body energy state. In humans the release of cytokines, that occurs in association with obesity and inflammatory diseases (especially in those with endotoxemia), and the release of non esterified fatty acids plays an important role in the development of IR. In dairy cow inflammatory diseases with intensive endotoxin/cytokine release (puerperal mastitis and metritis, clinical endometritis) are frequent complications in the puerperal phase. Our understanding on the relationship between periparturient metabolic disorders, insulin resistance and the poor reproductive performance in the modern dairy cow is limited, yet.

Our goal was to investigate the periparturient insulin pattern and IR in high lactating dairy cows in relation with some metabolic and reproductive malfunctions and to improve postpartum ovarian function through energy supplementations in cows under different management systems.

We showed that pancreatic ß-cell function and the biological potency of insulin is impaired in cows with long-term hyperketonemia. Short-term elevations in plasma free fatty acids and ketone bodies may not potentially induce further increase in peripheral tissue insulin resistance in the early lactation. However, severe inflammatory diseases like puerperal metritis may potentially further depress insulin secretion of the pancreatic ß-cells and the whole body insulin responsiveness of dairy cows, with long-term effects on metabolism and reproduction. Furthermore we also found that the revised quantitative insulin sensitivity check index (RQUICKI) as was assessed earlier only in healthy animals should be applied with cautions in the assessment of insulin sensitivity in dairy cows in different physiological and disease states *(Exp. 1)*. We found that top-dressing of pulverized propylene glycol (PGL) on the total mixed ration from d 14 before calving till

d 10 after calving was not effective in improving metabolic profile, insulin sensitivity, the time of first postpartum ovulation and pregnancy rate. Most probably the relatively good energy balance of the animals involved in our study limited the effectiveness of the supplement. However, the method of allocation and the absorbent incorporated for the PGL product used in the present study contributed to the inefficacy *(Exp. 2).* In contrast, in lean cows, which were kept under pasture condition, the pre-partum supplementation with cracked corn grain improved the energy balance, peripheral insulin concentrations and decreased the time to the first pp ovulation. *(Exp. 3).* It is obvious that the effectiveness of periparturient energy supplementation is greatly dependent on the initial energy state of the animals.

We hope that our results contribute to our understanding on dairy cow physiology and help to choose appropriate dietary tools in improving metabolic and reproductive performance.

Összefoglalás

Tejhasznú szarvasmarhában az ellés körül kialakuló inzulinrezisztencia (IR) központi szerepet játszik mind a tejtermelés energiaszükségletéhez való alkalmazkodásban, mind pedig egyes ellés körüli anyagforgalmi- és szaporodásbiológiai zavarok kórfejlődésében. Az inzulin a lipidmobilizáció és a ketonanyagok képződése ellen ható egyik legfontosabb anabolikus hatású hormon. Az anyagcserében betöltött szerepe mellett számos szaporodásbiológiai hatása is ismert: szérumszintje befolyásolja a hipofízis-petefészek-tengely működését, valamint a tüszők és a sárgatest szteroidgenezisét. Emberben bizonyított, hogy összefüggés van az elhízás, a plazma magas szabadzsírsav-koncentrációja, egyes gyulladásos mediátorok és az IR kialakulása között. A laktáció korai szakaszában szarvasmarhában gyakoriak a nagy mennyiségű endotoxin-felszabadulással járó szaporodásbiológiai zavarok, mint például a mastitis, vagy a puerperális metritis. Ugyanakkor az ellés körül kialakuló anyagforgalmi zavarok, az inzulinrezisztencia és a csökkent szaporodásbiológiai teljesítmény közötti kölcsönhatásokról szóló ismereteink ma még hiányosak.

Célunk volt tejhasznú szarvasmarhában az ellés körüli inzulinrezisztencia, valamint egyes anyagforgalmi és szaporodásbiológiai zavarok közötti összefüggések tanulmányozása. Vizsgáltuk továbbá, hogy különböző tartási körülmények között az ellés körül alkalmazott energia-kiegészítés milyen hatással van az inzulin szérumszintjére, a hasnyálmirigy β-sejtjeinek inzulintermelésére, az inzulinrezisztencia fokára, valamint a petefészek működésére.

Kimutattuk, hogy a tartós hyperketonaemia, valamint a puerperális metritis tovább csökkentik a hasnyálmirigy inzulin-elválasztását és annak biológiai hatását Holstein-Fríz tehenekben. Korábban csupán egészséges tehenekben vizsgált inzulinérzékenységi index (Revised Quantitative Insulin Sensitivity Check Index) eredményeink szerint csak körültekintően alkalmazható az inzulinérzékenységben bekövetkező változások mérésére eltérő élettani állapotú, illetve különböző puerperalis kórképben (szubklinikai ketosis, puerperalis metritis) szenvedő állatokban *(1. kísérlet)*. Kimutattuk továbbá, hogy az ellés körül a monodiétás takarmány kiegészítése porított propilénglikollal nem volt hatással az általunk vizsgált anyagforgalmi és endokrin paraméterekre, illetve nem befolyásolta az első

ovuláció idejét intenzív tartási körülmények között tartott nagy tejtermelésű tehenekben. Ennek hátterében nemcsak a kiegészítés módja, és a vivőanyag bendőbeli emésztést befolyásoló tulajdonsága állhat, hanem a vizsgálatba bevont állatok viszonylag jó energia-ellátottsága is *(2. kísérlet).* Ezzel szemben, legelőre alapozott takarmányozáskor a vemhesség utolsó heteiben gyenge tápláltsági állapotú tehenekben az ellés előtt alkalmazott kukoricadara alapú enegia-kiegészítés javította az állatok energia-egyensúlyát, növelte a perifériás inzulinszinteket, valamint elősegítette, hogy a petefészek működése az ellést követően mielőbb ciklikussá váljon *(3. kísérlet)*.

Reményeink szerint eredményeink hozzájárulnak az ellés körüli anyagforgalmi és hormonális változások jobb megértéséhez, illetve segítenek a megfelelő takarmányozási eszközök kiválasztásában a nagy tejtermelésű tehenek szaporodásbiológiai állapotának javításához.

1. Introduction

Reproductive performance in dairy cows has declined over the past several decades in association with remarkable increases in milk yields both in confined and in pasture-based systems. In the early weeks of lactation the metabolic demands of high milk production almost imperatively result in negative energy balance (NEB) characterized by dramatic changes in blood metabolites and hormones. NEB by itself is a physiological phenomenon; which may, however, postpone the time of first postpartum ovulation and estrus, can cause metabolic disorders (fat accumulation in the hepatocytes, increased production of ketone bodies) and decrease antimicrobial self-defense mechanisms. This later phenomenon predispose the affected cows for bacterial complications of uterine involution (puerperal metritis, clinical endometritis) and mastitis, and finally resulting in poor reproductive performance (Jorritsma et al., 2000; Suriyasathaporn et al., 2000; Butler 2000 and 2001; Jánosi et al., 2003; Huszenicza et al., 2004; Földi et al., 2006).

An important homeorhetic adaptation to the energy demand of gestation and lactation is represented by the reduced insulin secretion of the pancreatic ß-cells, paralleled by a decreased insulin responsiveness of the peripheral tissues to the action of this hormone (Bell, 1995; Bell and Bauman, 1997). Increasing number of studies are focusing on the role of insulin resistance (IR) in the development of periparturient metabolic and reproductive disorders in dairy cow (Butler et al., 2003 and 2004; Hayirli, 2006; Chagas et al., 2007ab; Balogh et al., 2008; Bossaert et al., 2008). Moreover, the link between obesity, release of pro-inflammatory cytokines and insulin resistance similar to the human diabetes type 2 has been reported to occur (Hirvonen et al., 1999; Kushibiki et al., 2000, 2001ab; Ohtsuka et al., 2001; Kulcsár et al., 2005a,b; Martens, 2007). Therefore there is an increased interest toward nutritional tools that limit the duration and magnitude of the NEB and regulate metabolic signaling, which are expected to have positive effect also on reproductive performance. The endocrine signals that most likely can inform the hypothalamic GnRH-producing neurons on the current state of body condition and energy balance involve insulin, insulin-like growth factor I (IGF-I) and leptin. Energy supplementation, as well as specific nutrients may interact with metabolism and reproductive performance (Roche et al., 2006). With some known limitations, energy intake can be increased by feeding

supplements based on starch or non-structural carbohydrates, or adding fat to the diet. Starch or glucogenic supplements, like propylene glycol (PGL) or glycerol – besides improving energy balance – reduce lipid mobilization and may have major impact on insulin secretion and on the magnitude of insulin responsiveness of the whole-body.

After reviewing the related literature, in the current thesis I wish to summarize our recent experiences regarding:

- with periparturient changes of pancreatic β-cell function and insulin resistance in healthy and diseased animals, and their interaction with metabolism and reproduction;
- how the insulin levels, glucose-induced insulin response and insulin-induced glucose reply may be influenced by
    - periparturient glucogenic feed additives (PGL),
    - prepartum administration of starch-rich supplements.

2. Review of literature

2.1. Physiology of negative energy balance and its effect on reproductive performance

2.1.1. Metabolic and endocrine physiology of postpartum negative energy balance

In the high yielding dairy cow the rapid increase in milk production after parturition is not paralleled by increased dry matter intake (DMI). At the same time the sensitivity of the adipose tissue to lipolytic signals (epinephrine and norepinephrine) is increased (Chilliard, 1993), while peripheral concentration of insulin is decreased during this period, acting toward more intensive lipid mobilization from body reserves (Bell, 1995; Hayirli, 2006). Circulating levels of non-esterified fatty acids (NEFA) derived from the adipose tissue tend to increase during late pregnancy, even in animals carefully fed to predicted energy requirement. In the liver NEFA can be completely oxidized into carbonic dioxide and water or partially oxidized into ketone bodies, while a part of it will be exported in the form of very-low density lipoprotein (VLDL) and can be utilized for milk fat synthesis in the mammary gland (Rukkwamsuk et al., 1999; Grummer et al., 2004; Bobe et al., 2004). As negative energy balance increases, more NEFA are released from body fat and the concentration of NEFA in blood increases. All lactating dairy cows encounter some degree of NEB after calving. Moderate increase in the concentration of acetoacetate, acetone and β-hydroxybutyrate (BHB) in blood and other biological fluids is detectable during early lactation in the majority of healthy postparturient animals (Baird, 1982; Leslie et al., 2000; LeBlanc et al., 2005). When NEB becomes decompensated, hyperketonemia with simultaneous hypoglycemia may develop (Baird, 1982; Rukkwamsuk et al., 1999). NEB, increased concentrations of NEFA and ketone bodies are highly associated with health disorders in dairy cattle. *Fatty liver* and *ketosis* are the most common metabolic disorders in the peripartum period. Factors involved in the etiology of fatty liver and ketosis are similar and in both disorders many of the important liver functions are impaired (Drackley et al., 2001; Hayirli et al., 2002).

Fatty liver or *hepatic lipidosis* refers to accumulation of lipids in hepatocytes. Triglyceride (TG) is the major type of lipid that accumulates in the liver of normal and obese dairy cows (Gaál T., 1983). It develops when the hepatic uptake of NEFA exceeds

the rate of oxidation and secretion of lipids by the liver. There is no difference in the capacity of complete hepatic NEFA oxidation between early- and mid-lactating cows. Pathogenesis of fatty liver in dairy cows is explained by the rapid lipolysis during NEB and the limited VLDL export capacity compared to other mammalian species (Drackley et al., 2001). Liver TG content is negatively correlated with plasma glucose and serum insulin concentrations (Studer et al., 1993) and positively with plasma NEFA and BHB (Húsvéth and Gaál, 1983). Current studies suggest close relationship between fatty liver, insulin resistance and the release of certain pro-inflammatory cytokines, like tumor necrosis factor-alpha (TNF-α; Ohtsuka et al., 2001; Hayirli, 2006). TNF-α is a polypeptide belonging to cytokine family. It is secreted mostly by macrophages, but adipocytes are also important source of TNF-α (Hotamisligil et al., 1993). Ohtsuka et al. (2001) reported that the severity of fatty infiltration of the liver during early lactation was positively correlated with increasing concentrations of TNF-α. Interrelations between cytokines and fatty liver will be discussed later in *Section 2.2.3*.

Ketosis is a metabolic disorder characterized by relatively high concentrations of the ketone bodies (acetoacetate, BHB and acetone) and a low to normal concentration of glucose in the blood (Brockman, 1979). Ketosis generally occurs 21–40 days after parturition and can occur both subclinically and clinically. To be manifested clinically, the cows normally have a low glucose level (hypoglycemia; Ingvartsen, 2006). Clinically manifested ketosis is characterized by hypophagia, decreased milk production, loss of body condition score (BCS), lethargy, hyperexcitability, hypoglycemia, hypoinsulinemia, hyperketonemia, hyperlipidemia, and depleted hepatic glycogen (Drackley et al., 2001; Bobe et al., 2004). The threshold used to define subclinical ketosis was selected at a concentration of 1200 μmol/L of BHB (Duffield et al., 1998; LeBlanc et al., 2005). Cows with blood BHB concentrations above this threshold value are at three-fold greater risk for developing clinical ketosis or displaced abomasum compared to cows with lower blood BHB concentrations. Ketone bodies impair immune function through suppressing mitogenic response of lymphocytes (Franklin et al., 1991).

Adaptation to pregnancy and lactation include changes in circulating hormone levels and their production as well as in periferic tissue sensitivity and response to insulin (Bell, 1995; Bell and Bauman, 1997). At the onset of lactation, beside changes of *catecholamines,*

elevated *cortisol* and *glucagon,* decreased *thyroid* hormone, *insulin* and *leptin* blood plasma concentrations are reported. *Growth hormone* (GH) concentration starts to increase obviously some days before calving, remains high for some days at the beginning of the lactation, and after a steadily decline, but will circulates at still elevated level in high-producing dairy cows (Reist et al., 2003). Simultaneously the GH-induced hepatic *insulin-like growth factor-I* (IGF-I) production is diminished and certain peripheral tissues reduce their sensitivity to the effect of insulin. Although the thyrotropin releasing hormone (TRH) induced thyroxine (T_4) response is only slightly altered, decreased T_4 and 3,3',5-triiodothyronine (T_3) levels and elevated concentration of the inactive thyroid metabolite, 3,3',5'-triiodothyronine (reverse-triiodothyronine, rT_3) are observed usually in the peripheral blood (Pethes et al., 1985; Huszenicza et al., 2002; Meikle et al., 2004). Huszenicza et al. (2002) could demonstrate significant reduction in TRH-challenged T_4 response only in severe cases of ketosis, proving the adaptive character of the periparturient T_4 decline. Also some recently discovered hormones produced by the adipocytes (*adiponectin, leptin*) and the gastrointestinal tract (*ghrelin*) have been proved to influence the dry matter intake (DMI) and energy metabolism, although our related knowledge is still limited in cattle (Chagas et al., 2007a). All these endocrine events are involved in regulation of shifting the metabolism from anabolic to catabolic direction.

Circulating *insulin* levels are reduced in ruminants during undernutrition (Peterson et al., 1993), and are lower during the lactation as compared to the dry period (Holtenius et al., 2003). Low plasma insulin reduces glucose uptake by insulin-sensitive tissues and facilitate the insulin-independent glucose uptake by the mammary gland during lactation (Lomax et al., 1979). In hepatocytes insulin seems to control the re-coupling of GH-induced IGF-I production through its positive effects on GHR expression. In the adipose tissue, however, both stimulatory (Rhoads et al., 2004) and inhibitory (Butler et al., 2003) effects of insulin on GHR were reported. Insulin is a key metabolic signal in coupling the GH-IGF-I axis in the early lactation. Insulin infusion into early postpartum dairy cows increased the GH receptor (GHR) and IGF-I mRNA contents in the liver, and the IGF-I concentrations in the blood (Butler et al., 2003; Rhoads et al., 2004).

Increasing body of evidences shows the pivotal role of the *GH - IGF-I axis* and hormones produced by β-cells of pancreatic islets and adipose tissue (*adipo-insular axis*:

insulin and leptin) in the adaptation to the periparturient metabolism (Blache et al., 2007; Chagas et al, 2007a; Lucy, 2008). As extensively overviewed recently by Lucy (2003 and 2008), in ruminants the pituitary GH is known to be responsible for galactopoiesis and for the persistency of lactation. The IGF-I is produced by the liver in response to GH. In plasma, IGF-I circulates connected with its binding proteins (IGFBPs) which inhibit the bioavailability and activity of IGF-I. The IGF-I acts as an endocrine signal that controls GH secretion through a negative feedback loop. Antagonizing the actions of insulin, GH has a nutrient partitioning effect through which the development of lean tissue and the production of milk are favored (Etherton and Bauman, 1998). In dairy cows, liver GHR expression (Butler et al., 2003) and plasma level of IGF-I (Meikle et al., 2004) decrease dramatically during the period before and immediately after calving: the hepatocellular loss of GHR causes a GH refractory state, while liver does not produce IGF-I in response to GH (uncoupling). The subsequent decrease in blood IGF-I concentration leads to diminished negative feedback and enhanced pituitary hormone production and increased circulating level of GH. In subcutaneous and visceral lipid depots, GH promotes lipolysis with rising NEFA content in plasma, while antagonizes lipogenesis and blocks insulin-dependent glucose uptake. Furthermore GH elevates the intrahepatic gluconeogenesis providing more glucose for lactose synthesis in the mammary gland, so supports further elevation in milk production (Drackley et al., 2001).

Leptin, a protein secreted by the white adipose tissue has significant role in long-term regulation of feed intake and reproduction. Its circulating level informs the hypothalamic region of central nervous system on degree of lipid saturation in the visceral and subcutaneous fat stores (reviewed by Chilliard et al., 2005; Zieba et al., 2005). In laboratory rodents and primates leptin is synthesized and released into the circulation in proportion to the amount of body fat, reflecting primarily the TG content of lipid depots. Similar tendencies were also reported in cattle (Delavaud et al., 2002 and 2004). The effects of nutrition on circulating leptin level may be combined with consequences of reproductive status (pregnancy, lactation). Gender-related and genetic differences may be significant: during the early weeks of lactation plasma leptin level failed to correlate with BCS in dairy cows (Holtenius et al., 2003; Wathes et al., 2007). Insulin, glucocorticoids, T_3 (but not T_4) and endotoxin exposure may increase its gene expression and/or plasma level, whereas

leptin can directly inhibit cortisol synthesis by adrenal cells. In pp cows NEB induces a sharp reduction in plasma leptin content. In the 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.

2.1.2. Reproductive consequences of the negative energy balance

Resumption of postpartum ovarian cyclicity in dairy cows

The re-establishment of a pulsatile luteinizing hormone (LH) secretion pattern is a key event in the return of ovarian cyclicity in pp dairy cows experiencing NEB (Beam and Butler, 1997). The regular formation of new follicular cohort from which the new dominant follicles (DF) will emerge is reported to proceed despite the average NEB of 31.38 MJ/day during the first 3-week period after calving (Beam and Butler, 1997). In non-suckling dairy cows the first FSH peak on d 4-5 after calving is followed immediately by the initiation of the first pp follicular wave producing the first DF. In early weeks of lactation the reduced activity of the GnRH pulse generator is expressed as reduced pulsatile LH support of follicular steroidogenesis necessary for induction of a preovulatory LH surge and subsequent ovulation. However, a seemingly low LH pulse frequency (2 pulses per 6 h) is apparently adequate to sustain the morphological development of DF by the 2nd wk pp. Possible signals which can modulate interaction in the hypothalamic-pituitary-ovarian axis have been focused primarily on blood metabolites (NEFA, glucose) and metabolic hormones (insulin:GH ratio, insulin, IGF-I, thyroid hormones and leptin).

Concerning the *NEFA* and *glucose*, however, quite contradictory observations were reported (Canfield and Butler, 1991). High peripheral concentration of NEFA during peripartum is reflected in the follicular fluid as well (recently reviewed by Leroy, 2008). Negative relationship between high follicular NEFA concentrations and 17β-estradiol (E2) has been demonstrated (Jorritsma et al., 2003). Furthermore, ovulatory cows had accumulated less liver TG and proportion to NEFA than non-ovulatory cows (Marr et al., 2002). It has been established that the brain is sensitive to hypoglycemia and administration of a glucose inhibitor interrupts estrus and ovulation (McClure et al., 1978). Glucose is the main energy substrate in the bovine ovary and during NEB low glucose and insulin does not support ovarian activity due to low utilizable fuel.

Recent studies draw attention to the role of *insulin* in decreasing the interval from calving to first ovulation both in beef and in dairy cows. Insulin interacts with reproduction both at hypothalamic and the ovarian level. Insulin-deficient states are associated with an impaired function of the hypothalamic-pituitary-gonadal axis, but the mechanisms underlying hypothalamic alterations are unknown. *In vitro* infusion with insulin dramatically increased the GnRH release of the perifused hypothalamic fragments from female adult ovariectomized rats (Arias et al., 2002) and studies in diabetic sheep indicated absolute requirement for insulin for normal LH pulsatility and induction of the LH surge (Bucholtz et al,. 2000). In sheep dietary treatments known to induce gonadotrophin release are associated with increased circulating concentrations of insulin. Early pp cows fed diets designed to increase plasma circulating insulin concentrations had reduced interval to first pp ovulation and more favorable conception rates after first service (Gong et al., 2002). However, under hyperinsulinemic-euglycemic conditions a 2.6-fold elevation in circulating insulin resulted in increased circulating 17β-estradiol (E2), without any apparent effect on LH pulsatility in dairy cows (Butler et al., 2004).

At the ovarian level insulin directly stimulates mitosis and steroid production in cultured granulosa (Guttierez et al., 1997), theca (Stewart et al., 1995) and luteal cells (Mamluk et al., 1999). Cell culture studies have shown bovine granulosa cells to be critically dependent on the presence of physiological concentrations of insulin (Gutierrez et al., 1997). Diet-induced increases in circulating concentrations of insulin increased E2 production in cultured granulosa cells from small antral (1 to 4 mm) follicles (Armstrong et al., 2002), demonstrating a direct action of metabolic hormones on follicle function. Different forms of insulin receptors are widely distributed throughout all ovarian compartments, including granulosa, thecal and stromal tissues in humans (Poretsky et al., 1999). Newly different forms of insulin receptors have been described in bovine granulosa and theca cells (Neuvians et al., 2003). However, our knowledge regarding the expression of insulin receptors in different physiological and pathological situation is limited, yet.

Circulating concentrations of *IGF-I* and one of its binding proteins (IGFBP-2) in the periparturient period were also good indicators of the capacity of energy-restricted cows to resume cycling after calving (Roberts et al., 1997). Furthermore, an increased insulin:GH ratio following parturition may be conductive to greater hepatic IGF-I production (McGuire

et al., 1995). Although some seemingly contradictory observation was reported in heifers, during the first 2 weeks pp Beam and Butler (1997) could detect significantly higher circulating IGF-I concentration in cows developing E2-active, ovulatory first-wave DF than in those with E2-inactive anovulatory first-wave DF. In cows the circulating IGF-I concentrations correlated with IGF-I levels in the follicular fluid of large follicles. Insulin and IGF-I acts synergistically to stimulate the in vitro steroidogenesis and proliferation of bovine thecal and granulosa cell cultures (Spicer and Echtnerkamp, 1995). Likewise, cows that ovulated within 35 days pp presented higher IGF-I concentrations as well as higher glucose and insulin and lower NEFA and BHB concentrations (Huszenicza et al., 2001). Reduced circulating insulin or IGF-I do not seem to be involved in the suboptimal GnRH and LH pulse frequency, but both are stimulatory to ovarian E2 output (Butler et al., 2004).

Spicer et al. (2001b) reported a direct stimulatory effect of T_3 and T_4 on thecal cell steroidogenesis in cattle with T_4 being a much weaker inducer of thecal cell P4 production than LH and T_3 having no effect on granulosa and thecal cell P4 production.

Leptin has also been reported to influence the genital functions in rodents, primates and in farm mammals (Barb and Krealing, 2004; Zieba et al., 2005). Results in rodents, non-human primates and in porcine and ovine models suggest that the suppression of GnRH / LH during fasting is mediated by central action of leptin in the pituitary of the brain (Smith et al. 2002; Barb and Krealing, 2004). Intracerebroventricular administration of leptin increased LH secretion in the fasted cow and ewe, but not in control fed animals, indicating that metabolic state is an important factor in modulating the response of hypothalamo-pituitary-ovarian axis to leptin (Barb et al., 2004). Leptin acts also directly in the ovary, and is supposed to influence the cell proliferation and steroidogenic activity. In a ewe model with ovarian autotransplant the passive immunization against leptin increased the E2 secretion, whereas the direct ovarian arterial infusion of low dose leptin decreased the E2 and stimulated the P4 production (Kendall et al., 2004). In an *in vitro* cell culture study, high doses of leptin both increase the insulin-induced proliferation of thecal cells, and inhibit steroidogenesis in bovine ovarian tissues (Spicer et al., 2001a). However, the correspondingly high plasma levels of leptin are exceptional in farm mammals, and perhaps never occur in pp dairy cows.

In the early weeks of lactation *inflammatory diseases* (puerperal metritis, clinical endometritis and severe forms of mastitis) with intensive *endotoxin and/or cytokine release* are thought to interact with the effect of NEB and NEB-related endocrine changes, postponing the time of the first pp ovulation, inducing anovulatory cysts, and influencing the cyclic ovarian function thereafter (Gilbert et al. 1990; Huszenicza et al., 1999 and 2005; Földi et al., 2006). In ruminant model the experimental endotoxin or cytokine (TNF-α) exposure has induced an elevation of plasma cortisol and GH levels, a reduction in plasma IGF-I level, a temporary increase in insulin release, and an enhanced inactivation of thyroid hormones (Soliman et al., 2002; Waldron et al., 2003). Simultaneously, glucose concentrations tended to increase initially and subsequently declined; also, there was a tendency for increased NEFA levels, while plasma BHB decreased dose-dependently (Waldron et al., 2003). Administration of endotoxin impairs both the basal LH pulsatility and formation of preovulatory LH peak in ruminants, as well as may induce early luteolysis of premature corpora lutea (CL). In a prospective study in a herd where these complications were observed to occur frequently, the endocrine and metabolic parameters of healthy cows compared to those affected by mild or severe cases of puerperal metritis (PM) were followed up (Kulcsár et al., 2005b, Földi et al., 2006). At the beginning (before the first pathognostic signs) the NEFA, insulin, IGF-I, leptin, T_4 and T_3 concentrations of severe PM, mild PM and healthy cows did not differ. The insulin, IGF-I, T_4 and T_3 levels tended to decline for several days, but increased thereafter, whereas the NEFA increased since the beginning, and remained altered for couple of weeks. All these changes were more pronounced in the metritis-affected (predominantly in the severe PM) cows. Simultaneously the plasma leptin level also decreased, but it remained at low (in mild PM and healthy cows) or at very low (in severe PM cows) level throughout the 5 weeks of sampling period. Significant delay in resumption of cyclicity and depressed re-conception rate were detected only in severe PM cows (Kulcsár et al., 2005b, Földi et al., 2006). Similar, but less marked inflammatory-related interference was seen in periparturient and pp changes of plasma insulin, IGF-I, thyroid hormones and leptin concentrations in cows affected by mastitis (Huszenicza et al., 2004, Kulcsár et al., 2005a).

Fertility in postpartum dairy cows

Poor *quality oocytes and embryos*, as well as the diminished character of *post-ovulatory/post-insemination P4 rise* have been supposed to decrease the first service conception rate (Mann and Lamming, 2001; Leroy et al., 2008). Progesterone (P4) is essential for pregnancy after breeding and must be present in blood in adequate amounts to support embryo development and survival. The levels of P4 increase over the first three ovulatory cycles in postpartum cows with less improvement in cows with greater NEB (Villa-Godoy et al., 1988). Lower P4 levels normally observed in high producing cows probably also reflects increased sexual steroid metabolism by the liver (Sangsritavong et al., 2002). A number of studies have revealed lower concentrations of P4 from d 10-12 following insemination both in milk and in plasma in inseminated cows in which pregnancy failed than in cows in which pregnancy was successfully established (Mann et al., 2005). However, the critical period for optimum P4 is demonstrated earlier, around d 5-6 post AI (Mann and Lamming, 2001). In a survey monitoring milk P4 concentrations in 1400 cows Starbruck et al. (2001) found close correlation between low P4 level on d 5 and pregnancy failure. The successful maternal recognition of pregnancy depends on the presence of a sufficiently well developed embryo producing adequate quantities of interferon-τ, which in turn, is dependent on appropriate stimulation by circulating P4 concentrations (Mann and Lamming, 2001).

Early embryonic mortality is a major cause of decreased fertility in the dairy cattle. It is estimated that in dairy cattle fertilization rate is around 80% (Peters, 1996). Some of the early embryo loss results from failure of embryo to prevent luteolysis. This is supported by studies conducted in dairy cows in which P4 supplementation from d 5-9 resulted insignificant increase in interferon-τ production on d 16 (Mann et al., 2005). An alternative approach is to increase endogenous P4 secretion by manipulation of the diet. Additional dietary fat may increase P4 concentration by serving cholesterol as precursor for steroid synthesis or by decreasing P4 clearance from blood (Staples et al., 1998).

Good *postpartum uterine involution* is also has critical importance for establishment of pregnancy. Leukocytes are the most important cellular elements of the antimicrobial cell defense mechanism. During the first 1 to 3 weeks of lactation the migration and phagocytic activity of neutrophil granulocytes are reduced (Suriyasathaporn et. al., 1999, 2000) which

increase the risk of uterine disorders, like puerperal metritis or clinical endometritis (Sheldon et al., 2005; Földi et al., 2006). Puerperal metritis dramatically delays involution and increase the time to re-conception after calving (Huszenicza et al., 1999; Földi et al., 2006).

Another possible carryover effect of early NEB may be that oocytes are imprinted by detrimental conditions within the follicle during their development over a period of 60-80 days (Britt, 1991). NEB associated low peripheral glucose, high NEFA and BHB concentrations are reflected even at follicular level, compromising the oocyte developmental capacity, which needs glucose for proper maturation (Leroy et al., 2006). Theca cell function was not influenced by BHB, but granulosa cell proliferation was increased and P4 and E2 production was decreased in a study of Vanholder et al. (2005). Leroy et al. (2006) found that an additive toxic effect of increased BHB concentration (1.8 mmol/l) on oocyte and embryo development *in vitro* when glucose levels were moderately low, similarly what is usually found in cows with subclinical ketosis. Severe NEB impaired oocyte developmental competence later, at 80-120 days of lactation, suggesting toxic effects of high periparturient NEFA concentrations (Kruip et al., 2001). While these results support concerns about early NEB affecting oocytes, results of another study showed that early embryo development is compromised even later during mid-lactation by ongoing metabolic effects associated with lower BCS ($\leq$2.5) in high genetic merit cows (Snijders et al., 2000). These collective results indicate a detrimental impact of NEB on oocyte competence for embryo development, but metabolic effects are not limited to follicular development during early lactation, it may be continuously manifested during high milk yield.

2.2. Periparturient insulin secretion and the phenomenon of insulin resistance in the dairy cow

2.2.1. Pancreatic insulin secretion

Insulin represents the most important antilipolytic, antiketogenic hormone involved in carbohydrate, protein and fat metabolism in ruminants and plays an important role in the central regulation of feed intake (Butler et al., 2004; Hayirli, 2006). By structure it is a polypeptide hormone consisting of amino acidic and basic chains connected by disulphide bridges. There are just minor differences in the chemical structure of the insulin secreted by mammals: human, cattle, sheep, pig, horse, rabbit, and dog (Hayirli, 2006). However, the biological potency of insulin varies among all species depending on nutritional condition, reproductive status, age, diet and by the concentration of serum levels of NEFA and ketone bodies (Elmahdi et al., 1997; Hayirli, 2006). Insulin is secreted by the ß-cells within the Langerhans islets of the pancreas in response to different stimuli. There are numerous factors that stimulate insulin secretion (Berne and Levy, 1993). These include nutrients (e.g. glucose, galactose, mannose, xylitol, glyceraldehydes, certain aminoacid, fatty acids, potassium and calcium), gastrointestinal hormones (e.g. glucagon, pancreatic polypeptide, gastric inhibitory peptide, secretin and cholecystokinin), parasympathetic stimuli (e.g. vagal activity), and drugs (e.g. sulpha drugs). Factors that suppress insulin-release include physiological conditions (e.g. fasting, exercise, obesity), gastrointestinal hormones (galanin, somatostatin), sympathetic stimuli (α-adrenergic activity), and other specific compounds (e.g. IL-1 and PGF2-α; Hayirli, 2006). Due to distinct features of metabolism in ruminants compared to monogastric species, the magnitude of insulin secretion in response to diverse nutrients is different in cows. For example, medium-chained fatty acids increased insulin secretion in ruminants, but failed to stimulate insulin secretion in rabbits or pigs (Horino et al., 1968). In ruminants short chain fatty acids (SCFA) also play an important role in stimulating the pancreatic ß-cell insulin secretion. SCFA infusion, except for acetate, raised the insulin level in the blood (Húsvéth et al., 1996). Relative rise was closely correlated with the length of carbon chain of the SCFA, that is, n-valerate caused the largest elevation of the insulin level, followed by n-butyrate and propionate. At the same time, acetate failed to cause a marked influence on the insulin level. These results of insulin

showed agreement with glucose concentration changes, with the exception of n-butyrate treatment, where the increase of plasma insulin concentrations after the infusion proved to be much larger than that of glucose, relative to the preinfusion value (Húsvéth et al., 1996).

Insulin acts to preserve nutrients by stimulating glycogenesis, lipogenesis, and glycerol synthesis and by inhibiting gluconeogenesis, glycogenolysis, and lipolysis (Brockman and Laarveld, 1986). In the liver insulin inhibits ketogenesis and substrates that are involved in the ketogenesis. Also it has been shown in alloxan-induced diabetes in sheep that insulin enhanced peripheral ketone utilization (Jarret et al., 1976). During early lactation low peripheral levels of insulin further accentuates lipid mobilization. In the peripheral tissue insulin facilitates glucose utilization. Within the muscle and adipose tissue facilitation of glucose uptake is realized through specific glucose transporters (GLUT; Komatsu et al., 2005). There are several isoforms of GLUT in various tissues. GLUT 4 is the only transporter that requires insulin for glucose uptake and it is present in skeletal muscle, in the heart muscle and in adipose tissue, thus in the insulin-sensitive tissues (DeFronzo et al., 1992; Zhou et al. 1999). The liver and the mammary gland are not insulin-sensitive organs; therefore their glucose uptake is independent from insulin.

The classical target organs for insulin action are muscle, adipose tissue and liver (Hayirli, 2006). Until approximately a decade ago, insulin was not thought to play a significant role in the regulation of ovarian function, however today is considered as one of the key metabolic molecule signaling in the hypothalamo-pituitary-ovarian axis (described in details in *Section 2. 1.2.).*

2.2.2. Insulin resistance; methods of its assessment

Insulin resistance (IR) describes a state when physiological levels of insulin produce less than normal biological response. IR is characterized by an altered response of insulin to glucose (*insulin responsiveness*), an altered response of glucose to insulin (*insulin sensitivity*), or both (Kahn, 1978). At the molecular level IR may be localized at pre-receptor, receptor or post-receptor level (Hayirli, 2006). IR at pre-receptor level may be caused by decreased insulin secretion, increased insulin degradation or both; receptor defect include decreased number of receptor or decreased binding affinity; post-receptor alterations comprise defect in intracellular signaling and translocation of the glucose

transporters (Hayirli, 2006). When insulin resistance is localized at receptor and post-receptor levels, the provision of exogenous insulin can not exert the physiological effects. Insulin secretion and signaling has been recently reviewed by Hayirli (2006).

Alterations of *insulin responsiveness* represent the decreased functional capacity of pancreatic β-cells, which can be tested by a time-related provisional increase after a standard-dose challenge with glucose, propionate, glucagon or other insulin-secretagogues (Hove, 1978; Holtenius and Traven, 1990; Samanc et al., 1996; Steen et al. 1997; Blum et al., 1999). The gold standard test for diagnosing insulin resistance in human medicine is the *hyperinsulinemic-euglycemic clamp test (HIEC)*. During the *euglycemic clamp*, the amount of insulin required to achieve the maximum response indicates *insulin responsiveness*, whereas the amount of insulin required to reach the half-maximal response indicates *insulin sensitivity* (Kahn, 1978). During the insulin infusion, glucose is continuously infused with a pump in order to maintain euglycemic levels. It has been used in limited, experimental, cases in dairy cows (Holtenius et al., 2000; Mashek et al., 2001; Sternbauer, 2005; Butler et al., 2006). This method provides precise information about insulin secretion and insulin sensitivity also in dairy cows; however, it can hardly be conducted under farm conditions (Butler et al. 2003).

The iv. *glucose tolerance test (GTT)* is a more practical and simple method than the HIEC test for determining glucose tolerance. In the GTT basal and peak insulin concentrations, plasma disappearance rate, half-life, time to reach basal concentrations, area under the curve for plasma insulin are parameters for evaluation of glucose tolerance (Hayirli, 2006). However, parameters obtained from GTT are not always easy to interpret. For example, it is not known whether faster glucose disappearance rate from the plasma is due to the enhanced glucose utilization or increased insulin production.

Propionates stimulate pancreatic insulin release in ruminants (Brockman, 1982; Samanc et al., 1996); therefore they can be used to challenge insulin response. However, severe adverse reactions have been reported when propionate was administered intravenously, including increased respiratory rate, heart rate and metabolic alkalosis. These physiologic changes might influence measured metabolic responses to the infusion, therefore its limited use (Bradford et al., 2006). Insulin response also can be evoked indirectly via glycogenolysis with epinephrine or glucagon injection. *Epinephrine* has

hyperglycemic effects via hepatic glycogenolysis. Increases of plasma glucose concentrations to the epinephrine challenge could reflect an increase of glucose production by the liver (rates of gluconeogenesis and glycogenolysis), reduction of glucose utilization by body tissues (for example, oxidation rates), or both. *Glucagon* via hepatic glycogenolysis and gluconeogensis has hyperglycemic effects which in turn stimulate insulin release. In addition, glucagon is an insulin secretagogue second to glucose. This is the base of the glucagon stimulation test (Kaneko, 1997). The response of insulin to glucagon was evaluated by Hippen et al. (1999). Insulin increase slightly preceded the increases in glucose concentrations, indicating a direct action of glucagon on pancreatic β-cells that stimulates insulin secretion independently of blood glucose. However insulin increase was observed in dose-independent manner. Taken these observations into account, alternative tests presented above have only limited value to evaluate pancreatic insulin secretion in cattle.

The whole-body *insulin sensitivity* can be estimated by a temporarily depressed glucose pattern after a standard-dose insulin load. *Intravenous insulin tolerance test (ITT)* involves the iv. administration of exogenous insulin. The plasma glucose decrement is monitored during the 30-60 minute period. After hypoglycaemia occurs, counter regulatory hormonal and metabolic response alter the plasma glucose concentration, thus hampering the interpretation of the results. In human populations the glucose response to exogenous insulin is in good correlation with clamp-derived indices of insulin sensitivity (Bonora, 1989).

In human epidemiological studies diverse homeostatic models of insulin sensitivity were developed for a more rapid and easy evaluation of insulin sensitivity. A promising method developed to measure insulin sensitivity in epidemiological studies in human populations, the *revised quantitative insulin sensitivity check index (RQUICKI*) was evaluated in Holstein cows by Holtenius and Holtenius (2007). RQUICKI implies the evaluation of the homeostatic energy balance based on plasma concentration of glucose, insulin and NEFA. Holtenius and Holtenius (2007) found significant negative linear relationship between the BCS and the revised insulin sensitivity index, but no validation to other sensitivity indexes was performed. Also, the RQUICKI seemed suitable to detect mild differences in IR in healthy lactating dairy cows (Balogh et al., 2008). However, there

are no data available on periparturient changes of RQUICKI in hyperketonemic cows, as well as in those affected by inflammatory diseases, where in the etiology of decreased insulin sensitivity other factors may be involved, not evaluated by the index.

2.2.3. Etiology of insulin resistance in the dairy cow

After calving dairy cows have depressed blood insulin concentrations and suffer from insulin resistance in order to increase glucose supply towards the mammary gland (Bell, 1995; **Table 2.2.3.1.**). This phenomenon may be most exaggerated immediately after parturition. Insulin response to glucose challenge was depressed, while glucose clearance rate increased pp compared to prepartum values (Holtenius et al., 2003; Bossaert et al., 2008). In peak- and late-lactating dairy cows the mammary gland expresses the non-insulin dependent GLUT 1 in approximately three times greater magnitude than in dry cows to facilitate entry of glucose into the udder (Komatsu et al., 2005). GLUT 1 was also found in the adipose tissue of late-lactating and nonlactating cows, but not during peak lactation. The abundance of the insulin sensitive GLUT 4 in skeletal muscle and in fat stores did not change as lactation progressed and was not different during the dry period (Komatsu et al., 2005). Sano et al. (1993) found that insulin responsiveness to glucose was reduced, but peripheral tissue sensitivity to insulin was unchanged despite higher metabolic clearance rate of insulin in late lactating (~150 days pp) compared to nonlactating cows. This interpretation is consistent with observations of diminished responsiveness but not sensitivity to insulin in vivo in terms of whole-body glucose utilization in lactating *versus* nonlactating goats (Debras et al., 1989). Lactating dairy cows had lower pancreatic insulin output and consequently lower hepatic insulin uptake than non-lactating ones (Lomax et al, 1979). They also showed reduced insulin responsiveness to iv. glucose and propionate challenges and a decrease in hepatic glucose output equal to the rate of glucose infusion. In turn, Blum et al. (1999) did not found differences in insulin responses to iv. glucose challenge, insulin metabolic clearance rate and insulin-dependant glucose utilization among early or mid- and late-lactating cows. Mashek et al. (2001) concluded from the results of a hyperinsulinemic-euglycemic study that mid-lactation cows had reduced tissue sensitivity to insulin compared to early-lactation cows. Insulin response was reduced pp compared to the dry period, but increased from d 14 to 42 after calving (Bossaert et al., 2008). Contradictory results were obtained in lactating beef cattle (Sano et al., 1991). Both the

responsiveness of insulin to glucose and the tissue responsiveness to insulin were enhanced during lactation in beef cows, indicating that nutrients were deposited to peripheral tissues also during lactation. This metabolic difference between beef and dairy cows may partly explain why dairy cows are more prone to loose weight during lactation.

Table 2.2.3.1. Possible homeorhetic hormones and glucose-related tissue responses in pregnancy and lactation (after Bell, 1995).

State	Hormone	Putative action	Tissue response
Mid pregnancy	Progesterone	↑insulin sensitivity	↑ adipose glucose uptake ↑ adipose lipogenesis
Late pregnancy	Placental lactogen Estrogens	↓ insulin sensitivity and responsiveness	↓ glucose uptake by adipose and muscle ↓ lipogenesis ↑ muscle glycolysis and lactate release
Lactogenesis, early lactation	Prolactin Estrogen Cortisol Somatotropin	↓ insulin sensitivity and responsiveness	↑ liver gluconeogenesis ↓ glucose uptake by adipose tissue and muscle ↓ adipose lipogenesis - In the muscle: ↓ protein synthesis ↑ protein degradation ↑ amino acid release

Insulin resistance is a *multi-factorial phenomenon* in monogastric species as well as in ruminants. As discussed above, the pregnancy and lactation is in very close relation with the circulating insulin concentrations. Beside lactation, the genetic strain, reproductive state, body condition, certain hormones, inflammatory conditions, as well as nutrition may be the main factors altering the insulin secretion of pancreas and the sensitivity of peripheral tissues to this hormone.

Genetic aspects

Cows with genetically higher milk production traits are likely to have stronger IR state than low producing dairy cows. Holstein cows exhibited lower basal insulin concentration and lower glucose-induced insulin response after parturition than in late pregnancy. In contrast, early lactating Japanese Black cows had the same basal plasma glucose, insulin concentrations and similar magnitude of the glucose-induced insulin response as in late gestation (Shingu et al., 2002). Under pasture system, high producing North American

Holstein-Friesian (HF) cows had slower glucose-turnover rate than New Zealand HF which had lower milk production, indicating more severe insulin resistance (Chagas et al., 2009). A polymorphic site of the GH gene (*Alu*I polymorphism) that results in an amino acid change at position 127 of the protein chain (leucine, L to valine, V; Lucy et al., 1991) has been linked to milk production traits with various outcomes. HF cows heterozygous for *Alu*I polymorphism seem more likely to develop IR during early lactation than leucine homozygous cows (Balogh et al., 2008).

Swali and Wathes (2006) found lower pp concentrations of insulin in cows originating from sires with high genetic merit of milk. They estimated a moderate heritability of insulin concentration (h^2= 0.43). Guttierez et al. (2006) also found lower insulin in cows with high genetic merit for milk yield compared with low genetic ones, although their energy balance did not differed. Japanese Black beef cows with much lower milk production had significantly higher glucose induced insulin secretion than Holstein cows, but had similar ability to inhibit insulin-stimulated glucose utilization in peripheral tissues (Shingu et al., 2002). Most recently, Bossaert et al. (2009) found higher insulin levels in neonatal Holstein-Friesian calves compared to Belgian Blue breed, selected for double-muscling. The higher pancreatic insulin secretion in HF calves was accompanied by slower glucose clearance after GTT. The muscle proportion of the Belgian Blue is roughly 20% higher than in normally muscled cattle, which may account for the enhanced glucose clearance in this breed. However, apart from muscle differences the higher basal glucose and insulin and lower revised QUICKI in HF calves suggest that an innate difference toward repartitioning glucose toward other tissues, like the mammary gland exist (Bossaert et al., 2009). Similarly, New Zealand cows with lower genetic merit had lower glucose fractional turnover rate than their North American counterparts with higher genetic milk merit. Insulin increment and insulin AUC was not affected in that study (Chagas et al., 2009).

Non-esterified fatty acids

Increased plasma NEFA level has been associated with lower glucose-induced insulin responsiveness in a four-day fasting model of non-lactating, non-pregnant dairy cows (Oikawa and Oetzel, 2006), furthermore with decreased glucose and insulin clearance after glucose load in lactating HF cows (Bossaert et al., 2008). High NEFA levels impair insulin actions at various levels. NEFA inhibit insulin-stimulated glucose uptake in skeletal muscle

and suppress glycogenolysis in liver. The inhibition of glucose uptake by NEFA in insulin-sensitive tissues involves intracellular signaling pathways in the liver and in peripheral tissues (e.g. abnormality in GLUT 4 translocation, receptor down regulation, decreased coupling between stimulated receptors and glucose transport) and the suppression of GLUT 4 abundance (reviewed by Hayirli, 2006). Rat adipocytes exposed to high levels of free fatty acids *in vitro* showed IR within 4 hours (especially when incubated with palmitate, even at low concentrations), through the inhibition of GLUT 4 activation, in this manner affecting insulin-mediated glucose transport (van Epps-Fung et al., 1997). Free fatty acids acutely enhance glucose-induced insulin secretion (Stein et al., 1997), but chronically increased levels desensitize insulin secretory capacity of pancreatic β-cells. Therefore free fatty acids provoke IR that was proved by higher basal insulin and glucose concentrations and decreased glucose infusion rates (Mason et al., 1999). Direct deleterious effect of NEFA on pancreatic ß-cells has been demonstrated in human (Zhou and Grill, 1995) and rat (Maedler et al., 2001) pancreatic cells.

Growth hormone

GH has well known diabetogenic effects by enhancing lipolysis in adipose tissue and gluconeogenesis in the liver (Bell, 1995; Block et al., 2001; Ingvartsen, 2006). After chronic GH treatment blood glucose was increased and insulin resistance occurred within a few hours in insulin-sensitive tissues, but GLUT 4 translocation was not disturbed in hamster ovary cells (Yokota et al., 1998). Four-day treatment with GH releasing factor diminished glucose turnover rate during hyperinsulinemic-euglycemic clamp test in late lactation cows, but not during early lactation, possibly related to rates of insulin-stimulated glucose uptake in adipose tissues, which are very low during early lactation (Rose et al., 1996).

Leptin and other hormones secreted by adipose tissue

Insulin is known to stimulate leptin production by adipocytes and in turn, leptin can act on insulin secretion (indirectly or directly via its receptors in the pancreas) in a stimulatory manner during feed restriction, but inhibiting further insulin output after re-feeding (Houseknecht et al,. 2000; Chilliard et al., 2001; Amstalden et al., 2002; Block et al., 2001; Zieba et al., 2005). Leptin enhance insulin sensitivity, glucose utilization and energy

expenditure in skeletal muscle, stimulates fatty acid oxidation in the muscle and liver and lipolysis in adipose tissue, and inhibits lipogenesis in hepatocytes and in fat stores. The effect of insulin on leptin secretion was studied by performing euglycemic-hyperinsulinemic clamps in mid-lactating dairy cows. After 96 h of hyperinsulinaemia, plasma leptin was increased significantly. These data indicate that insulin regulates plasma leptin concentration in lactating dairy cows (Block et al., 2003).

In laboratory rodents other adipose tissue secreted hormones, such as resistin and adiponectin has been recently described as potential mediators of IR. *Adiponectin* is considered as an endogenous insulin sensitizer. It signals *via* AMP kinase, a stress-activated signaling enzyme implicated in a variety of metabolic responses, including suppression of hepatic gluconeogenesis, glucose uptake in exercising skeletal muscle, fatty acid oxidation, and inhibition of lypolysis. *Resistin*, another recently discovered adipose-secreted polypeptide hormone, has proved to be a mediator of insulin resistance in mice. The administration of the anti-resistin antibody to mice with diet-induced obesity, IR and hyperglycemia was partially reversed and improved the sensitivity to exogenous insulin (Steppan and Lazar, 2002). However, the current knowledge about the putative role of these hormones is limited in the human medicine, and their possible involvement in the pathogenesis insulin resistance is still remaining to be elucidated in ruminants.

Steroid hormones and prolactin

There is extensive experimental evidence that *sexual steroids* and insulin interact in their actions on tissues. Addition of E2 to culture medium of isolated rat adipose tissue cells increased maximum insulin binding; addition of P4 and cortisol decreased glucose transport and maximum insulin binding, while addition of prolactin and placental lactogen decreased glucose transport without changing the maximum insulin binding (Ryan and Enns, 1988). At physiological levels, testosterone and E2 are thought to be involved in maintaining normal insulin sensitivity. However, outside of the "physiological window" these steroids may promote IR (Livingstone and Collison, 2002). Polycystic ovarian syndrome is one of the most common causes of infertility in women, which is associated with hyperinsulinemia and excessive androgen production, and IR (Poretsky et al., 1999). Hyperinsulinemia in patients with this condition is believed to stimulate ovarian androgen production, and there is also evidence that androgens act directly on peripheral tissues to

promote insulin resistance. The molecular basis of this IR has been reported to involve reduced insulin receptor autophosphorylation, reduced expression and translocation of insulin-responsive glucose transporters and defects of the insulin signaling pathway distal to the insulin receptor (Livingstone and Collinson, 2002). However, Opsomer et al. (1999) could not prove a relationship between IR and cystic ovarian disease in high lactating dairy cows. Basal insulin and glucose levels, and glucose response to an iv. GTT was not different between cystic cows and their matched controls, but insulin secretion was significantly reduced in cows with anovulatory cysts. Other authors also confirmed that cows with ovarian cyst are likely to have lower peripheral insulin concentrations compared to healthy cows (Vanholder et al., 2005; Braw-Tal et al., 2009).

Glucocorticoids increase the conversion of amino acids to glucose and restrict peripheral glucose utilization (Bell and Baumann, 1997). Dexamethasone-induced insulin resistance in man is reportedly related to both reduced whole-body insulin-dependent glucose oxidation and to non-oxidative glucose disposal (Tappy et al., 1994). Treatment with a single dose of flumethasone treatment in heifers increased plasma glucose and insulin levels by 2 mmol/l and 16.5 mU/l, respectively, 24 hours post treatment. At 72 hrs post-flumethasone injection, these effects were abolished except for a persistent 10% increase in plasma glucose concentration (Sternabuer et al., 1998).

Malnutrition

Chronic malnutrition decreased pancreatic islet number and islet size in a study of Tse et al. (1998) lead to reduced insulin secretion in rats. Insulin secretion rate to glucose was lower in malnourished rats than in well-nourished ones during oral GTT (Reis et al., 1997). Prepartum cows on restricted pasture feeding tended to have lower insulin responses to glucose 2 weeks pp than their herd mates on *ad libitum* diet (Chagas et al., 2008). From these previous reports it seems that low plasma insulin levels found in dairy cattle after calving could be the consequence of disturbed pancreatic islet function and islet regression, which is the result of feed depression commonly encountered shortly before and following parturition and its hormonal and metabolic consequences (Hayirli, 2006).

Diet

Fat supplementation

Dietary fat is able to modulate whole-body insulin sensitivity in humans and rodents (Storlien, 2000; Delarue, 2004). There is also evidence that feeding fats to ruminants may induce IR (Chilliard and Ottou, 1995; Gaynor et al., 1996; Pires et al., 2007). Glucose clearance was impaired during GTT in lactating cows infused with 640 g of TG into the duodenum. Also, glycemia was not influenced despite higher insulin concentration (Chilliard and Ottou, 1995). In non-lactating, non-pregnant dairy cows short-term hyperlipidemia following iv. tallow infusion increased basal glucose and insulin levels as well as decreased glucose clearance rate during both GTT and ITT (Pires et al., 2007a). Insulin sensitivity could be reinstituted by lowering NEFA and thus enhancing glucose clearance rate despite lower insulin secretion (Pires et al., 2007b). Lactating cows infused with TG had greater insulin concentration during GTT but achieved the same glucose concentration as uninfused cows (Gaynor et al., 1996).

The chain length and degree of saturation of the different fatty acids may have different role in modulating whole-body insulin sensitivity. Saturated FAs were more insulinogenic compared to polyunsaturated fatty acids (PUFA) in rats fed different type of fatty acids (Stein, 1997). Compared to saturated fatty acids, polyunsaturated n-3 fatty acids may prevent IR in humans and rodents (Clarke, 2000; Delarue 2004; Storlien, 2000). There is only limited evidence on the effect of different FA on insulin sensitivity in dairy cows. Short term abomasal infusion with linseed oil rich in the n-3 fatty acid C18:3 had an insulin sensitizing effect in non-lactating, non-gestating cows (Pires et al., 2008). In a second experiment when lipolysis was stimulated by feed restriction, linseed supplementation increased the antilipolytic effect of insulin in the adipose tissue (Pires et al., 2008). Abomasal infusion of fish oil increased key intermediates in the insulin signaling cascade in muscle (Gingras et al., 2007). The mechanism how the diffeent FA modulates IR is not fully understood, but may relate to altered membrane phospholipid fatty acid composition, membrane fluidity and stability, changes in lipogenic gene transcription, and direct interference with insulin signaling (Lee et al., 2006).

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of the octadecadienoic linoleic acid (C18:2). The *trans*-10*, cis*-12 (*t*10,*c*12) and *cis*-9*, trans*-

11*(c*9,*t*11*)* CLA are the most studied isomers, having different biological effects (Baumgard et al., 2002). In humans commercial products of CLA are being widely promoted for their weight loss effect and action on body fat mass. However, contradicting effect of CLA on IR has been published. The possible effect of CLA on blood insulin and insulin sensitivity is confused by marked differences in species response. In genetically diabetic Zucker rats dietary CLA restored insulin sensitivity (Houseknecht et al., 1998). By contrast, mice fed CLA-supplemented diets developed mild insulin-resistance in parallel with depletion of the adipokines leptin and adiponectin, and increased hepatic steatosis (Poirier et al., 2006). In a subsequent study, the reduction of leptin and adiponectin induced by CLA coincided with a pro-inflammatory state marked by an increased expression of IL-6, TNF-α, and macrophage infiltration in adipose tissue, likely contributing to hyperinsulinemia (Poirier et al., 2006). Recent studies suggest that CLA may improve insulin sensitivity throuhg the activation of peroxisome proliferator-activated receptor (PPAR) family (Brown et al., 2003). The PPAR-gamma is a nuclear hormone receptor with important role in lipid metabolism in different tissues. Activation of the PPAR-gamma increased insulin sensitivity when human preadipocytes was treated with *cis*-9, *trans*-11 CLA, but not when *trans*-10, *cis*-12 CLA (Brown et al., 2003).

Baumgard et al. (2002) found no difference in the plasma glucose response to an insulin challenge and only a minimal decrease in the adipose tissue response to an epinephrine challenge when cows in established lactation were abomasally infused 5 days with CLA. De Veth et al. (2006) reported no effect of 16 days supplementation with *t*10,*c*12 *CLA* supplementation on glucose clearance after insulin challenge or epinephrine induced NEFA responses in lactating cows after 16 days of supplementation, despite of effect on milk synthesis.

Carbohydrate feeding

In experimental work conducted on rats, diets rich in simple sugars were associated with IR compared to complex carbohydrates (Storlien et al., 2000). HIEC test combined with tracer administration showed impaired action of insulin at the level of liver and individual skeletal muscle in rat, when starch was substituted with either sucrose or fructose (Berne and Levy, 1993).

Interaction between fatty liver, inflammation and insulin resistance

There is increased body of evidence that that some NEB-related metabolic malfunctions and organic diseases, such as excessive lipid accumulation in the liver, ketosis, abomasal displacement, cystic ovarian disease and laminitis are in closed relationship in the peri- and post-parturient IR in dairy cow (Hove, 1978; Sakai, 1993; Hendry et al., 1999; Opsomer et al., 1999; Holtenius et al., 2000; Ohtsuka et al., 2001; Pravettoni et al., 2004; Hayirli, 2006). Studies in human and animal models suggest a causal relationship between elevated NEFA concentrations, the release of pro-inflammatory cytokines (e.g. TNF-α, interleukin 6; IL-6) and IR (Martens, 2007).

Cytokines and inflammatory conditions

In humans the release of cytokines, that occurs in association with obesity and inflammatory diseases (especially in those with endotoxemia), plays an important role in the development of IR (Ruan and Lodish, 2003). An interaction between cytokines and IR has been recently explored by exogenous administration of recombinant TNF-α to growing steers (Kushibiki et al., 2001ab) or in naturally occurring fatty liver disease (Ohtsuka et al., 2001). Endotoxin as well as TNF-α are known to create a catabolic state depressing DMI, milk and milk protein yield, transiently increasing plasma GH, NEFA and IGFBP-1 concentrations and decreasing IGF-I and T_3 plasma levels in lactating cows and in sheep (Kushibiki et al., 2003). TNF-α also interferes with peripheral insulin sensitivity in rats and humans (Lang et al., 1990; van der Poll et al., 1991) and in cattle (Kushibiki et al., 2001ab). Steers treated with daily injections of TNF-α had higher basal insulin levels, higher or normal basal glucose concentrations, less reduced glucose nadir and smaller glucose area under the curve (AUC) after ITT than in control animals reflecting a state of IR (Kushibiki et al., 2001a,b). Conversely, their insulin responses following an iv. glucose challenge increased significantly compared to control steers. Administration of lypopolisacharides (LPS) to steers resulted in a temporary hyperglycemia followed by decreased basal glucose and increased insulin levels. IR was also proven from the iv. GTT conducted at 6 and 24 hours after LPS treatment (McMahon et al, 1998). TNF-α mRNA expression is increased in adipose tissue of obese rats and humans; thus may serve as a link between IR and TNF-α, commonly found in fat subjects and obesity (reviewed by Ruan and Lodish, 2003). This may, at least in part, explain why cows with high BCS have decreased tissue sensitivity to

insulin as showed by Holtenius et al. (2003) and Holtenius and Holtenius (2007). TNF-α might be also an important mediator of IR in the complex of hepatic lipidosis.

Cytokines and obesity

Obesity is one of the most important factors in the pathogenesis of IR in human patients suffering from diabetes type 2. In humans obesity is accompanied by chronic activation of inflammation and sustained release of the inflammatory cytokines discussed above. Abdominal fat accumulation has been shown to play a crucial role in the development of so-called metabolic syndrome in humans which is characterized by hyperglycemia, hyperinsulinemia and vascular disorders. The possible reasons for linking obesity to IR it appears to be the levels of NEFA, TNF-α and IL-6 (Martens, 2007). Fürll et al. (2008) found a close positive correlation between NEFA and TNF-α in heifers with high backfat thickness. The antepartal TNF-α concentrations correlate with backfat thickness. These authors also investigated the relationship between TNF-α in healthy cows *vs*. in those affected by different type of puerperal diseases on d 3 antepartum and on d 10 pp. TNF-α was increased in cows with puerperal diseases. In cows with retained placenta, mastitis and abomasal displacement the TNF-α correlates positively with antepartal NEFA. Postpartum increase in glucose disappearance rate was smaller in cows fed high energy diet before calving due to IR in fat cows (Holtenius et al., 2003). BCS was also significantly and negatively correlated with the RQUICKI in the same dataset (Holtenius and Holtenius, 2007). Obese heifers developed IR that was reflected in higher basal insulin concentrations with euglycemia and higher insulin responsiveness to glucose meanwhile glucose fractional removal rates were similar to lean animals (Mc Cann and Reimers, 1986).

Fatty liver and hyperketonemia

Accumulation of fat in the liver was directly related to decreased insulin sensitivity as was found by Ohtsuka et al. (2001). Lipid accumulation can be preceded by increased plasma concentrations of TNF-*α* (Ametaj et al., 2002) and of certain acute phase proteins, such as haptoglobulin and serum amyloid A (Katoh, 2002). The severity of hepatic lipidosis was directly associated with the degree of decreased insulin responsiveness (Oikawa and Oetzel, 2006). Severe fatty liver was associated with approximately 3-times higher TNF-α value than in cows with mild lipidosis. Cows with fatty liver had increased plasma NEFA

and BHB levels, decreased insulin concentrations and a compromised insulin-stimulated blood glucose response (ISBGR; Oikawa and Oetzel, 2006). ISBGR was negatively related to NEFA, BHB and liver TG, and positively to insulin levels. *In vitro* cultures of bovine hepatocytes loaded with TG had lower insulin clearance rates and impaired insulin- and/or glucagon-stimulated albumin synthesis than normal hepatocytes (Strang et al., 1998).

Hyperketonemia initiates a state of insulin resistance in lactating dairy cows. Spontaneously ketotic and fasted cows had markedly reduced insulin secretory capacity after GTT than healthy animals, and glucose clearance was also significantly lower in fasted cows than in the two other groups (Hove, 1978). Similar results of Sakai et al. (1996) showed that ketotic cows had depressed pancreatic β–cell function and decreased glucose and insulin disappearance rates after glucose and xylitol challenge. In agreement with above reports, several authors found lower than normal insulin responses to glucose, propionate or glucagon stimulation in ketotic cows (Sakai et al., 1993; Samanc et al., 1996, Steen et al., 1997). Hyperketonemia at the beginning of lactation is associated with hypoglycemia, hypoinsulinemia, metabolic acidosis, high NEFA and glucagon levels (Baird, 1982; Holtenius et al., 1993) and with hepatic lipidosis (Grummer, 1993). Van Putten et al. (1985) conducted a study to elucidate the mechanism by which ketoacidosis causes insulin resistance *in vitro*. Exposure of adipocytes to low pH and ketoacids for 48 h resulted in decrease in pH from 7.4 to 6.9 and a 50% reduction in insulin binding. Insulin enhances utilization of ketone bodies like acetoacetate and BHB by extrahepatic peripheral tissues in induced diabetes in sheep (Jarret et al., 1976). Sakai et al. (1993) showed that subcutaneous injection of 200 IU of slow release insulin with concomitant infusion of dextrose compared with infusion of dextrose alone for 4 d enhanced the effectiveness of treatment for ketosis and shortened the recovery period compared to iv. injection of glucose alone.

Abomasal displacement

Peripheral glucose concentrations were increased in cows with left displacement of abomasum at the time of the clinical manifestation of the disease, despite of concurrent subclinical ketosis (Vörös et al., 1986ab). Cows with left displacement of the abomasum showed impaired glucose tolerance and heterogeneity of insulin responses to glucagon stimulation (Holtenius and Traven, 1990), increased basal insulin and glucose levels and

low and slightly fluctuating myoelectric activity of the abomaso-duodenum up to 7 days after surgical correction (Pravettoni et al., 2004). Myoelectric patterns of non-insulin resistant patients were higher and improved progressively after surgery. Abomasal displacement that usually occurs shortly after calving in dairy cows has a complex etiology that still needs clarification (Doll et al., 2009).

2.3. Effect of periparturient energy supplementation on metabolic and reproductive performance in dairy cow

Metabolic and endocrine responses to diet interact with production responses and reproduction in ruminants (Diskin et al., 2003; Roche, 2006; Chagas et al., 2007a). Potential sites of action of nutrition on reproduction include the hypothalamic-pituitary axis, the ovarian and the follicular level (Diskin et al., 2003). Signals which regulate the follicular growth and maturation, time of first postpartum ovulation, the resumption of ovarian cyclicity, furthermore the development and quality of oocytes produced are linked to changes both in energy intake and to specific nutrients. Short-term changes in energy intake influence both oocyte morphology and development in ewes (O'Callaghan et al., 2000). Dairy cows are also highly sensitive to energy input through the diet and there are many reports in the literature on the relationships of energy balance and fertility (Butler, 2000; Cavestany et al., 2005). Though, maintaining energy intake in the transition period is challenging both in the total mixed ration (TMR) and in the pasture fed systems. Regulation of feed intake in dairy cattle is a complex process and is not the scope of this thesis to describe these mechanisms. Multifarious interrelations among brain, BCS and metabolic signals have been recently reviewed by Chagas et al. (2007a). Nutritional and metabolic inputs interact with the reproductive axis and the somatotropic axis at several levels (**Figure 2.3.1.**). Dietary changes may induce rapid responses in peripheral levels of metabolites (glucose, NEFA) and hormones (GH, IGF-I, insulin, thyroid hormones and leptin) with important role in regulation of follicular growth (Gong et al., 2002; Holtenius et al., 2003; Lucy, 2008). Similarly, under grazing conditions, Roche et al. (2005) showed that the level of feeding – not referring to energy density and/or concentrate supplementation - during the last month before calving increased prepartum leptin, IGF-I and glucose, but not during postpartum period. Whatever the mechanism, it is obvious that

the reproductive success of the dairy cow is linked to body energy reserves and metabolic responses to nutrition (Roche, 2006; Chagas et al., 2007a).

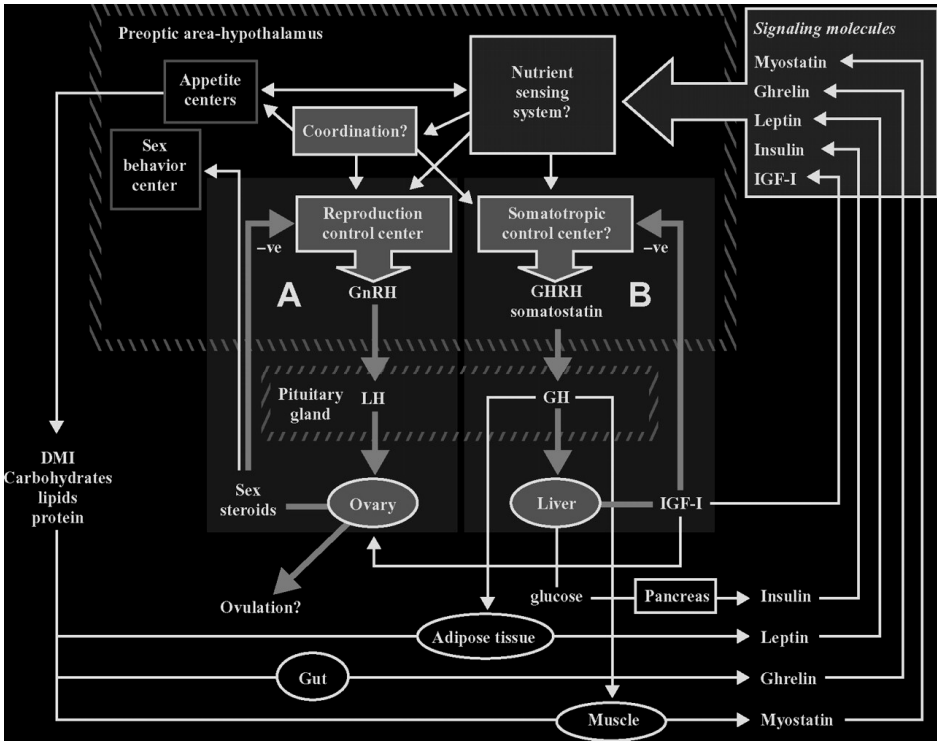


Figure 2.3.1. Interaction between nutrition, metabolic signals and reproduction. The feedback-regulated systems that control the reproductive axis (shaded area A) and the somatotropic axis (shaded area B) interact at several levels and thus link nutritional and metabolic inputs into the reproductive process (Chagas et al., 2007a).

The endocrine signals that most likely can inform the hypothalamic GnRH-producing neurons on the current state of body condition and energy balance are insulin, IGF-I and leptin. Considering the central role of insulin in development of lipid related metabolic disorders and its function in the reproduction axis, surging peripheral insulin concentrations and/or altering the tissue sensitivity to insulin would be an alternative means to improve ovarian function. Exogenous treatment with insulin was attenmpted, but its use is limited by the fact that can cause severe hypoglycemia and eventually depress feed intake. Hayirli et al. (2002) evaluated the use of slow release insulin to determine whether there is a dose of insulin that decreases plasma NEFA and liver TG, without decreasing plasma glucose concentration in healthy animals. Any dose of insulin may cause hypoglycemia (<1.5

mmol/L). However, the authors concluded that 0.14 IU/kg body weight of slow release insulin was prophylactic against hepatic lipidosis and ketosis during the periparturient period. Compounds which should sensitize the peripheral tissues to the action of insulin by lowering NEFA (e.g. nicotinic acid) were scarcely evaluated in dairy cows. Administration of nicotinic acid has been suggested for the treatment of ketosis due to its antilipolytic properties. However, the occurrence of NEFA rebound after large doses of nicotinic acid depress feed intake (Thornton and Schultz, 1980) and potentially can aggravate fatty liver, ketone production, and IR (Pires and Grummer, 2007). Alternatively, long-acting niacin analog, the acipimox which works by preventing fatty acid mobilization from adipose tissue could be applied to lower plasma NEFA, but to our knowledge, this compound has not been tested in ruminants. Chromium (Cr^{3+}) potentiates the action of insulin and chromium deficiency is associated with impaired glucose tolerance, which is reversed by chromium supplementation. Chromium-methionine chelate supplementation attenuated insulin sensitivity prepartum and enhanced glucose tolerance postpartum, but not prepartum (Hayirli et al., 2001).

Alternative option to reduce lipid mobilization and elevation of NEFA can be achieved through increasing energy intake. Energy intake can be achieved by supplements with glucogenic properties (e.g. propylene glycol or glycerin) or by including fat in the dairy ration. Increased peripartal energy intake can be achievied through feeding supplements based on starch or non-structural carbohydrates. The inclusion of dietary starch shortens the duration of NEB, thereby increasing the nadir BCS, and the level of circulating IGF-I, potentially enhancing reproductive succes (Chagas et al., 2007a). Gong et al. (2002) demonstrated that feeding a high-starch diet to dairy cows for the first 50 days post partum enhanced circulating insulin concentrations. Moreover it is known that feeding a higher quantity of easily fermentable carbohydrates during the prepartum transition period prepares the microbial population to lactation diets, promotes development of ruminal papillae, increases absorptive capacity of the rumen epithelium, and reduces lipolysis by delivering more glycogenic precursor to the liver (Grummer 1995; Rabelo et al., 2005). However, feeding higher amount of easily fermentable carbohydrates or without a proper adaptation period will results lowering the ruminal pH and increase the risk of ruminal acidosis (Oetzel, 2007). Compared to starch, feeding propylene glycol (PGL)

showed a higher molar proportion of rumen propionate, other then lactate, and elicited a higher insulin response (Fronk, 1975). Glycerol and propionates are also used as gluconeogenic feed supplements, though it is not clear if there are substantial metabolic differences between the effect of these different glucogenic precursors. Staufenbiel et al. (2007) reported higher DMI in cows fed prepartum with glycerol compared to PGL cows; and even though, reproductive parameters were superior in the later group. It is not clear if these differences were due to direct effect of the PGL. Propionates seem to have less significant metabolic effect than PGL or glycerin. Stokes and Goff (2001) did not detect metabolic responses (decreased NEFA and/or BHB concentrations in plasma) in response to oral administration of a sizeable (0.68 kg) bolus of Ca-propionate. They speculated that there may also be differences in either ruminal metabolism or absorption kinetics of PGL and the propionate supplements. Moreover, feeding glycerol consistently decreased DMI pp in dairy cows, possibly because of its hygroscopic properties and consequently lowering the rate of passage (DeFrain et al., 2004; Ogborne, 2006).

Including different fats to the diet of the dairy cows is another common practice to increase energy intake and in this way decrease the duration or magnitude of NEB. Furthermore, the type of fatty acid included in the diet can have dramatic impact on reproduction physiology – particularly fat source rich in polyunsaturated fatty acids (PUFA) are able to modulate metabolic and reproductive physiology. When fat is added to the diet of beef or dairy cattle, positive effect on follicular growth, on size of the DF, embryo quality and on pregnancy rate is frequently observed (Staples et al., 1998; Santos et al., 2008). The mechanisms by which fat supplementation alters reproductive physiology are not completely understood. Generally both n-6 and n-3 PUFA have effect on follicular growth and oocyte quality, although feeding n-6 PUFAs in late gestation and early lactation enhanced uterine prostaglandin (PG) secretion and uterine involution (Cullens et al., 2004), while feeding n-3 PUFAs in early lactation suppressed the uterine PG secretion, improved the embryo quality and maintained pregnancy (Bilby et al., 2006). Increased steroidogenesis, enhanced luteal function, oocyte/embryo quality, modulation of the uterine PG synthesis and maternal recognition of the pregnancy are considered important pathways of fat intake regulatory mechanism.

2.3.1. Effect of propylene glycol supplementation on production responses and on metabolic and endocrine profile

Propylene glycol (*PGL; 1,2-propanediol*) is a 3-carbon compound alcohol ($C_3H_8O_2$) derived from propylene. It contains two hydroxyl groups, located on carbons 1 and 2. PGL is a colorless or nearly odorless, clear, viscous liquid. As an oral drench PGL has been used for many years for the prevention and treatment of ketosis in dairy cows (Johnson, 1954). PGL can be administered *via* three ways: by drenching intraruminally, feeding together mixed with the concentrate, or incorporated as a part of the TMR. Oral drenching of liquid PGL is probably the most effective way to prevent and treat ketosis in fresh cows. Liquid drenching, however, requires skilled personnel, labor intensive and very stressfull to the animals.

Experiments with sheep and cows have shown that PGL is highly digestible and it disappears quickly from rumen via three different routes: *absorption, fermentation* or *direct passage* to the intestine (Kristensen and Raun, 2007). Early data on PGL metabolism suggested that PGL was mainly absorbed in intact form through the ruminal epithelium and absorbed into the liver, and just to a lesser extent, fermented into propionate before absorption (Emery et al., 1967). However, recent studies have shown that *ruminal fermentation* is considerable in cattle, and hepatic extraction of PGL is of lesser importance (Kristensen and Raun, 2007). Microbial degradation of PGL results in propanal (propionaldehyde), propanol and propionate. PGL is absorbed into the liver just in small amounts. PGL has been shown to decrease the ruminal acetate to propionate ratio, thereby resulting in a ruminal pattern of volatile fatty acid that is more glucogenic while the pH usually is not affected (Nielsen and Ingvartsen, 2004). However, pH was consistently lower when cows were orally drenched with PGL compared with cows fed dry PGL as part of the TMR (Chung et al., 2009). Even though, pH was above the critical value which can cause ruminal acidosis (pH > 5.5). The effect of PGL on butyrate is inconsistent, which may be related to the type of diet fed or the amount of PGL administered (Nielsen and Ingvartsen, 2004).

In the liver lactate appeared to be the main product of *hepatic metabolism* of PGL (Kristensen and Raun, 2007). Lactate is a common substrate in gluconeogenesis in ruminants and enters the tricarboxylic acid (TCA) cycle via pyruvate and is converted to

oxaloacetate. PGL that is metabolized to propionate in the rumen can also be converted into glucose via gluconeogenesis in the liver (**Figure 2.3.1.1.**). In ketosis the oxaloacetate is used for gluconeogenesis, and the acetil-CoA from β-oxidation is not able to enter to the TCA cycle. PGL increase the hepatic oxaloacetate concentration and decrease ketone bodies and liver TG concentration. Factors that determine the rate of PGL fermentation in the rumen are not completely known, but probably relates to the roughage/concentrate ratio and ruminal microflora activity.

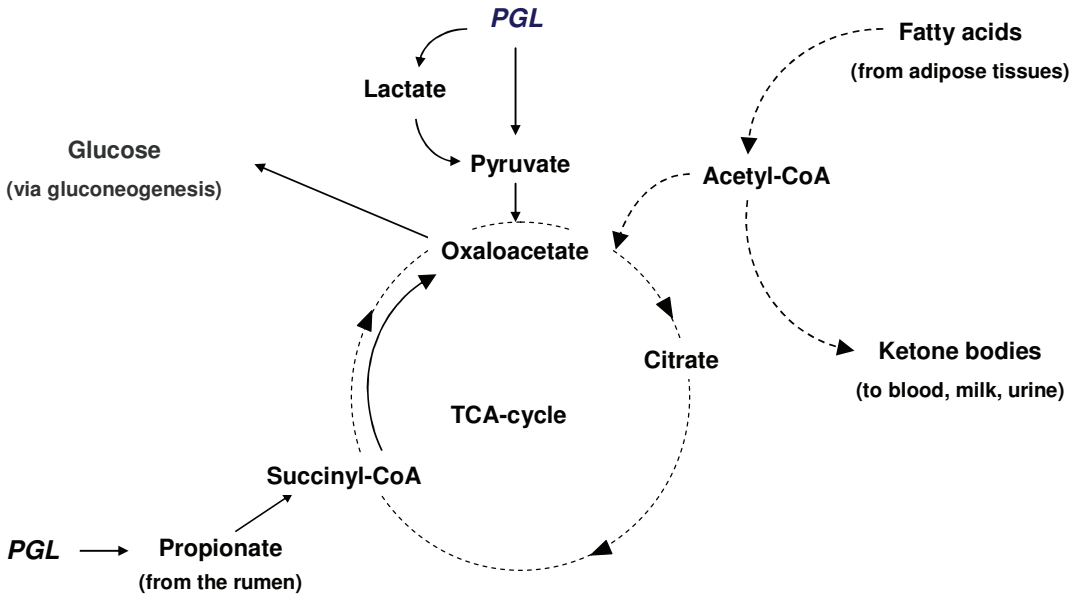


Figure 2.3.1.1. Metabolism of propylene glycol (PGL) and its interaction with the ketogenesis in the liver of cattle. Gluconeogenic pathways are shown by the solid lines. (Nielsen and Ingvartsen, 2004)

In general, PGL supplementation has little impact on *milk production* and *milk composition* as summarized by Nielsen and Ingvartsen (2004). However, supplemented to cows during early lactation showed a tendency to increase milk yield and decrease milk fat percentage; this later effect is probably due to decreased acetate/propionate ratio in the rumen and lower plasma NEFA concentration, and consequently lowered uptake of NEFA by the mammary gland (Nielsen and Ingvartsen, 2004). By sparing energy from milk synthesis cows mobilize less from adipose tissue and in this manner the energy status is improved. Milk fat percentage was also reduced in studies of Hoedemaker (2004) and

Chagas et al. (2007ab). Percentages of milk lactose was increased when cows were fed with 161 g (mixed in TMR) or 200 g oral or ruminal drench (Chung et al., 2009), while feeding 200 to 400 g/d had no effect on milk lactose in mid-lactation (Cozzi et al., 1996; Shingfield et al., 2002). Studies conducted in mid-lactation have not observed any effect on milk production (Cozzi et al., 1996; Shingfield et al., 2002).

PGL is an *unpalatable* feed additive in itself. Miyoshi et al. (2001) observed that lactating cows decreased their feed intake after 1–2 days of top-dressing 518 g PGL per day, due to its low palatability. In one recent study where a commercial product containing 55% dry PGL was used in the periparturient period not influenced DMI before parturition, but increased it with 2.6% after parturition (Moallem et al., 2007a, 2007b). It does not appear that PGL limits the consumption of concentrates containing up to 10% PGL (Fisher et al., 1973; Christensen et al., 1997). Feeding 161 g/d dry, pulverized PGL (absorbed on silicon dioxide) mixed in TMR did not affect the DMI when compared to unsupplemented cows or those receiving PGL as oral or ruminal drench (Chung et al., 2009).

PGL is a feed supplement with high energy content. Assuming that all the PGL is absorbed in the cow, the amount of digestible energy would be 23.7 MJ gross energy/kg DM (Miyoshi et al., 2001). Available studies consistently demonstrated that PGL supplementation increase glucose and insulin and decrease plasma NEFA and ketone body levels and the accumulation of TG in the liver (Studer, 1993; Grummer et al. 1994; Christensen et al. 1997; Rukkwamsuk et al. 2005). However, the magnitude of responses varies among trials: only minor changes were found in some studies, while significant differences were proved in several others. Time of sampling from PGL administration, dosage, physiological status of the animals and allocation method can be related to these differences. A drench dose of approximately 500 mL/d or more is usually used as a prophylactic treatment for clinical ketosis in dairy cows (Nielsen and Ingvartsen, 2004). A lower amount of PGL (e.g. 118 mL/d), however, is often drenched to cows after parturition as a preventive for subclinical ketosis. The effect of PGL dose on plasma levels of NEFA and BHB depends on the physiological state of the animals, thereby the level of these metabolites before drenching. In dry cows PGL had no effect on liver TG (Bremmer et al., 2000), whilst administration of 1063 g PGL 10 d before calving until calving significantly decreased TG content of the liver by 32% and 42% on d 1 and 21 pp, respectively (Studer

et al., 1993). Drenching 400 ml of PGL once daily from d 7 before calving until d 7 after calving reduced serum NEFA and TG accumulation in the liver (Rukkwamsuk et al., 2005). It has been shown that drenching PGL is more effective in increasing insulin and decreasing plasma NEFA and BHB concentrations than fed in the TMR (Christensen et al., 1997; Chung et al., 2009). The reason for the less pronounced effect of PGL in a TMR based system is that PGL is taken up over a longer period of time, compared with drenching. However, mixing pulverized PGL into the TMR distributes energy at a slower rate for a longer period of time while top-dressing dry PGL onto the TMR stimulates a surge of insulin similar to drench of liquid PGL.

PGL is a potent stimulator of plasma *insulin* concentration. Insulin concentration increased by 200-400% within 30 min using dosages ranging from 307 to 1036 g (Studer et al., 1993; Christensen et al., 1997). The time of sampling after PGL administration and the method of administration may affect the plasma or serum insulin concentration. Some authors (Grummer et al., 1994; Christensen et al., 1997; Miyoshi et al. 2001) measured serum insulin within the first 3 hour after administration of PGL, while others (Cozzi et al., 1996; Rukkwamsuk et al., 2005) sampled >4 hours after allocation, thereby after the expected insulin peak. Plasma insulin was greater for oral or ruminal drench of PGL compared to PGL mixed in the TMR (Christensen et al., 1997; Chung et al., 2009). Studies which evaluated the response of glucose and insulin with frequent blood sampling after PGL administration had shown that plasma insulin peak occurs earlier than glucose (Studer et al., 1993; Grummer et al., 1994). Brockman (1982) reported that propionate stimulates directly the pancreatic insulin production. When relatively large amount of PGL is fed, the propionate produced in the rumen can saturate the liver, escaping hepatic removal and stimulating pancreatic secretion of insulin (Chung et al., 2009). The magnitude of the response in blood insulin concentration also is highly dependent on the amount of feed ingested (Armentano et al., 1984). To prevent postprandial increases of blood insulin concentrations, and therefore interfering with the effect of PGL treatment, Chung et al. (2009) used a 12x feeding/d frequency, which provide a steady state for glucose production and utilization. The effect of PGL on blood *glucose* concentration is limited compared to its influence on insulin. An explanation is that the large insulin peak elicited by PGL administration rapidly reduces plasma glucose concentration.

There are only few reports regarding *IGF-I* response to PGL supplementation. Hoedemaker et al. (2004) reported that peripartal drop in the IGF-I concentrations occurred later in cows fed diet enriched at 10% PGL than in controls cows, without PGL supplementation. In the control group, this decrease occurred earlier (1 wk antepartum) than in the PGL fed group (at parturition), and mean IGF-I concentrations were higher in the PGL group than in the control from 1 wk antepartum until 1 wk postpartum. Formigoni et al. (1996) also reported a positive effect on postpartal IGF-I concentrations after administration of PGL during the transition period in the treated *vs*. the control group.

2.3.2. Effect of propylene glycol supplementation on reproductive performance

Experiments focused on reproductive consequences of PGL supplementation have divergent results. On d 96 pp lower proportions of cows was acyclic in cows drenched with PGL than in controls (Formigoni et. al., 1996). Drenching daily 500 ml of PGL (which corresponds to 31.38 MJ) Miyoshi et al. (2001) reported decreased interval to first postpartum ovulation (32 vs. 44 days). First pp luteal phase was longer in cows supplemented with PGL (13.4 vs. 7.3 days), reflecting better luteal function. Drenching the same amount of PGL to dairy cows from d 10 before parturition until d 25 pp had no effect on the proportion of follicles which became ovulatory, cystic or atretic. Despite of improved energy status PGL supplementation failed to increase mean LH or LH pulse frequency on d 10 pp (Butler et al., 2006). PGL supplementation had no effect on steroidogenic capacity of the DF (P4, androstendione and E2 secretion) in spite of increased insulin secretion (Moallem et al., 2007a,b). Hoedemaker et al. (2004) studied the effect of peripartal PGL supplementation mixed into concentrate on metabolism, neutrophil function, health and fertility in 8 dairy herds with high milk production. Plasma NEFA and BHB were lower and IGF-I started to decrease later and it remained higher in the PGL supplemented groups in all farms. Furthermore, incidence of cows in subclinical ketosis was lower in the PGL enriched group. Even though, no long term effect on health and fertility was observed. Based on P4 concentration on week 3 pp 47.9% of PGL treated and 46.3% of control cows had luteal activity. Even if PGL supplementation lowered NEFA and BHB concentration, the count and functional activity of neutrophil granulocytes were not affected (Hoedemaker et al., 2004).

The inconsistent efficacy of PGL between the studies to some extent maybe related to

the different energetic conditions of the animals supplemented. This is supported by studies conducted under pasture based systems by Chagas et al. (2007a,b, 2008). Feeding PGL in pasture based system has shown positive effects of energy supplementation in cows and heifers having poor BCS at the time of calving (Chagas et al., 2007b). Holstein heifers were allocated into low (BCS ≤2.8) and high (BCS ≥3.4) BCS group at the time of calving. Half of the low BCS group was drenched twice daily with 250 ml PGL for 16 weeks after calving. The time of first pp ovulation and the length of the pp anoestrus were significantly lower in cows with low BCS supplemented with PGL, but was the same as in the group with good BCS without PGL supplementation. All cows from PGL group, 92% of cows from high BCS group and only 66% of the non-supplemented group became pregnant. Reduction of the pp anoestrus period was attributed to the increase in LH pulse frequency on weeks 2 and 5 pp (Chagas et al., 2007b). In another study conducted by Chagas et al. (2008) under grazing condition, the plasma insulin was higher in feed restricted dairy cows drenched twice daily with 250 ml of monopropylene glycol for 150 d than not treated controls. However, there was no difference in interval from calving to the first ovulation or in the liver GH receptor and plasma IGF-I levels. It was concluded that in term of reproduction PGL has no benefit in cows calving at optimal BCS.

Glucogenic supplementation may have positive effects on the resumption of ovarian cyclicity via increasing insulin concentration; however, recent studies reported unfavorable effects on oocyte and/or embryo quality (Adamiak et al., 2005; Fouladi-Nashta et al., 2005). Oocyte quality is also affected by an interaction between feeding level and body condition: a high level of feeding is beneficial to oocytes from animals of low body condition, but detrimental to oocytes from animals of moderate to high body condition (Adamiak et al., 2005). Cows were fed isoenergetic diets with low or high starch diet and after synchronized estrus oocytes were collected with ultrasound guided ovum pick up and were fertilized in vitro (Fouladi-Nashta et al., 2005). HS diet adversely affected oocyte quality and the number of cleaved embryos. However, information regarding to the effect of PGL drenching on oocyte quality are very scarce. One study evaluated the effect of PGL supplementation on metabolic hormones interacting with fertility and on oocyte quality in pp dairy cows (Rizos et al., 2008). 500 ml of PGL was drenched from d 7 pp to HF cows. Oocytes were recovered by ultrasound guided ovum pick up starting on approximately d 30

pp and submitted to *in vitro* fertilization. Treatment with PGL had no effect on follicular dynamics, mean days to emergence of the first cohort of follicles postpartum, or days to dominance and duration of dominance for any follicular wave recorded postpartum. There was also no difference in interval to first ovulation or in size of the preovulatory follicle between treatments. Oocyte quality was measured by blastocyst development after *in vitro* fertilization; despite a higher cleavage rate and blastocyst yield in treated cows, the difference was not significant (Rizos et al., 2008). An effect on oocyte developmental competence remains to be proved.

3. Aims of the studies

Our goal was to investigate the periparturient insulin pattern and insulin resistance in high lactating dairy cows in relation with some metabolic and reproductive malfunctions and to improve postpartum ovarian function through energy supplementations in cows under different management systems. The aims of the experiments were:

(i) to determine the glucose-induced insulin responsiveness and the whole body-insulin sensitivity in cows showing different forms of periparturient ketone pattern with and without puerperal metritis, and the interrelation between results of these challenge tests with the revised quantitative insulin sensitivity check index (RQUICKI) and different metabolic and hormonal parameters in *TMR-fed* dairy cows (*Exp.1.*);

(ii) to study the effects of periparturient propylene glycol (PGL) supplementation (provided, as a pulverized product) on glucose-induced insulin responsiveness, whole-body insulin sensitivity, some metabolic and hormonal parameters, the time of the first postpartum ovulation and pregnancy rates in *TMR-fed* dairy cows (*Exp. 2.*);

(iii) to investigate the effects of prepartum energy supplementation with cracked corn grain on postpartum milk production and reproductive performance in cows under a *pasture-based* dairy production system (*Exp. 3.*).

4. Materials and methods

4.1. Farm conditions

Description of the common housing, management and nutrition systems of the experiments are given here, while specifications on the circumstances of the given study are detailed under the trial (*Section 5.1., 5.2.* and *5.3.*). All studies were performed in accordance with the rules and under the permission and control of the local state veterinary services. All of them were carried out following the guidelines given by the code of ethics of the Szent István University, Faculty of Veterinary Sciences Animal Welfare Board.

Exp. 1 and *2* were conducted under intensive conditions in TMR-fed large scale dairy herds. *Exp. 3* was carried out in Uruguay, under pasture-based conditions. In all the animals of the 3 herds calved year-round in continuously used maternity barns. Calves were weaned immediately after parturition. Cows were milked twice daily, according to the regular farm management. Drinking water was available *ad libitum*. Diet in *Exp. 1* and *2* consisted of corn silage, concentrate, alfalfa and grass hay, vitamin and mineral premix offered in form of TMR. Composition of the daily rations was calculated in accordance with the NRC (1999 or 2001) recommendations. TMR was offered twice a day under the trial. In *Exp. 3* cows assigned into experiment were separated in paddocks of natural pasture and received concentrate supplementation according to the experimental design described in details. Further information is provided in *Section 5.1., 5.2.* and *5.3.*

4.2. Reproductive management

In *Exp. 1* and *2* the course of uterine involution was controlled on a regular basis by palpation per rectum (PR) and vaginoscopy by the herd veterinarian, and bacterial complications (puerperal metritis, clinical endometritis, pyometra) were treated with commercially available antimicrobials, uterotonica and luteolytica. In *Exp. 1* cows were artificially inseminated (AI) $\geq$ 50 days after calving when showing signs of heat. Pregnancy checks were carried out at 45-60 days post AI by rectal palpation. Pre-Ovsynch protocol was used for synchronization of ovarian cyclicity from day 35 after calving in *Exp. 2*. Twenty four hours after the last GnRH injection the cows were artificially inseminated (fixed time AI). Pregnancy was confirmed with transrectal ultrasonography performed

between days 35 and 40 after the fixed time AI. Non-pregnant cows after the first AI were inseminated later at estrus and pregnancy rates were followed until day 150 after calving. Only healthy cows with no detectable puerperal complications were involved in *Exp. 3*, where cows were inseminated as in *Exp. 1*, and pregnancy was checked as in *Exp. 2*.

4.3. Sampling

Depending on the actual design the sampling schedule was different in each study (see also *Section 5.1., 5.2*. and *5.3*.). Here only the technical aspects are detailed.

Blood collection and processment for assaying metabolites and metabolic hormones

All blood samples were taken from the *jugular vein* into tubes containing NaF (for blood glucose analysis), furthermore into tubes preserved with Na_2-EDTA (*Exp. 1* and *2*) or heparin (in *Exp. 3*) (for assaying the NEFA, BHB, urea, insulin, IGF-I, T4, T3 and leptin content). The samples were cooled and centrifuged within 60 min; plasma was harvested immediately, and stored at +4 ^{o}C (if assayed <48 h), or -20 ^{o}C or -60 ^{o}C (if assayed later).

Monitoring the resumption of cyclic ovarian function

For monitoring the time of first pp ovulation and resumption of ovarian cyclicity the P4 content was determined in serially collected milk (*Exp. 1* and *2*) and blood (*Exp. 3*) samples. Milk samples were collected in vials containing potassium bichromate and kept on +4 $^{\circ}$C until analysis, within 14 days after collection. Blood samples were taken and processed as seen above.

Intravenous glucose and insulin tolerance tests (GTT, ITT)

Simultaneous glucose (GTT) and insulin (ITT) tolerance tests were performed in *Exp. 1* and *2*. (**Fig. 4.3.1**.). The initial blood collection was followed by a standard-dose glucose infusion (0.5 g/kg body weight glucose, Glucose 40% inf., Human Ltd., Gödöllő, Hungary) in the *v. epigastrica superficialis* during an average time of 6 min. Blood samples were collected at 5, 15, 30, 45, 60, 75, 90, 120, 150, 180, and 210 min after finishing glucose infusion. After the blood collection in minute 210 rapid-acting human recombinant insulin (0.1 IU/kg, Humulin R inj., 40 IU/ml, Eli Lilly Co., Indianapolis, IN) was injected into the jugular vein. Further blood samples were collected at 240, 270, 300, 330, 360, 420 and 480 min after insulin injection. Metabolites and hormones were determined from basal samples

and insulin (Na_2-EDTA) and glucose (NaF) from each sample.

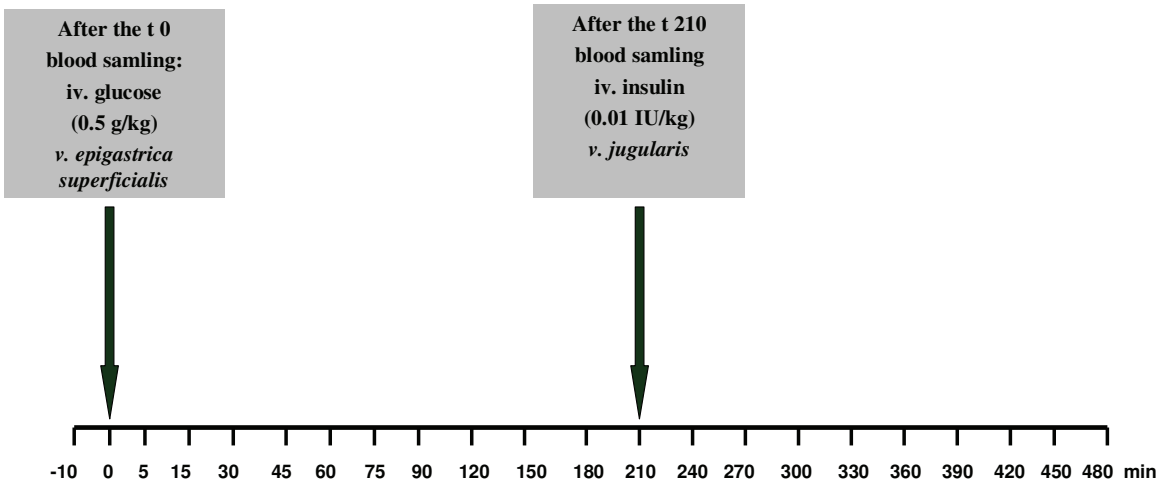


Figure 4.3.1. Simultaneous intravenous glucose (GTT) and insulin tolerance (ITT) test.

Liver biopsy

In *Exp. 2* a subset of 16 cows were selected for liver biopsy. Biopsy was performed in the 11th intercostal space by percutaneous needle biopsy under local anesthesia (10 ml of procain-hydrochloride, Minocain™; Kon-Pharma GmbH, Hannover, Germany). Approximately 700-800 mg of hepatic tissue was collected from each animal, and thereafter stored at -50°C until analysis of total lipid content.

4.4. General introduction of laboratory procedures

All of the endocrine assay systems used in these studies were validated previously for bovine plasma: the binding pattern of serially diluted plasma or milk samples (with known high quantity of the hormone analyzed) was parallel to that of the standard curves; the recovery rates of added known quantity of hormones to standard bovine plasma samples (n=6 in each case) varied between 95 and 106%. Using triplicates of quality control samples with known (low, intermediate and high) quantity of the analyzed hormone, the reproducibility (intra- and inter-assay coefficient of variation, CV%) of each assay run was checked continuously. The results were accepted only if the actually measured concentrations of quality control samples were within the 95% confidence limit.

The *glucose*, *NEFA, BHB, urea* and *total cholesterol* were analyzed with commercial kits based on enzymatic reactions. The technical aspects of various assay procedures may be different in the various studies and are detailed in *Sections 5.1*., *5.2*. and *5.3*.

Total lipid was extracted from the *liver samples* according to the method described by Folch et al. (1957). Half a gram of the liver biopsy sample was homogenized with 10 ml of 2:1 (v/v) mixture of chloroform-methanol after which 2.0 ml 0.9% NaCl was added, mixed and allow to stand for 2 h prior to phase separation. The chloroform-methanol extract was evaporated to dryness in a water bath at 50 °C under N_2 flow then the residue was weighed in a laboratory scale. Total lipid content of the sample was expressed as gram per 1 kg of wet liver weight.

4.5. Statistical procedures

All data are presented as means and standard error of the means (±SEM). The results were usually subjected to 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 ($P<0.05$), post hoc tests were performed for further comparisons. Temporal patterns were usually tested by repeated measures ANOVA. Interrelations were estimated by using Pearson's correlation. To analyze the time to first postpartum ovulation and the first visible estrus, the Kaplan-Meier survival analysis was performed.

Parameters measured during the GTT in *Exp. 1* and *2* included: basal insulin and glucose, insulin area under the curve (Ins AUC), insulin peak (the maximal insulin response; Ins peak), insulin increment (Ins peak- Ins basal), glucose clearance rate (glucose CR) and glucose half-time (glucose $T_{1/2}$).

Basal glucose and insulin were calculated by averaging values from samples t-10 and t0. The insulin AUC in the first 60 min after glucose infusion was calculated by trapezoidal model and corrected for the basal values. The insulin response to glucose was illustrated by insulin peak (maximal insulin concentration observed after the glucose load; Ins peak), insulin increment (insulin peak-basal insulin; Ins Inc) and the insulin area under the curve (Ins AUC).

Glucose clearance rate (glucose CR) and the glucose half-life (glucose $T_{1/2}$) was measured according to Kaneko (1989):

Glucose CR= [(ln glucose t5 – ln glucose t60)/ (t60-t5)] x 100 = % min

Glucose $T_{1/2}$= (0.693/k) x 100 = min.

During the the the ITT in *Exp. 1* and *2* the insulin stimulated blood glucose response was calculated with the formula according to Oikawa and Oetzel (2006):

ISBGR (%) = [(glucose t0 - glucose t30) /glucose t0] x 100

Statistical analysis was done using Microsoft Excel®, SPSS (version 8.0®; SPSS Inc., Chicago, IL), the Proc Mixed (Statistical Analysis System, SAS Institute, Cary NC, USA 2000) and the R 2.4.1. free-ware statistics programs. Further details of statistical methods are also given at the description of studies (*Sections 5.1*., *5.2*. and *5.3*.).

5. Description and results of experiments

5.1. Pancreatic insulin secretion and whole-body insulin sensitivity in cows with different forms of hyperketonemia with or without puerperal metritis (*Exp. 1*)

Materials and Methods

Farm condition; experimental animals

The study was conducted in a large-scale dairy herd with about 900 Holstein Friesian cows. Groups of cows (70 to 100 animals in each) were established about once a month in accordance with the stage of lactation and the average daily milk production. Cows were kept and fed in a free-stall housing system without pasturing. During the 8-week dry period, in the periparturient days, and in the first four weeks after calving cows were placed in separated groups. Daily ration was calculated in accordance with the NRC (2001) recommendations.

On day 259-265 of gestation 31 healthy multiparous (parity: 3 to 5) cows producing 8331±193 kg of fat corrected milk in the previous 305-day lactation, and calving in February and March were selected for the study. The animals (n=31) were separated from the other herd mates in a paddock (for 14-16 d), 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 d). The group of late-pregnant cows received the same diet (consisting of 3.5 kg commercial concentrate mixed into 15 kg of corn silage, with free access to meadow hay available *ad libitum*) for 14-16 d (**Table 5.1.1**.). When the cows were placed in boxes of 3-4 animals in the maternity unit (herein denominated as transition cows) growing quantity of concentrate and corn silage was given. The quantity of concentrate was incremented gradually (about 1.0 kg in every other day until the end of the sampling period, with the maximum of 9.0 kg on day 7 after calving). Corn silage was increased from 15 to 25 kg after calving. Meadow hay was available *ad libitum* throughout this period. All cows left the maternity unit 8 days after calving, and were moved into the group of fresh milkers. Later during the lactation, these cows were kept together with the other herd mates and received the same feeding and reproductive management.

Table 5.1.1. Daily ration of milking cows fed during the dry period and in the first 100 d of lactation, and thereafter.

	Dry cow I	**Dry cow II**	**Transition cow**	**Fresh cow**	**Peak lactation** (39 kg/d)	**Late lactation**
Components of daily ration (kg/day/cow)						
Corn silage	12	15	15* / 25**	25.0	25.0	25.0
Meadow hay	Ad libitum	Ad libitum	Ad libitum	-	-	-
Alfalfa hay	-	-	-	4.5	7.0	7.0 ⇒ 4.5
Concentrate I	1.0	3.5	-	-	-	-
Concentrate II	-	-	3.5 ⇒ 9.0***	9.0	10.0	9.0 ⇒ 3.5
Wet sugar beet pulp	-	-	-	1.5	2.0	1.0
Corn gluten	-	-	-	1.8	2,2	0,7
Molasses	-	-	-	0.5	0.5	0.5
Additives	-	0.15	0.15	0.4	0.2-	-
Ingredients						
Dry matter (kg/d)	≈ 11.0	≈ 12.0		20.2	22.7	22.7 ⇒20.0
NE_L (MJ/d)	59.6	74.1		139.5	163.9	Decreasing
Crude protein (g/d)	1208	1594		3247	3972	Decreasing

Dry cow I: first 5 weeks of the dry period;
Dry cow II: last 3 weeks of the dry period;
Transition cow: kept in the maternity unit, between d about 4-5 prepartum to day 8 postpartum;
Fresh cow: from calving to first test milking on d 28-36 of lactation;
* Before calving
** After calving
*** The quantity of concentrate increased continuously (with about 1.0 kg in every alternate day till the end of the sampling period, with the maximum of 9.0 kg on day 7 after calving)
Concentrate I: 28.5% corn, 30% barley, 28% solvent-extracted sunflower meal, 13,5% mineral-vitamin premix
Concentrate II: 55.5% corn, 10% full-fat soybean, 10% barley, 5% corn distiller's dried grain, 4% solvent-extracted sunflower meal, 13.5% mineral-vitamin premix, 2% *Saccharomyces cerevisiae* living culture
Additives: niacin, cobalt-chloride and *Saccharomyces cerevisiae*
NE_L net energy lactation.

Clinical examination and sampling

On d 1 to 7 after calving, the uterine involution was checked once daily with vaginoscopy and rectal palpation, and also the rectal temperature was measured. If malodorous, reddish-brown, watery (putrid) vaginal discharge was present, animals were diagnosed as affected by *puerperal metritis*. These cows were treated with intrauterine administration of 2-4 g oxytetracycline (Tetra-Bol™ 2000 tabl., CP-Pharma GmbH, Burgdorf, Germany) repeated once a day for 3 to 5 d. Cows with pyrexia (≥ 39.5 °C) received also intramuscular oxytetracycline therapy (10 mg/kg body weight, repeated once 48 h later; Engemycin™ inj., Intervet, Angers, France). C*linical endometritis* (presence of purulent vaginal discharge after pp day 14 onwards) was treated with 0.5 g cefapyrin (Metricur™ intrauterine inf., Intervet, Angers, France) combined with repeated administration of synthetic PGF2α (0.25 mg cloprostenol, Estrumate™ inj., Schering-Plough Animal Health, Union, NJ, USA). All cows showing estrus on day about ≥50 were inseminated (AI; unless the vaginal discharge was mucopurulent or purulent at estrus), and those not returning to estrus were checked for pregnancy by rectal palpation on day 45-60 after AI. The presence of systemic signs (fever, anorexia, depression, etc.) and concurrent diseases (i.e. mastitis) were recorded.

Individual milk yield was recorded on d 7 and once between d 28-36 and d 60-70 after calving. Body condition score (BCS) was recorded at the time of inclusion in the study (d -18 to 25), on d 7 and between d 28-35 pp. BCS of cows was estimated using a scale from 1 (emaciated) to 5 (fat) according to Edmondson et al. (1989). Body weight was recorded at inclusion and on d 7 pp.

Blood samples were taken regularly: on d 259-265 of gestation, again two times 7 days apart, subsequently once a day until d 7 after calving, and thereafter two times again, on d 25-35 and 60-70 of lactation for assaying the NEFA, BHB, insulin, IGF-I and leptin. For monitoring the resumption of ovarian cyclicity milk samples were taken 2-3 days apart (three times a week) from d 8-10 after calving for 10-12 wk for P4 determination.

Glucose and insulin tolerance test (GTT and ITT)

Simultaneous glucose (GTT) and insulin (ITT) tolerance tests were performed between d 18-22 before delivery (e.g. late gestation), on d 7 after calving (e.g. early lactation), and again on d 60-70 of lactation. The initial blood collection with 10 min apart

(t-10 and t0, respectively) and thereafter performed as described in *Section 4.3.*

Laboratory assays

Insulin content was quantified as free insulin with a commercial ^{125}I-IRMA kit developed for human samples and validated for bovine plasma samples (BI-Insulin IRMA kit; CIS Bio International Ltd.). The sensitivity of the assay was 3.16 pmol/L; intra- and inter-assay CV was between 1.3 to 5.6% and ≤8.5%, respectively. Leptin concentration was quantified by a local version adapted by Kulcsár et al. (2006) of the ruminant-specific, homologous, double-antibody ^{125}I-RIA of Delavaud et al. 2002 (sensitivity: 0.032 nmol/l; inter- and intra-assay CV: 12.2, 5.6 and 6.1%, furthermore 10.1, 4.6 and 5.3% in ranges of quality control samples with "low", "medium" and "high" leptin content, respectively). Neutralized acid-ethanol extracts of bovine plasma were analyzed for IGF-I using a heterologous ^{125}I-RIA (Nikolic et al., 2001). The mean sensitivity, intra- and inter-assay CVs were 0.20 nmol/L, ≤6.2 and ≤12.0 %, respectively. The glucose, NEFA and BHB were analyzed with commercial kits based on enzymatic reactions (Glucose: kit no. 40841, Diagnosztikum Ltd., Budapest, Hungary; NEFA: kit no. FA 115, Randox Laboratories Ltd., Ardmore, UK; BHB: kit no. RB 1007, Randox Laboratories Ltd., Ardmore, UK). The P4 content of previously defatted bovine milk samples was determined with a microplate ELISA developed for equine plasma (Nagy et al., 1998), which was modified and validated for assaying bovine skim-milk (Huszenicza et al., 1999 and 2005). Sensitivity was 0.18 nmol/L. The inter- and intra-assay coefficients varied between 10.3 and 12.3%, and between 5.2 and 11.6%, respectively.

Statistical analysis

The general aspects of data analysis are detailed in *Section 4.5*.

The revised quantitative insulin sensitivity check index (RQUICKI) was calculated as 1/ [log (basal glucose) + log (basal insulin) + log (basal free fatty acid)] (Holtenius and Holtenius, 2007). Parameters measured during the GTT and ITT performed between d -18-22 before delivery and on d 7 and plasma metabolites and hormones were subjected to repeated measures analysis. The fixed effect included time and group based on the periparturient BHB patterns and their interaction. Cow was used as random variable. If the overall analysis proved significant difference ($P<0.05$), post hoc tests were performed for further comparisons. Pearson's correlation was used to evaluate the association between

basal parameters (NEFA, BHB, insulin, glucose, leptin, IGF-I, RQUICKI, BCS, BCS loss), and the parameters calculated during the GTT and ITT.

Results

Data of 3 cows showing moderate clinical signs of mastitis on days 1, 2 and 4 after calving were excluded from the final analysis. Finally data from 28 cows were evaluated. In 6 of them puerperal metritis was diagnosed: the first pathognomonic signs (malodorous, reddish-brown, watery vaginal discharge with fever, anorexia and depression) were observed on d 2 to 6 after calving, and were still existing on day 7, when the GTT and ITT were conducted.

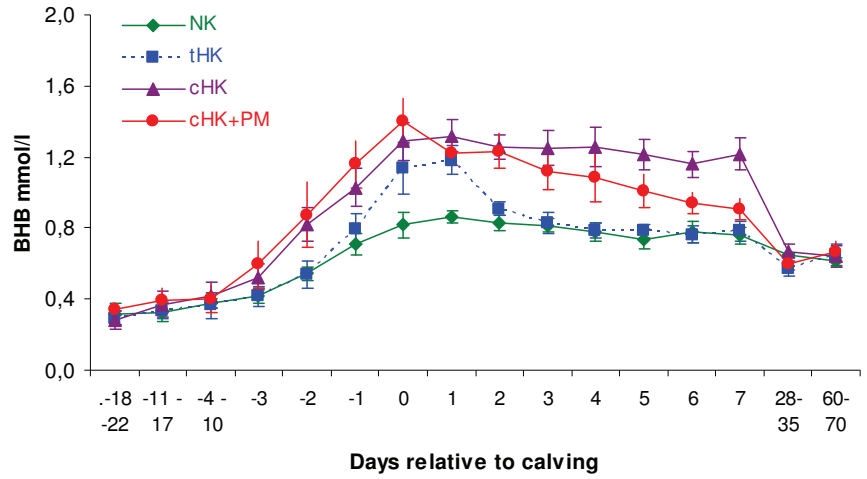
Cows were in moderate to good condition during late gestation ($BCS_{d\text{-}18\ to\ -25}$: 3.75±0.04; range: 3.50 to 4.25). BCS started to decrease during early weeks of lactation ($BCS_{d\ 7}$: 3.31±0.05, P<0.001, $BCS_{wk\ 5}$: 2.66±0.06, P<0.001). On d 7 after parturition, cows presenting clinical signs of puerperal metritis had lower BCS than the other 22 animals (3.08±0.11 *vs.* 3.37 ±0.08; P= 0.03). On d 28-35 pp this difference became more pronounced: compared to their BCS in late pregnancy, the PM cows showed significant BCS loss, whereas the BCS of others decreased only in a moderate form (BCS_{loss}: 1.42 ±0.1 *vs*. 1.0 ±0.01; P=0.008). The milk production recorded on d 7, 28-36 and 60-70 pp was 26.4±1.3, 38.3±1.5 and 36.4±1.7 L/day, respectively. Cows showing metritis in the early puerperal phase produced less milk on d 28-35 than the others (29.9 ±3.5 kg vs. 40.6 ±1.3 kg, P=0.02).

Periparturient BHB patterns; related changes in plasma metabolites and hormones

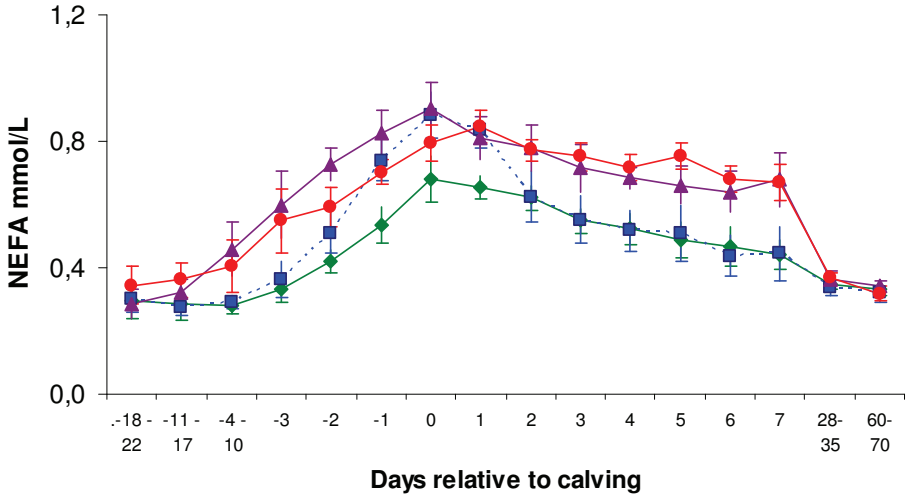
During the late pregnancy the plasma BHB was low (<0.75 mmol/L) in all cows, and it started to increase 1 to 3 d before calving, with wide individual variations. Based on the periparturient plasma BHB patterns and the occurrence of puerperal metritis the 28 cows could be classified into four *groups (**Fig. 5.1.1A**). In* 9 animals the BHB levels did not exceeded the 1.20 mmol/L threshold value on the sampling days (*normoketonemic* cows, NK), while in 7 cows were elevated only on the day of, and/or one day after calving (cows with *transient hyperketonemia*, tHK). In 6 cows, however, the ketone body elevation became significant 2 d before calving (BHB levels compared to those on day 18-22 before calving: P<0.05), the BHB levels reached threshold value of 1.20 mmol/L on day -2 to 0 (day 0 = day of calving) and remained elevated through the first 7 d after calving, till the

end of the intensive (once-a-day blood) sampling (cows with *continuous hyperketonemia*, cHK; n=6). In another 6 cows clinical signs of *puerperal metritis* were observed together with long-term hyperketonemia (cHK+PM). In cows with uncomplicated uterine involution, neither the BCS or its changes, nor the milk production interacted with various BHB patterns by themselves.

The plasma *NEFA* profile followed almost the same pattern as BHB levels, except in cHK or cHK+PM cows, which presented early increases in plasma NEFA concentration (some on day 4-10 before calving*) (**Fig. 5.1.1B**).* During late pregnancy the plasma *glucose* was 3.40±0.05 mmol/L, and remained almost unchanged until d 3 before calving. A moderate decline was observed thereafter. On d 28-35 and 60-70 the plasma glucose returned to concentrations similar to those observed in early dry period in all cows (**Fig. 5.1.1C** and **Table 5.1.2**). Plasma *IGF-I* levels started to decrease gradually from 3 wk before calving, and reached the nadir between d 3 and 7 after calving (**Fig. 5.1.2D** and **Table 5.1.2**). *Insulin* levels were usually high at the beginning of sampling, but presented a wide range of variation (7.0 to 25.0 pmol/L). Insulin started to decrease before parturition in all cows (**Fig. 5.1.2E** and **Table 5.1.2**). In PM cows, insulin levels started to decrease more abruptly only on d 2-3 after calving, and reached the same level than their cHK mates on d 3-4 after parturition, i.e. at clinical manifestation of metritis. On d 28-35 after calving, plasma insulin levels reached the initial values in all cows except cHK. Plasma *leptin* levels started to decrease before parturition in all cows, but remained higher throughout the study in NK cows. Cows with only transient HK also had low leptin concentrations (**Fig. 5.1.2F** and **Table 5.1.2**).



A



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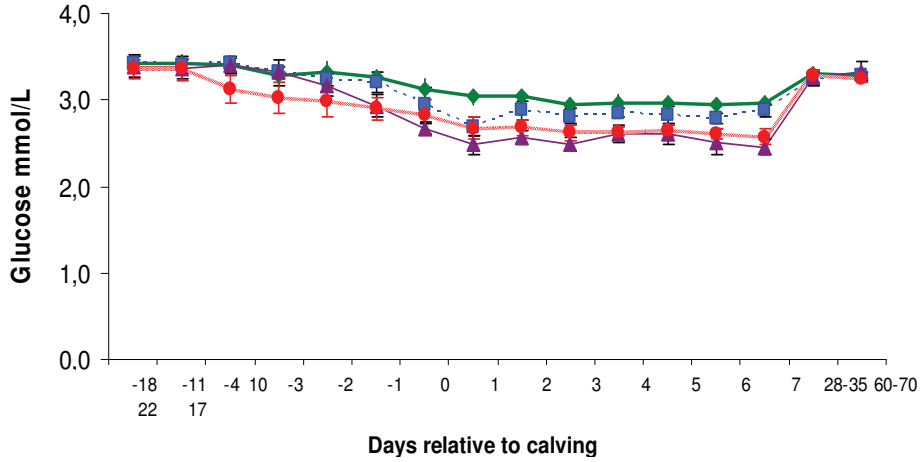
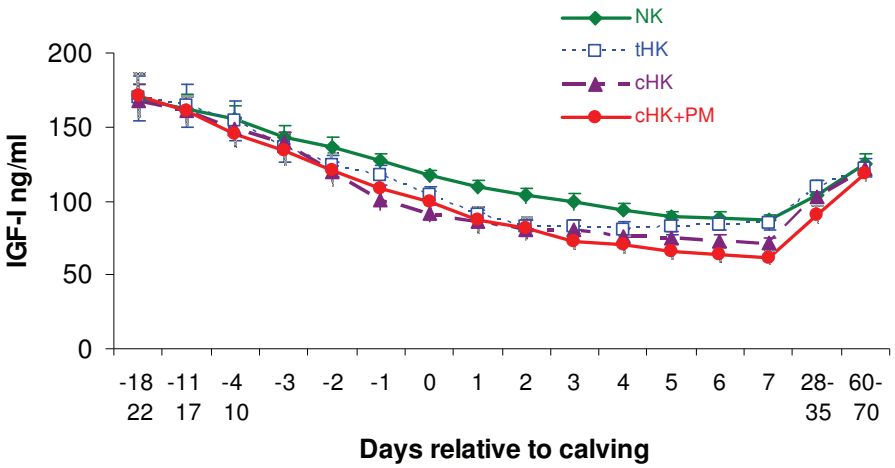
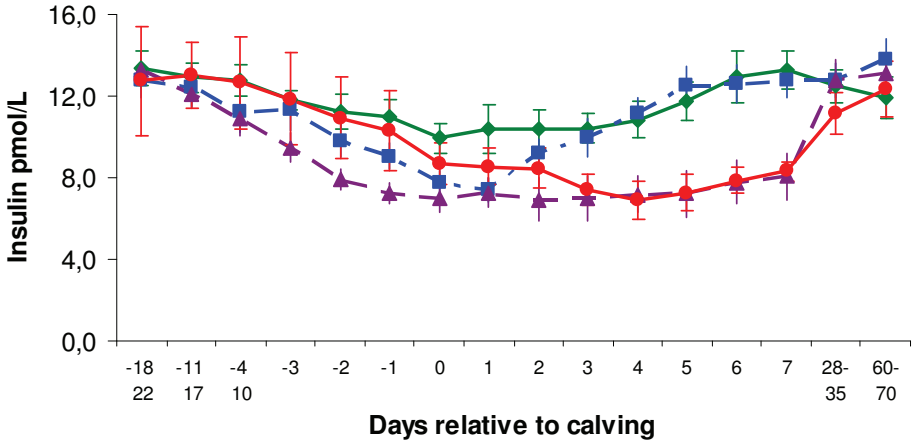


C

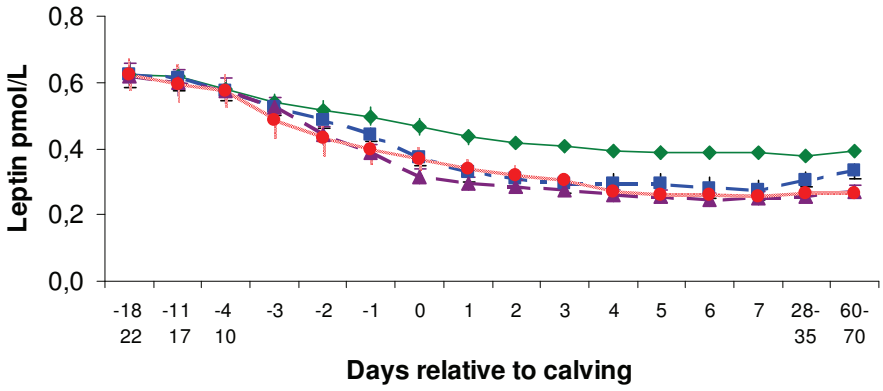
Figure 5.1.1. Periparturient changes of plasma BHB (A), NEFA (B) and glucose (C) concentrations around parturition in normoketonemic (NK), transiently hyperketonemic (tHK), continuously HK (cHK) and continuously HK cows with puerperal metritis (cHK+PM).



D



E



F

Figure 5.1.2. Periparturient changes of plasma IGF-I (D), insulin (E) and leptin (F) concentrations around parturition in normoketonemic (NK), transiently hyperketonemic (tHK), continuously HK (cHK) and continuously HK cows with puerperal metritis (cHK+PM).

Table 5.1.2. The mean (±SEM) concentrations of plasma metabolites and hormones on days relative to parturition in the normoketonemic (NK), transiently hyperketonemic or (tHK), continuously HK (cHK) cows without or with puerperal metritis (cHK+PM).

	Days relative to calving	Treatment group					Fixed effect		
		NK	tHK	cHK	cHK+PM	±SEM	Group	Time	Group x time
BHB	-18 to -22	0.32	0.29	0.28	0.34	0.02	P<0.001	P<0.001	P<0.001
(mmol/L)	-11 to -17	0.32	0.34	0.37	0.40	0.02			
	-4 to -10	0.38	0.36	0.42	0.40	0.03			
	-3 to -1	0.56	0.58	0.79	0.88	0.05			
	0 to 7	0.80	0.90	1.25	1.11	0.05			
	28 to 35	0.65	0.57	0.66	0.60	0.02			
	60 to 70	0.61	0.65	0.64	0.67	0.03			
NEFA	-18 to -22	0.30	0.29	0.35	0.31	0.03	P<0.001	P<0.001	P=0.18
(mmol/L)	-11 to -17	0.28	0.32	0.36	0.31	0.02			
	-4 to -10	0.29	0.46	0.41	0.35	0.03			
	-3 to -1	0.54	0.72	0.62	0.56	0.04			
	0 to 7	0.60	0.73	0.75	0.65	0.03			
	28 to 35	0.34	0.37	0.37	0.35	0.01			
	60 to 70	0.32	0.35	0.32	0.33	0.01			
Glucose	-18 to -22	3.41	3.43	3.38	3.36	0.05	P<0.001	P<0.001	P=0.44
(mmol/L)	-11 to -17	3.42	3.41	3.36	3.35	0.05			
	-4 to -10	3.41	3.41	3.40	3.12	0.06			
	-3 to -1	3.29	3.25	3.14	2.97	0.06			
	0 to 7	3.00	2.83	2.55	2.66	0.06			
	28 to 35	3.31	3.25	3.26	3.28	0.04			
	60 to 70	3.29	3.28	3.33	3.25	0.03			
Insulin	-18 to -22	13.38	12.80	13.32	12.75	3.73	P<0.001	P<0.001	P=0.02
(pmol/L)	-11 to -17	12.91	12.44	12.12	13.03	3.36			
	-4 to -10	12.77	11.27	10.87	12.67	3.1			
	-3 to -1	11.34	10.05	8.23	11.02	2.47			
	0 to 7	11.23	10.40	7.29	7.92	2.60			
	28 to 35	12.48	12.79	12.75	11.17	3.8			
	60 to 70	11.93	13.83	13.13	12.30	3.7			
IGF-I	-18 to -22	168.9	169.5	167.4	171.1	5.8	P<0.001	P<0.001	P=0.46
(ng/mL)	-11 to -17	162.5	164.7	161.1	160.7	5.2			
	-4 to -10	155.0	153.8	148.2	144.9	5.0			
	-3 to -1	135.5	125.5	119.8	121.0	4.0			
	0 to 7	104.6	91.6	85.4	82.5	3.2			
	28 to 35	103.9	110.0	102.4	90.7	2.4			
	60 to 70	125.6	121.9	121.8	118.0	2.7			
Leptin	-18 to -22	0.63	0.62	0.62	0.62	0.02	P<0.001	P<0.001	P=0.98
(nmol/L)	-11 to -17	0.62	0.61	0.60	0.60	0.02			
	-4 to -10	0.58	0.58	0.57	0.58	0.02			
	-3 to -1	0.52	0.48	0.45	0.44	0.02			
	0 to 7	0.41	0.31	0.28	0.30	0.02			
	28 to 35	0.38	0.31	0.26	0.26	0.01			
	60 to 70	0.39	0.33	0.27	0.26	0.01			

Challenge tests of pancreatic function and whole-body insulin sensitivity

During the late gestation and early lactation these challenge tests were conducted in all the cows evaluated. However – because of technical reasons – on day 60-70 of lactation those were done only in a restricted number of animals (NK= 3, tHK= 1, cHK= 3, cHK+PM= 3).

Glucose tolerance tes

Insulin AUC and *maximal insulin response to glucose* (Ins peak) was significantly lower in early lactation than in late-pregnancy (P<0.001). *Insulin clearance rate* (Ins CR) was lower on d 7 than in late gestation (P=0.01, **Table 5.1.3**.) and lower on d 7 in the cHK and cHK+PM group than in NK group (P<0.001). In early lactation, there were no differences in the Ins peak, Ins AUC and Ins CR between NK and tHK cows; however these measurements were significantly lower in both cHK and cHK+PM (P<0.001; **Table 5.1.3, Figs. 5.1.3A** and **5.1.3B**). *Glucose clearance rate* (glucose CR) was lower on d 7 than in the dry period (P=0.001) (**Table 5.1.3**). There was no effect of ketonemic status on the *glucose* $T_{1/2}$ (P=0.39).

Insulin tolerance test

The standard low-dose insulin treatment reduced the circulating blood glucose levels in cows in all testing periods. The lowest glucose concentration was usually reached 30 minute after the insulin administration. The only exception was observed in the cHK+PM cows: on d 7 after calving their glucose nadir was observed only at the 60 minute. The *insulin stimulated blood glucose reduction* (ISBGR) during the first 30 minute after the insulin injection was less obvious after parturition than during the dry period (67.4 ± 0.8 vs. 47.9 ± 2.4%, P<0.001). Furthermore, it was significantly lower during the early lactation of cHK and cHK+PM than in NK and tHK cows (P<0.001; **Fig. 5.1.4**.). Seven days after calving significant negative correlation was found between the BHB status and ISBGR (r=-0.76, P<0.001), furthermore there were a strong negative correlations between ISBGR and BCS loss and NEFA, and very strong positive correlations between ISBGR and plasma levels of IGF-I, leptin and basal glucose concentration (**Table 5.1.4.).**

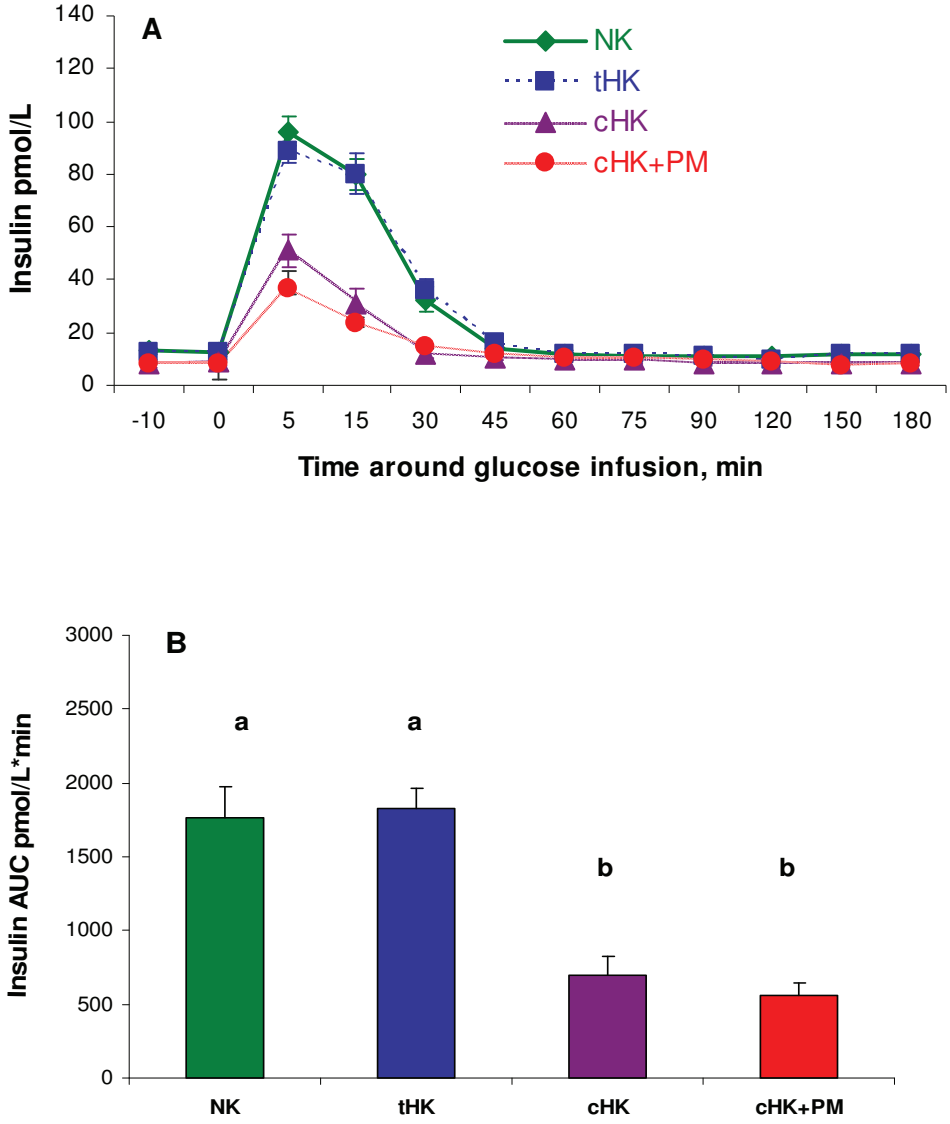
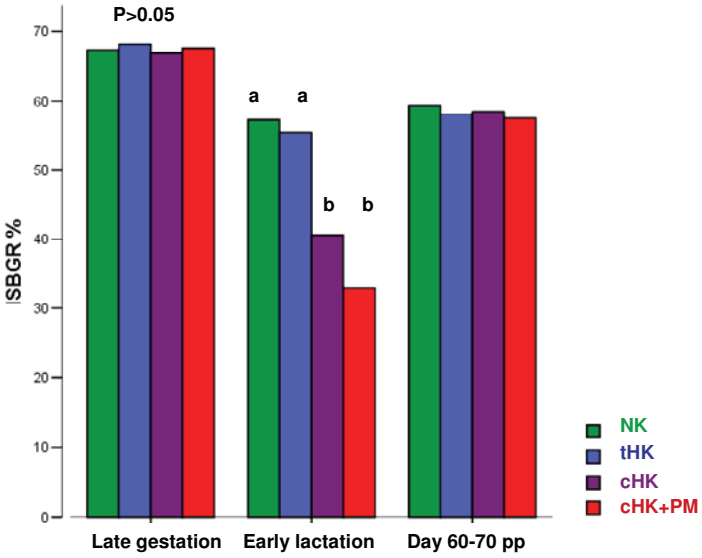


Figure 5.1.3. Mean ±SEM plasma insulin after glucose infusion (A) and the insulin area under the curve in response to glucose (B) on d 7 after calving in normoketonemic (NK), transiently hyperketonemic (tHK), continuously HK (cHK) and cHK cows affected by puerperal metritis (cHK+PM). Different letters (a,b) above columns means significant difference at P<0.001 level.

Table 5.1.3. Different measurements (mean ± SEM) of the GTT and ITT around parturition in the normoketonemic (NK), transiently hyperketonemic (tHK), continuously HK (cHK) and continuously HK cows with puerperal metritis (cHK+PM). P-values represent variance analysis of parameters between d -18-22 and on d 7 pp.

Parameters		Time around parturition			Fixed effects P		
	Group	D -18-22	D 7	D 60-70	Group	Time	Time x Group
GTT							
Ins AUC	NK	2169 ± 161	1762 ±210	2240 ±393	*0.001*	*<0.001*	*<0.001*
(pmol/L*min)	tHK	2101 ± 180	1824 ±140	2562			
	cHK	2291± 172	696 ±132	2109 ± 314			
	cHK+PM	2186 ± 257	560 ± 89	1674 ± 185			
Ins peak	NK	102 ±8.5	97.1 ± 5.4	117.0 ±13.8	*0.005*	*<0.001*	*<0.001*
(pmol/l)	tHK	104 ± 4.7	89.9 ± 5.2	127.3			
	cHK	112 ±10.5	51.0 ± 6.2	109.7 ±15.7			
	cHK+PM	109 ±12.7	37.3 ± 6.0	97.0 ±13			
Ins increment	NK	84.1 ±6.1	98.8 ±64	104.7 ±14.2	*0.01*	*<0.001*	*0.007*
(pmol/L)	tHK	77.4 ±5.1	102.2 ±8.7	114			
	cHK	42.7 ±5.9	109.4 ±10	97.5 ±16.6			
	cHK+PM	29.0 ±5.9	105.8 ±12	83.4 ±15.2			
Ins CR	NK	3.51 ±0.1	3.83 ±0.1	4.11 ±0.06	*0.01*	*0.02*	*0.001*
(% min)	tHK	3.69 ±0.2	3.60 ±0.2	4.40 ±0.04			
	cHK	3.65 ±0.2	3.00 ±0.2	3.88 ±0.30			
	cHK+PM	3.70 ±0.2	2.20 ±0.2	3.92 ±0.40			
Glucose CR	NK	1.54	1.58	1.58	*0.01*	*0.001*	*<0.001*
(% min)	tHK	1.62	1.65	1.62			
	cHK	1.64	1.64	1.57			
	cHK+PM	1.65	1.65	1.58			
Glucose $T_{1/2}$	NK	45.0 ±1.3	44.1 ±1.2	43.8 ±0.8	*0.39*	*0.006*	*0.16*
(min)	tHK	42.8 ±0.7	41.9 ±0.2	42.7			
	cHK	41.7 ±0.8	42.4 ±1.1	44.0 ±0.4			
	cHK+PM	41.1 ±0.3	41.9 ±0.4	43.8 ±1.1			
ITT							
ISBGR	NK	67.3 ±1.7	57.1 ±2.5	59.3 ±0.4	*0.004*	*<0.001*	*<0.001*
(%)	tHK	68.1 ±1.6	55.3 ±3.7	58.0			
	cHK	66.8 ±1.9	40.6 ±1.2	58.5 ±0.4			
	cHK+PM	67.7 ±1.9	33.0 ±4.9	57.4 ±1.0			
Basal sample							
RQUICKI	NK	0.56	0.51	0.53	*0.86*	*0.05*	*0.48*
	tHK	0.54	0.52	0.51			
	cHK	0.55	0.55	0.54			
	cHK+PM	*0.55*	*0.54*	*0.54*			

Figure 5.1.4.
Insulin stimulated blood glucose reduction (ISBGR, %) in late gestation (d -18 to -25 before calving), in the early lactation (d 7 after parturition) and on days 60-70 pp in normoketonemic (NK), transiently hyperketonemic (tHK), continuously HK (cHK) and HK cows affected by puerperal metritis (cHK+PM). Different letters means significant difference at P<0.05 level.



Revised quantitative insulin sensitivity check index (RQUICKI)

There was a highly significant time effect on RQUICKI value (P<0.001; **Fig. 5.1.5**). The numeric value of the index was the lowest between d 1 prepartum and day 4 pp. There was no difference in the RQUICKI in cows with different form of HK with or without PM (P=0.41). The RQUICKI was in negative relation to plasma levels of NEFA, IGF-1 and leptin (**Table 5.1.4**). However, there was no correlation between the RQUICKI and BCS or any parameters of the GTT and ITT.

Figure 5.1.5.
Revised quantitative insulin sensitivity check index (RQUICKI) around parturition in normoketonemic (NK), transient hyperketonemic (tHK), continuously HK (cHK) cows and in HK cows affected by puerperal metritis (cHK+PM).

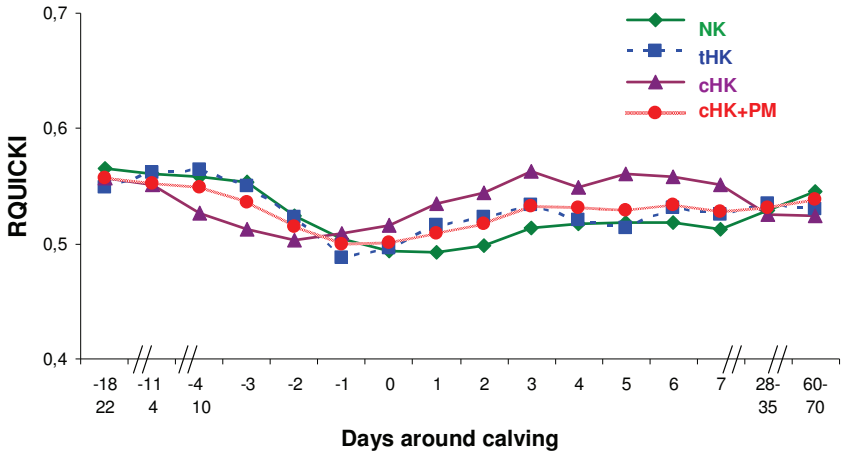


Table 5.1.4. Correlations between the measurements of the intravenous glucose tolerance test (GTT) and the insulin tolerance test (ITT) performed in the early lactation with different metabolic parameters on day 7 and the day of first pp ovulation.

	Ins AUC	Ins peak	Ins increment	Glucose CR	Glucose $T_{1/2}$	ISBGR	RQUICKI
BCS loss	-0,60 **	-0,70 **	-0,69 **	-0,36	0,35 *	-0,51 **	0,13
NEFA	-0,38 *	-0,46 *	-0,41 *	-0,18	0,19	-0,38 *	-0,44 *
BHB	-0,40	-0,36	-0,33	-0,18	0,17	-0,28	0,25
Glucose	0,07 **	0,69 **	0,68 **	0,04	-0,05	0,63 **	-0,25
Insulin	0,39 *	0,56 **	0,48 *	0,30	-0,32	0,45 *	-0,36
IGF-I	0,67 **	0,73 **	0,73 **	0,53 **	-0,54 **	0,79 **	-0,42 *
Leptin	0,47 *	0,57 **	0,55 **	-0,13	-0,14	0,48 **	-0,48 **
Ov day	-0,64 **	-0,75 **	0,72 **	-0,07	0,07	-0,41 *	0,24

*P<0.05 **P<0.01.

Reproductive parameters

All cows ovulated within 63 d pp. In normoketonemic cows the first pp ovulation occurred earlier than in cows affected by different types of HK (d 26.2±3.0, 34.7±4.0, 44.0±4.0, 50.1±5.0 for NK, tHK, cHK and cHK+PM, respectively; P=0.002). Cows with continuous HK with or without PM ovulated later than normoketonemic cows (P= 0.012 and 0.001), but not cows with transient HK (P=0.32; **Fig. 5.1.6**).

The time of the first observed estrus, number of AI per conception and the day of re-conception was not different between groups (**Table 5.1.5**). The day of the first pp ovulation negatively correlated with the Ins AUC and ISBGR on day 7 pp and positively with BCS loss and basal NEFA concentrations (**Table 5.1.4**).

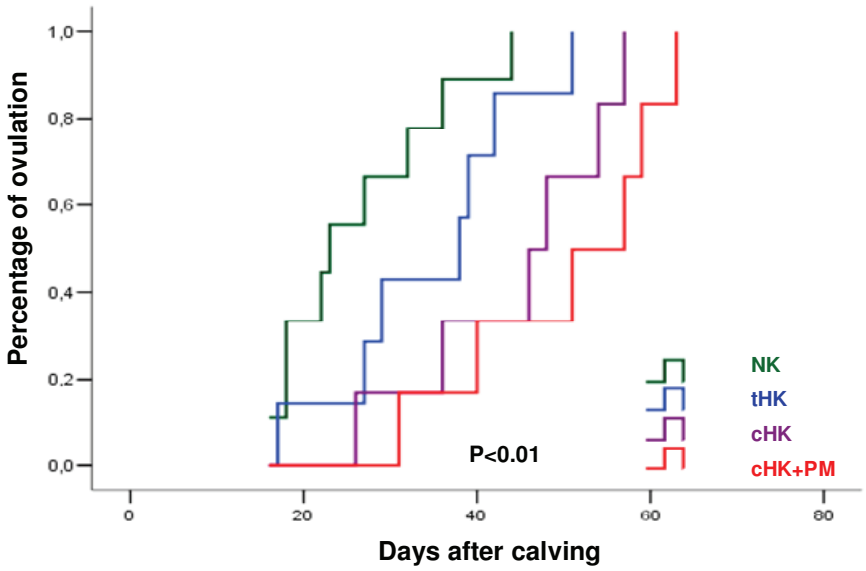


Figure 5.1.6. Kaplan-Meier survival analysis of first postpartum ovulation in normoketonemic (NK), transiently hyperketonemic (tHK), continuously HK cows, without (cHK) or with puerperal metritis (cHK+PM).

Table 5.1.5. Reproductive parameters (mean ±SEM) in the normoketonemic (NK), transiently hyperketonemic (tHK), continuously HK (cHK) and continuously HK cows with puerperal metritis (cHK+PM).

	Group				Fixed effect
	NK	tHK	cHK	cHK+PM	Group
Day of ovulation	26.2 ± 3	34.7 ± 4	44.0 ± 4	50.1 ± 5	<0.01
First estrus	73.0 ± 3.6	72.0 ± 4.9	80.0 ± 7.3	87.0 ± 4.1	0.16
AI/conception	2.0 ± 0.2	2.4 ± 0.3	2.0 ± 0.4	3.0 ± 0.4	0.25
Day of reconception	108.8 ± 8.8	110.8 ± 12	112.5 ± 12	139 ± 14	0.36

Discussion

The main purpose of the present study was to determine the time related changes in periparturient insulin secretion and whole-body insulin sensitivity in dairy cows with different patterns of hyperketonemia and in cows with severe clinical signs of puerperal metritis. IR may be exacerbated by excessive lipid mobilization with consequent rise in the plasma NEFA and ketones both in non-pregnant, non-lactating (Oikawa and Oetzel, 2006;

Pires et al., 2007) or lactating (Bossaert et al., 2008) dairy cows. In feed-restricted, non-lactating Holstein cows, short-term reduction of plasma NEFA concentrations by infusion with nicotinic acid was accompanied by enhanced clearance of glucose during GTT despite of lower insulin concentration (Pires et al., 2007). Beside excessive lipid mobilization different inflammatory conditions with intensive release of pro-inflammatory cytokines (such as TNF-α) also can affect glucose homeostasis and lipid metabolism in the dairy cow (Oikawa and Oetzel, 2006; Kushibiki et al., 2001). Puerperal metritis (PM) is a frequent bacterial complication of the early puerperium, which was accompanied by elevated plasma levels of TNF-α and acute phase proteins (haptoglobin, α_1-acid glucoprotein), increased NEFA contents, and low insulin concentrations in some earlier studies (Hirvonen et al., 1999; Kulcsár et al., 2005a,b). The relationship between elevated plasma NEFA concentrations, hyperketonemia and inflammatory cytokines was discussed in detail in *Section 2.2.3.*

Based on the BHB pattern and the clinical signs of puerperal mastitis we could assign the cows in four groups: normoketonemic throughout the study, cows with only transient or cows with long-term elevation of ketone bodies with or without PM. Periparturient profile of plasma metabolites and metabolic hormones, as well as of resumption of cyclic ovarian function followed trends identical to several earlier studies (Holtenius et al., 2003, Walsh et al., 2007). In accordance with findings of Holtenius et al. (2003) and Bossaert et al. (2008), in early lactation we observed decreased insulin response to glucose infusion (insulin responsiveness). Similarly, the glucose decrease after insulin injection was lower in early lactation than in late pregnant or mid lactating animals. In our study the insulin response to GTT in the early lactation was further impaired in animals developing long term HK, with or without puerperal metritis, but not in cows with only transient increase in the BHB levels. Likewise, more pronounced tissue unresponsiveness to insulin in continuously HK cows was reflected by the lower ISBGR. There was no difference in the plasma NEFA dynamics in the blood between the cHK cows and the cHK+PM after parturition. It may be expected that hyperketonemic cows with severe clinical signs of PM should have even stronger evidence of IR as a result of an additional effect of cytokine production to the insulin unresponsiveness. Insulin AUC and Ins peak after glucose- and the insulin-induced blood glucose level decrease numerically was lower during the early lactation in cows

affected by PM than in cHK group without PM. However, these differences were not statistically significant; therefore a study with a larger number of animals would be necessary to permit the elucidation of the relationship.

Cows experiencing subclinical ketosis on first week pp were at greater risk of anovulation and the duration of elevated BHB was negatively associated with pregnancy after first service pp (Walsh et al., 2007). Puerperal metritis is also associated with delayed resumption of ovarian function (Huszenicza et al., 1998). In the present study, the time of the first ovulation was recorded significantly later in hyperketonemic cows with or without PM, than in normoketonemic or only transiently HK cows. The time of first ovulation was negatively correlated with Ins AUC, Ins peak and ISBGR, indicating reduced insulin responsiveness in cows with delayed ovulation. Insulin has important role in differentiation of ovarian granulosa cells (Spicer et al., 1995) and its plasma level correlates well with the resumption of ovarian cyclicity in some studies (Beam and Butler, 1998). Increased concentrations of NEFA in the follicular fluid can have detrimental effects on bovine oocyte and embryo development (Leroy et al., 2008). The increased NEFA concentrations together with the decreased tissue insulin-responsiveness observed in the cows with cHK in our study may have contributed to the delayed ovulation; however the design of the study does not allow the demonstration of a direct causative relationship.

The revised qunatitative insulin sensitivity check index (RQUICKI) originally developed to measure insulin sensitivity in human epidemiological studies has been evaluated in healthy Holstein cows (Butler et al., 2004; Balogh et al., 2008). In human studies involving different insulin-resistant states, a close correlation was found between the RQUICKI and the insulin sensitivity index obtained with the hyperinsulinemic clamp test (Rhabasa-Lhoret et al., 2003). In cattle, there was a significant negative linear relationship between BCS and the RQUICKI, but no correspondence to other sensitivity indexes was assessed (Holtenius and Holtenius, 2007). In our earlier study (Balogh et al., 2008) the RQUICKI positively related to the insulin clearance rate. However, only healthy cows were evaluated in these two experiments cited, and the periparturient changes in BHB pattern were not followed up. In the present study the cHK cows had decreased peripheral insulin sensitivity and glucose responsiveness to insulin, but there was no correlation between the RQUICKI and any of the measured parameters during the GTT and ITT.

Moreover, the RQUICKI did not correlate with the BCS measured on d 7, d 25 or the BCS loss. In the present study a highly significant effect of time on RQUICKI was noted, which suggests that it is affected by the considerable changes of plasma metabolic and hormonal concentrations around the date of calving. These observations altogether suggest that the RQUICKI has a low discrimination power in diagnosing decreased insulin sensitivity in cows affected by various metabolic diseases. Additional studies are necessary to further assess the RQUICKI in dairy cows with different nutritional, metabolic and diseased conditions.

In conclusion, we showed that pancreatic ß-cell function and the biological potency of insulin is impaired in cows with long-term hyperketonaemia. Short-term elevations in plasma free fatty acids and BHB may not potentially induce further increase in peripheral tissue insulin resistance in the early lactation. Severe inflammatory diseases like puerperal metritis with intensive release of pro-inflammatory cytokines potentially further depress insulin secretion of the pancreatic ß-cells and the whole body insulin responsiveness of the dairy cows, with long-term effects on metabolism and reproduction. However, further studies with larger number of animals are needed to improve these observations. The homeostatic model RQUICKI should be applied with cautions in the assessment of insulin sensitivity in dairy cows in different physiological and disease states.

5.2. Effect of periparturient dry propylene glycol supplementation on metabolic and reproductive performance in Holstein–Friesian cows *(Exp. 2)*

Material and methods

Housing, animals, feeding and design

The study was conducted in a commercial large-scale dairy herd with Holstein-Friesian cows. During the first 6 weeks of the dry period the group of cows were kept in a separated pen, and fed with maize silage (10 kg / cow) and grass hay (*ad libitum*). Two to three weeks before calving all cows were moved to boxes (4 to 6 in each) of the maternity unit, where they were kept until d 7-10 postpartum, and were fed with total mixed ration (TMR; *composition before calving*: 10 kg maize silage, 4 kg alfalfa hay, 4 kg grass hay and 1.8 kg inert fat enriched concentrate; energy content: 6.5 MJ/kg NE_l, crude protein: 15.5%; *composition after calving*: 12 kg maize silage, 3 kg alfalfa silage, 2 kg alfalfa hay, and 7.5 kg inert fat enriched concentrate; energy content: 6.85 MJ/kg NE_l, crude protein: 19.0%). On d 7-10 postpartum the cows left the maternity unit; thereafter they were kept in groups of 70 to 80 individuals, and were fed with TMR (energy content: 7.23 MJ/kg NE_l, composition in the subsequent about 8-10 weeks: 14 kg maize silage, 6 kg alfalfa silage, 3.5 kg alfalfa hay, and 11.5 kg inert fat enriched concentrate). Later the quantity of concentrate was slightly decreased according to the actual milk production.

On day 14 before the expected calving date 51 multiparous Holstein-Friesian cows were selected and assigned in two treatment group based on parity and the previous 305-d fat corrected milk (FCM) yield. From day 14 before the expected parturition until d 10 postpartum the PGL group received daily top-dressing of 350 g of pulverized propylene glycol (VMD Ltd, Budapest, Hungary; composition: 75% propylene glycol, 25% fumed silica[1]; the liquid PGL was adsorbed on fumed silica, producing a white, fine powder) on the TMR. Animals in the control (CTL) group did not receive PGL. In the supplemented group the daily 350 g PGL should provide an approximately 5.25 MJ of extra energy/day/animal, which is similar to the amount of PGL administered in previous studies that proved to be effective in improving metabolic status and/or reproduction (Grummer et al., 1994, Christensen et al., 1996; Miyoshi et al., 2001).

Blood sampling started after commencement of PGL treatment from the jugular vein on weeks -2, -1 before, and 1, 2, 3, 5, 7 after parturition for BHB, NEFA, insulin, IGF-I, T_3, T_4 and glucose analysis. Blood samples were taken approximately within 3 hours after the morning feeding for metabolite and hormone analysis. Individual daily milk production was recorded beginning from d 10 postpartum until d 100 of lactation. The milk content of protein, lactose and fat were analyzed for each cow once a month. The body condition score (BCS) of animals were assessed on a scale ranging from 1 to 5 as described by Edmonson et al (1989) between d 7-10 postpartum.

Liver sample collection; intravenous glucose and insulin tolerance test

Between d 7-10 after calving, a subset of 16 cows (CTL n=10; PGL n=6) were selected for *liver biopsy* and simultaneous *intravenous glucose and insulin tolerance tests*. The sampling procedures (detailed in *Sections 4.3.* and *4.4.*) were carried out after the morning milking. After the liver biopsy, the simultaneous standard intravenous glucose and insulin tolerance tests were performed as described in *Section 4.3.* Metabolites and hormones were determined from basal samples (t_0) and insulin and glucose were determined from each sampling time.

Ovarian function and reproductive parameters

The time of first pp ovulation was determined by individual milk P4 profile from milk samples collected three times weekly from d 10 pp until d 100 pp. P4 was assayed by a microplate ELISA method (Nagy et al. 1998; modified and validated for assaying P4 in bovine skim milk by Huszenicza et al., 1999 and 2005), within 14 days after collection. Luteal function was confirmed when P4 concentrations were $\geq$1.5 nmol/L for two or more consecutive samples. Sensitivity was 0.2 nmol/L. The Pre-Ovsynch protocol was used for synchronization of ovarian cyclicity from day 35 after calving. The protocol consisted in pre-synchronization with two prostaglandin F2α injections at 14 days interval (500 µg Cloporostenol, Estrumate®, Schering-Plough Animal Health, Union, NJ, USA), followed by GnRH (150 µg Gonadorelin, Fertagyl®, Intervet, Angers, France), PGF2α and GnRH injections on d 0, 7 and 9 after the second PGF2α treatment. 24 hours after the last GnRH injection the cows were artificially inseminated (fixed time AI). Cows were inseminated

[1] SiO_2; commercially available as HDKN20

around d 70 pp. Pregnancy was confirmed with rectal ultrasonography[2] performed between d 35-40 after the AI. Non-pregnant cows after the first AI were inseminated and pregnancy rates were followed until d 150 after calving.

Blood metabolites and hormones analyses

NEFA and BHB were analyzed with enzymatic assays (D-3-Hydroxybutyrate kit, Kat. # RB 1007 and NEFA kit, Kat. # FA 115, from Randox Laboratories Ltd, Ardmore, UK). Glucose was analyzed by glucose oxidase-peroxidase (GOD-POD) reaction (Glucose kit, Kat. # 40841, Diagnosztikum Ltd., Budapest, Hungary). T_4 and T_3 were determined by ^{125}I-Spec RIA coated tube kits (Institute of Isotopes Co., Ltd. Budapest, Hungary). The sensitivity was 1,5 nmol/L for T_4 and 0,24nmol/L for T_3. The intra-assay CV was 6.4 - 8.1 % for T_4 and 6.0 - 8.3% for T_3. The inter-assay CV was ≤5.8% and ≤6.5% respectively. The plasma insulin content was quantified as free insulin with a commercial ^{125}I-labelled radioimmunometric sandwich assay kit (BI-Insulin IRMA kit; CIS Bio International Ltd – Subsidiary of Schering S.A., Gif-Sur-Yvette, France; sensitivity: 0,43 pmol/L; intra- and inter-assay CV: from 1.3 to 5.6% and ≤8.5%, respectively). The IGF-I 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 inter-assay CV: from 3.4 to 6.6% and ≤7.0%, respectively).

Milk yield and component

Milk yield was measured daily and milk components (protein, lactose and fat) were determined at the Livestock Performance Testing Ltd. (Gödöllő, Hungary) with equipment based on infrared absorption principle (Bentley 2000 equipment; Bentley Instruments Inc. Chaska, USA).

Statistical analysis

The basic methods of data analysis are described earlier (*Section 4.5.*). Statistical differences in milk yield, plasma metabolites and hormone concentrations were analyzed by analysis of variances for repeated measures. The main effects tested diet (CTL and. PGL), time (week) and their interactions. Parameters measured during the GTT and ITT were

[2] SC-200; equipped with 5/7,5 MHz linear array transrectal transducer (Pie Medical Equipment B.V., Maastricht, The Netherlands).

compared with two-paired t-test. The number of days from calving to the first pp ovulation was analyzed using the Kaplan-Meyer survival analysis. The number of cows remained pregnant after the fixed AI and on the 150 day postpartum was compared with exact Fisher test.

Results

Milk production and composition, BCS

Daily average milk yield in the first 12 weeks of lactation and in milk protein and lactose content did not differ between treatments (**Table 5.2.1**). On week 5 pp the average weekly milk yield was slightly higher in PGL than in CTL cows (33.1±1.5 vs. 36.4±1.4 kg/day; P=0.1). PGL group tended to have higher milk fat production compared to the CTL group (P=0.09). On d 7-10 there was no difference in BCS score between the CTL and PGL group (CTL_{BCS}: 2.9 ±0.07 and PGL_{BCS}: 3.0 ±0.01; P=0.51).

Table 5.2.1. Milk yield and milk composition (mean ±SEM) in the control (CTL) and in the propylene glycol (PGL) supplemented group during the first 12 weeks after parturition. The effect of time, treatment (T) and their interaction (T*W) are presented.

Variables	Week	Treatment		P-value		
		Control	PGL	Time	T	T*W
Milk fat %	4	3.57 ±0.22	3.85±0.20	0.08	0.09	0.97
	8	3.25 ±0.23	3.64 ±0.28			
	12	3.13± 0.24	3.19 ±0.26			
Milk protein %	4	2.89 ±0.06	2.90 ±0.08	**	0.26	0.44
	8	2.86 ±0.05	2.97 ±0.05			
	12	2.94 ±0.12	2.97 ±0.05			
Milk lactose %	4	4.69 ±0.08	4.64 ±0.09	0.52	0.94	0.66
	8	4.74 ±0.03	4.76 ± 0.07			
	12	4.57 ±0.18	4.74 ± 0.06			
Average milk yield (kg/day) in first 12 week pp		32.85 ±0.3	33.81 ± 0.4	***	0.1	0.55

**P<0.01; ***P<0.001

Plasma metabolites, hormones and hepatic lipid content

Mean ± SEM for plasma metabolite and hormone contents are presented in Table 5.2.2. Plasma NEFA concentration started to increase on week 2 before parturition in both groups but there was no observable effect of treatment (**Fig. 5.2.2A**). Plasma BHB content started to increase after parturition in both groups and it was numerically higher in the CTL group on week 1 before- and after calving. However, these differences were not statistically significant (**Fig. 5.2.2B**). There was no effect of time or treatment on plasma glucose concentrations (**Fig. 5.2.2C**). Insulin level was significantly higher the PGL group during the supplementation prepartum ($P<0.01$), but not in the first week postpartum (**Fig. 5.2.2D**). Pulverized PGL supplementation had no effect on plasma T_3, T_4 and IGF-I levels (data not shown).

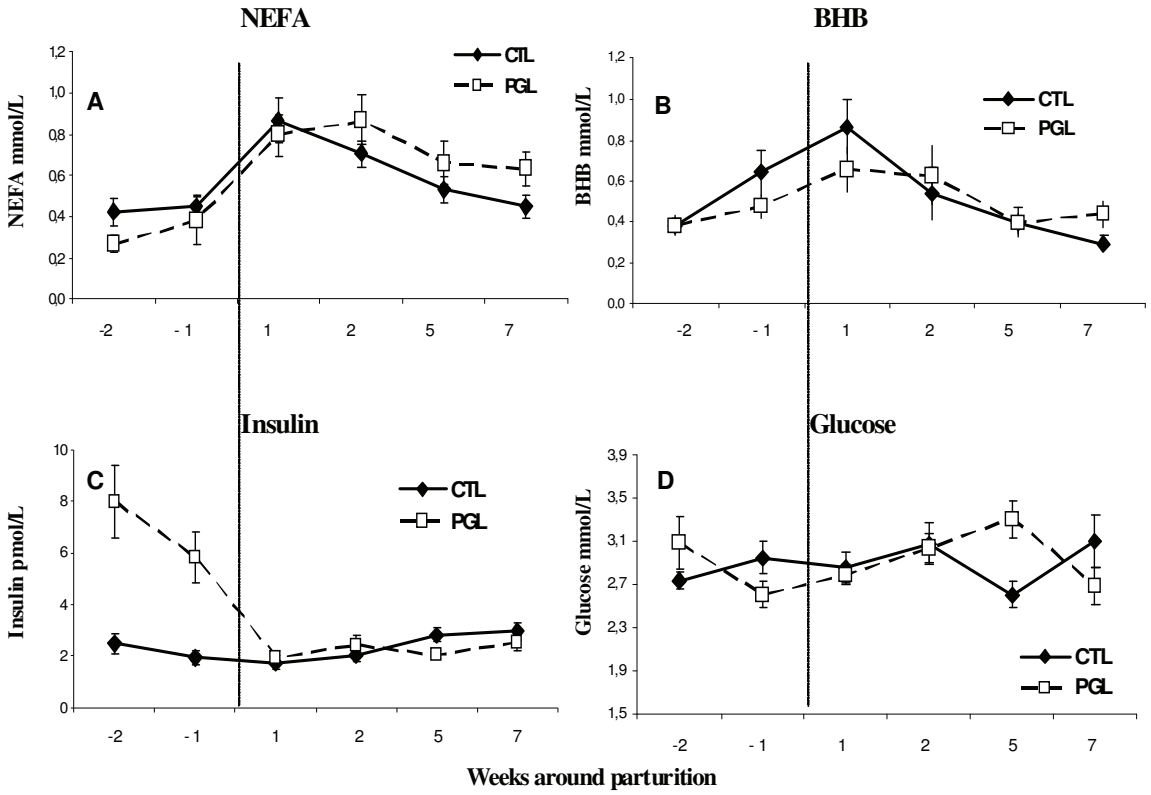


Figure 5.2.2. Plasma metabolites and hormones (mean ±SEM) in the control (CTL) and in propylene glycol (PGL) supplemented group around parturition.

Table 5.2.2. Plasma metabolites and hormones (mean ±SEM) in the CTL and in the in the propylene glycol (PGL) supplemented group. Effect of treatment (T), week (W), and their interaction (T*W) are presented.

Variable		Treatment		P-values		
	Week	Control	PGL	W	T	T*W
NEFA	-2	0.42 ± 0.07	0.27 ± 0.04	***	0.52	0.19
mmol/L	-1	0.45 ± 0.05	0.38 ± 0.11			
	+1	0.87 ± 0.11	0.79 ± 0.10			
	+2	0.71 ± 0.06	0.87 ± 0.12			
	+5	0.53 ± 0.06	0.66 ± 0.10			
	+7	0.45 ± 0.05	0.63 ± 0.08			
BHB	-2	0.39 ± 0.04	0.38 ± 0.05	***	0.67	0.39
mmol/L	-1	0.64 ± 0.11	0.48 ± 0.06			
	+1	0.86 ± 0.13	0.66 ± 0.11			
	+2	0.54 ± 0.12	0.63 ± 0.14			
	+5	0.40 ± 0.07	0.39 ± 0.05			
	+7	0.30 ± 0.03	0.44 ± 0.06			
Insulin	-2	19.3 ± 2.7	62.47 ± 11.0	***	***	***
pmol/L	-1	15.1 ± 2.2	45.51 ± 7.5			
	+1	15.2 ± 2.6	15.19 ± 2.10			
	+2	16.2 ± 2.1	20.90 ± 3.3			
	+5	19.7 ± 1.5	16.08 ± 1.4			
	+7	23.3 ± 2.5	19.9 ± 2.5			
Glucose	-2	2.73 ± 0.09	3.09 ± 0.24	0.63	0.73	0.01
mmol/L	-1	2.95 ± 0.15	2.61 ± 0.13			
	+1	2.86 ± 0.14	2.79 ± 0.09			
	+2	3.08 ± 0.20	3.04 ± 0.13			
	+5	2.60 ± 0.12	3.30 ± 0.17			
	+7	3.10 ± 0.23	2.69 ± 0.17			

***P<0.001

Hepatic total lipid content was not different between the CTL and the PGL group on d 7 after parturition (105.9 ±48.9 vs. 76.7 ±28.02 g/kg, P= 0.18, **Fig. 5.2.3**).

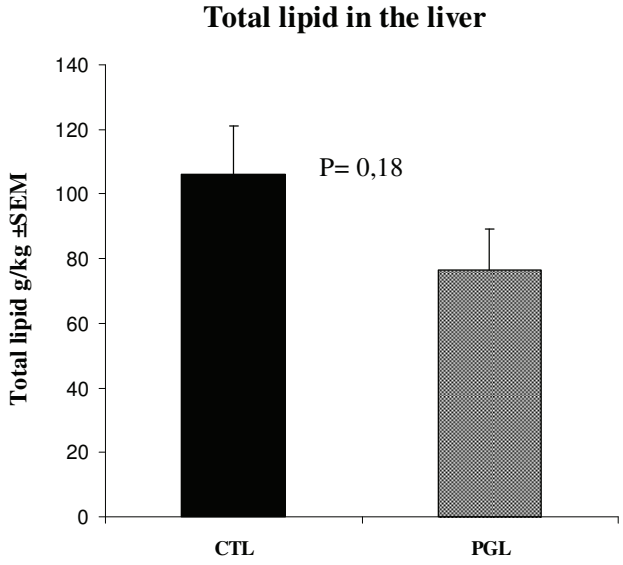


Figure 5.2.3.

Total liver lipid content (mean±SEM) in the control (CTL) and in the propylene glycol (PGL) supplemented group measured from liver biopsy samples taken on day 7 pp.

Standard intravenous glucose and insulin tolerance test in early lactation

After the glucose infusion test plasma insulin peaked at min 5 in the PGL group and about min 15 in CTL group, and returned to the baseline value around 60 minutes later in both treatment groups (**Fig. 5.2.3.).** The supplementation had no effect on the glucose $T_{1/2}$, glucose CR or on the insulin secretion reflected by the peak insulin concentrations and insulin AUC in response to glucose (**Fig. 5.2.4**.). After the insulin challenge, plasma concentration of glucose decreased and reached minimal values at 30 min in both treatment groups. The concentration of glucose returned to the baseline value around 150 min after insulin administration (**Fig. 5.2.5.**). There was no difference in the insulin stimulated blood glucose decrease between the groups (**Table 5.2.3.**).

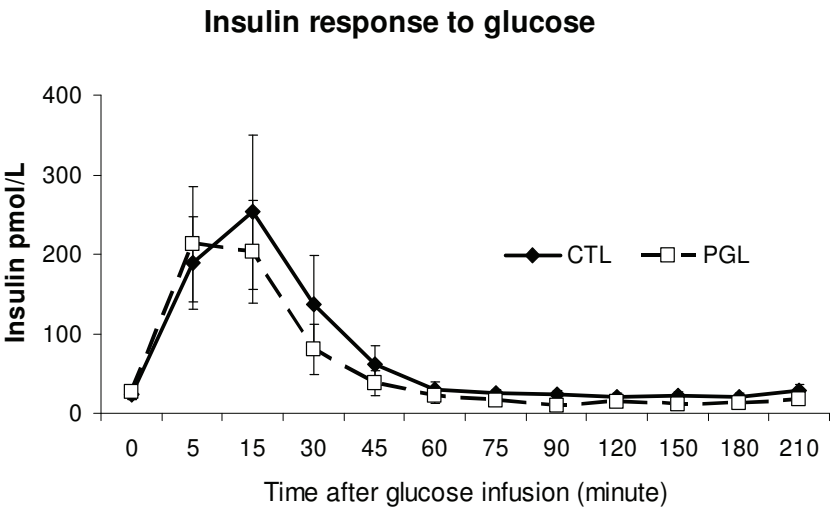


Figure 5.2.4.

Insulin response to glucose during the glucose tolerance test in the control (CTL) and in the propylene glycol (PGL) group.

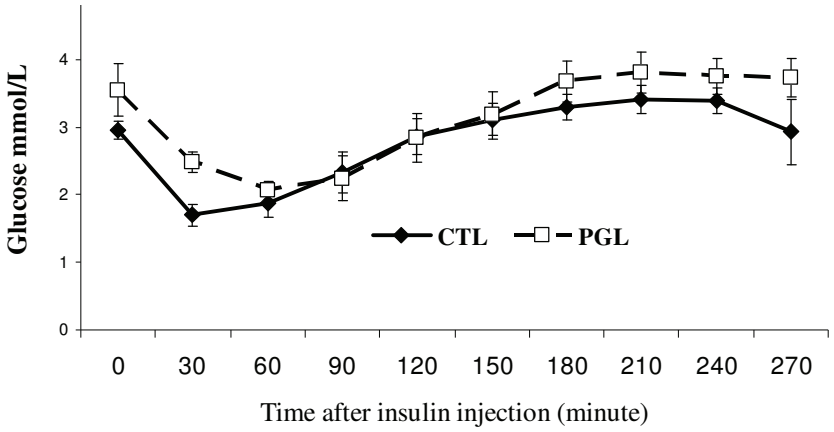


Figure 5.2.5.

Glucose response to insulin during insulin tolerance test in control (CTL) and in propylene glycol (PGL) group.

Table 5.2.3. Parameters (mean ± SEM) measured during the intravenous glucose and insulin tolerance test on d 7 postpartum in the control (CTL) and in the propylene glycol (PGL) supplemented group.

Variable	CTL	PGL	P
GTT			
Ins peak (pmol/L)	263.5 ±95.2	228 ±75.6	0.45
Ins increment (pmol/L)	239.8 ±89.2	201.5 ±73.2	0.69
Ins AUC (pmol/L *min)	1419 ± 411	1587 ± 533	0.81
Glucose CR (%/min)	1.50 ± 0.1	1.70 ± 0.15	0.16
Glucose $T_{1/2}$ (min)	35.6 ± 2.5	40.9 ± 1.4	0.12
ITT			
ISBGR (%)	41.4 ± 5.8	27.5 ± 4.9	0.16

Ovarian function and reproductive performance

All cows ovulated within 75 d after parturition. There was no difference in the interval between calving to first postpartum ovulation between the CTL and PGL group (d 34.1±12.9 vs. d 34.9±14.5, P=0.98). Pregnancy rate after the fixed time insemination was 10.7% in the CTL and 17.6% in the PGL group (P=0.45). The number of pregnant cow till day 150 after parturition in the CTL and PGL group was 16.1% and 36% (P=0.56).

Discussion

The aim of this study was to asses the metabolic and reproductive effects of pulverized PGL administration top dressed on the TMR from day 14 before calving until d 10 pp. PGL absorbed from the rumen has significant glucogenic property and is frequently used in liquid form to alleviate the negative effects of the NEB which occurs in high-yielding dairy cows around calving (Nielsen and Ingvartsen, 2004; further details are introduced in *Sections 2.3.1.* and *2.3.2.*). Drenching is a labor intensive work and produce stress to the animals; therefore administration of PGL which may be used top dressed or mixed with the TMR could be a more convenient approach in the dairy practice.

A tendency toward increased milk yield can be observed in some of the studies using PGL in the periparturient and/or early lactation periods (Fonseca et al., 1998). Fonseca et al. (1998) found small increase in the fourth and fifth weeks of lactation of cows that received PGL pre- and postpartum. We observed higher milk production in the PGL supplemented group on week 5 after calving, but this difference was not significant. However, there was a tendency toward increased milk fat percentage in the PGL supplemented group, but no effect of PGL supplementation was found in milk protein and lactose content, which results are in agreement with several other studies (Studer et al., 1993; Formigioni et al., 1996; Moallem et al., 2007b; Rizos et al., 2008).

Individual DMI was not possible to measure in the present study, therefore it cannot be excluded that the current form of pulverized PGL (absorbed on fumed silica) decreased the palatability of the diet. There are just few reported studies where DMI was measured individually. Miyoshi et al. (2001) observed lower feed intake of lactating cows after 1–2 days of top-dressing PGL addition with a dose of 518 g/day. In a recent study, where a commercial product containing 55% dry PGL was allocated in the periparturient period,

DMI was not affected before parturition, but increased it with 2.6% after parturition in the supplemented group (Moallem et al., 2007a,b).

PGL supplementation generally decreases the plasma NEFA and BHB concentrations and increase glucose and insulin levels (Nielsen and Ingvartsen, 2004). Studer (1993) drenching 1036 g PGL per day during the prepartum period and demonstrated a decrease in NEFA concentration. In the present study the plasma BHB was lower in the PGL supplemented cows on week -1 before and on week 1 after calving. The moderate effect in case of BHB and the lack of effect on NEFA concentration in our study can be the consequence of the reduced BHB and NEFA levels. BHB concentration measured during the sampling period was lower than 1.2 mmol/L, the threshold level of subclinical ketosis (LeBlanc et al., 2005). Prepartum administration of PGL significantly decreased the TG content of the liver after parturition (Studer et al., 1993). Pickett et al. (2003) allocated 518 g PGL per day orally for the first 3 days after calving, and found that the TG content of the liver was decreased by 44% on day 7 postpartum compared to control cows, but the difference was not significant. A similar tendency observed in our current study: the total lipid content of the liver was slightly lower in the PGL group, but this difference was not significant. There was no effect of PGL administration on the plasma T_3 and T_4 levels around parturition.

The magnitude and the duration of the insulin response after PGL supplementation is dose dependent and it is affected by the method of administration and by the blood sampling time. Insulin response to PGL fed as part of TMR was lower and lasted shorter than after PGL drench or mixed with the concentrate (Christensen et al., 1997). In our study (evaluating the base-line plasma insulin in samples taken 2 to 3 h after morning feeding) the dry PGL supplementation increased insulin concentration prepartum but not postpartum. The lack of insulin increase in response to PGL on the week after parturition possibly due to the fact that pancreatic insulin release in response to propionate or glucose is less obvious in early lactation than in the non-lactating state (Lomax et al., 1979; Sano et al., 1993). In our study cows were sampled for base-line plasma insulin at the suspected peak of postprandial increase: perhaps also this sampling schedule may cover the less clear PGL-related differenced. However, neither the feeding management of the herd, nor the daily schedule of sampling could have been changed in the current experiment.

Around parturition not just the peripheral concentrations of the insulin is depressed, but also the sensitivity of the peripheral tissues (mostly the adipose and muscular tissue) to the action of the insulin is shut down (Holtenius et al., 2003; Bossaert et al., 2008). This insulin resistant state can be exacerbated by elevated NEFA and ketone body levels in the blood as described previously in *Section 2.2.3*. and confirmed by the *Exp. 1*. Therefore by decreasing lipid mobilization with the means of glucogenic supplementation is an attractive choice to augment the biological effect of insulin and increase glucose availability for peripheral tissues. The pancreatic insulin response to glucose infusion was not affected by the treatment. However, glucose cleareance rate had a tendency to be slower in the PGL group. Glucose disappearance must be interpreted cautiously, mostly in early lactation, when the glucose uptake of the mammary gland is increased. Similarly, the glucose response to exogenous insulin (ISBGR) had a tendency to be lower, indicating a more insulin sensitive state of the peripheral tissues compared to control group. This is in accordance with the observations of Kristensen and Raun (2007), that glucose was not increased when cows were infused with PGL, even under increased serum insulin concentrations. The authors suggested that the increased *glucose* concentration observed after PGL treatment is caused by decreased glucose utilization rather than increased glucose production, likely induced by PGL and its metabolites (e.g. propanolol), by a decreased ratio of ketogenic to glucogenic metabolites in the blood from PGL metabolism, or both (Kristensen and Raun, 2007).

In our study the mean time from calving to the first pp ovulation was the same in the control and in supplemented group. As insulin concentrations has been positively related to reduced interval to first postpartum ovulation (Beam and Butler, 1998) attempts have been made to use hyperinsulinemic diets with the purpose of "fooling" the cow into a virtual anabolic condition. Additionaly, gluconeogenetic precursors may improve energy balance and reduce lipid mobilization and ketone bodies, which are proved to have negative effect on reproduction (Reist et al., 2000; Jorritsma et al., 2003). The inconsistent effect of PGL administration on the time of first pp ovulation may be due to the differences in the dose and the method of PGL administration, and also in variances in the physiological state and body condition of the animals.

There are just a few studies which reported the effect of periparturient PGL administration on fertility. In spite of reducing the pp anestrous interval, the PGL drench

had no effect on pregnancy rate and the calving interval in high-yielding Holstein cows (Miyoshi et al., 2001). This is agreement with the study of Chagas et al. (2007b), where there was no difference in pregnancy rate after the first 6 week of the mating period in control heifers and those which received PGL drench. In our study any positive effect of the dry PGL supplementation was observed on pregnancy rate after the fixed time AI and on the 150 d pp compared to the controls. The lack of effect of PGL on fertilization may be attributed to the relatively small effects on energy balance and on ovarian function. On the other hand, there is not enough evidence about the effect of different energy sources on the oocyte and embryonic quality. Some recent studies suggest that diets which are favorable to follicular growth are not necessary the same with the conditions needed for the oocyte and embryonic development (Adamiak et al., 2005). Diet rich in starch adversely affected blastocyst numbers and the percentage of fertilized oocytes in lactating dairy cows (Fouladi-Nashta et al., 2005). Further studies would be necessary to evaluate the long-term effect of periparturient PGL supplementation on oocyte quality and on fertilization.

5.3. Effect of prepartum energetic supplementation on productive and reproductive characteristics, and metabolic and hormonal profiles in dairy cows under grazing conditions (*Exp. 3*)

Material and methods

Housing, animals, feeding and design

Twenty multiparous Holstein cows with an average body weight of 578±14 kg and BCS of 2.5±0.1 (scale 1 to 5, Edmonson et al., 1989) were selected from the experimental herd of the dairy farm research station of INIA La Estanzuela (Colonia, Uruguay). The cows were expected to calve at the beginning of the austral autumn (March) and 28 days before the individual expected calving date, cows were separated from the herd and allocated in a different paddock of natural pastures. At day 21 before the expected calving date, cows were assigned to two prepartum treatment groups, considering BCS and days in dry period. Treatments were as follows: **Energy group** (n = 10) that received (at 8:00 AM) 3.5 kg/day of cracked corn grain [82% organic dry matter digestibility (ODMD), 9% neutral detergent fiber (NDF) and 7.7% acid detergent fiber (ADF); 13.5 MJ NE_l] each cow in individual feeders, and **Control group** (n = 10) without additional concentrate. Cows were offered also bales of hay of improved pasture [legumes (white clover, *Trifolium repens*), and Gramineae (rye grass, *Lolium multiflorum*; 54.5% ODMD, 12.4% crude protein (CP), 60.1% NDF and 47.2% ADF] ad libitum, and both groups were kept in separate paddocks of improved natural pastures (11.8% CP, 58.2% NDF and 34.7% ADF; 9.9 MJ NE_l). After parturition, both groups were group-fed and received the same diet, that consisted in 4.0 kg/day of a commercial concentrate (19.3% CP, 28.3% NDF and 14.1% ADF; 7.1 MJ NE_l) administered twice a day individually during milking time. This was complemented with 12 kg/day corn silage (73.9% ODMD, 6.2% CP, 41.1% NDF and 28.3% ADF; 10.6 MJ NE_l) and the animals had access to a daily strip of improved pastures consisting in a mixture of alfalfa (*Medicago sativa*), white clover and tall fescue (*Festuca arundinacea*) (62.5% ODMD, 17.7% CP, 52.4% NDF and 38.3% ADF; 9.2 MJ NE_l). Nutrient composition was calculated on a dry matter basis. Under this pasture-based system, grazing is done in different paddocks, which are managed under a rotational system. The estimated distance walked per day was 2 km during the prepartum and 2-2.5 km during the postpartum period,

depending on the location of the pasture strip (this distance does not include the one corresponding to grazing). Body condition score was determined weekly from the beginning of the experiment until week 5 postpartum by the same operator. Milk production was recorded daily and then averaged for each week. For determinations of milk composition (fat and protein content), a composite sample of four consecutive milking of each week was taken during the first 5 weeks postpartum.

Blood samples were obtained weekly in the morning before the administration of concentrate from d -28 to d 35 (parturition: d 0) for assaying metabolites and metabolic hormones, and twice a week starting at the 2nd week postpartum for analyzing P4. Sampling for P4 was continued until the time of pregnancy detection.

Determination of ovarian cyclicity

Reinitiation of ovarian cyclicity was monitored by twice a week by transrectal ovarian ultrasonography[3] and assaying P4 in the blood. Ovulation was determined by disappearance of the largest follicle followed by the formation of a corpus luteum which was confirmed by plasma P4 concentrations, from samples taken twice a week. The re-initiation of ovarian cyclicity was defined as the day when P4 increased from basal concentrations in two consecutive samples of >1.6 nmol/L or one sample of >3.2 nmol/L (Meikle et al., 2004).

Assay procedures

The serially collected plasma samples were assayed for metabolites, metabolic hormones and P4.

Urea was analyzed by the Urease UV method with a Weiner (Weiner Laboratories, Rosario, Argentina) kit # 861237004; *total cholesterol* with the CHOD-PAP method with a Weiner kit # 861231904; *NEFA* were analyzed by the ACS-ACOD (acyl-CoA synthetase & acyl-CoA oxidase) method with a NEFA-C kit # 994-75409 of Wako Chemicals (Richmond, VA 23237, USA); *BHB* with 3-HBDH-NAD+3-hydroxybutyrate dehydrogenase-NAD+ method (Ranbut, Randox Laboratories Ltd., Crumlin, Antrim, UK). For quality controls Lyotrol™ N and P was used, as well as internal controls of the veterinary laboratory "Miguel Rubino" (DILAVE, Uruguay). The intra-assay coefficient of

[3] With a 5.0 MHz linear probe (Aloka 500; Aloka, Tokyo, Japan)

variation was ≤ 3.7% for all the parameters, and the inter-assay CV was ≤ 9.6%.

Thyroxine (T_4) and *3,3',5-tri-iodothyronine* (T_3) were determined by ^{125}I-Spec RIA coated tube kits (Institute of Isotopes Co., Ltd. Budapest, Hungary). The sensitivity was 0.5 nmol/L (T_4) and 0.19 nmol/L (T_3). The intra assays CV were 6.4 - 8.1% for T_4 and 6.0 - 8.3% for T_3. The inter assays CV were ≤5.8% and ≤6.5%, respectively. Plasma *insulin* content was quantified as free insulin with a commercial ^{125}I-labelled radioimmunometric sandwich assay kit (BI-Insulin IRMA kit; CIS Bio International Ltd – Subsidiary of Schering S.A., Gif-Sur-Yvette, France; sensitivity: 0.86 pmol/l; intra- and interassay CV: from 1.3 to 5.6% and ≤8.5%, respectively). Plasma *IGF-I* concentrations were determined with a ^{125}I-labelled two-site immunoradiometric method (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). Plasma *leptin* concentration was quantified with a ruminant-specific ^{125}I-RIA of (Delavaud et al., 2002) adapted and modified by Kulcsár et al. (2006).

Progesterone (P4) was determined by radioimmunoassay using a commercial kit (Coat-a-Count; DPC, Diagnostic Products Co., Los Angeles, CA, 90045, USA). The intra- and inter-assay coefficients of variation were 6% and 11%. The sensitivity was 0.1 nmol/L.

Milk fat and *protein* content was analyzed by the Mojonnier method the Kjeldahl method, respectively (with a Bentley 2000 equipment; Bentley Instruments Inc., Chaska, MN, USA).

Statistical analysis

Milk production, BCS, plasma metabolites and hormones concentrations were analyzed by the Proc Mixed model (Statistical Analysis System, SAS Institute, Cary NC, USA 2000) and the model included treatment, weekly observations and their interactions. Cow within treatment was set as random effect. Least square means were compared with LSD procedure with a significance level of 5%. Previous lactation milk production, BCS at the beginning of the experiment and number of days dry were tested as covariables but the effect was not significant, therefore they were not included in the analysis. Ovarian cyclicity was analyzed by Proc GLM analysis (Statistical Analysis System, SAS Institute, Cary NC, USA 2000) with the prepartum treatment as the fixed effect. Proportion of cows ovulating in the early postpartum was analyzed by Chi square procedure. Results are

presented as least square means ± pooled standard error. Pearson's correlation coefficients were calculated to study relationships between variables.

Results

Body Condition Score (BCS)

BCS was similar in both groups at the beginning of the trial, but from 7 d prepartum through remaining the experimental period, cows in the *Energy* group had higher BCS than cows in the *Control* group (**Table 5.3.1**, P<0.01), except for those observed at calving and at the first week postpartum, when BCS was similar in both groups.

Table 5.3.1. Fixed effects included in the model for measured parameters in cows under grazing conditions. Fixed effects are treatment, experimental week and their interaction

Variable	Treatment	Week	Treatment*Week
Body condition score	**	***	*
Milk production	*	***	
Non-esterified fatty acids	*	***	.15
β-hydroxybutyrate		**	***
Cholesterol	*	***	
Urea	.15	*	**
Insulin	**	*	***
IGF-I	.09	***	*
Leptin	.08	***	
3,3'5 triiodothyroinine	.12	***	
Thyroxine		***	

*P<0.05;**P<0.01;***P<0.001

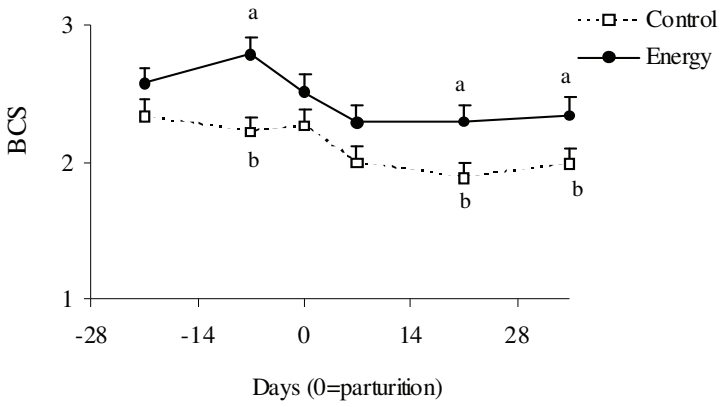


Figure 5.3.1.

Evolution of BCS in **Control** and **Energy** (prepartum cracked corn addition) groups in dairy cows under grazing conditions.

In the *Energy* group, cows increased their BCS during the first two weeks of treatment and there was a decrease from the week prior to parturition to one week postpartum (P<0.01; **Fig. 5.3.1**). Thereafter it remained steady during the last weeks of the trial, but from weeks 3 to 5 postpartum it was higher than in the *Control* group. BCS in the *Control* group decreased only in the first week postpartum and did not recover to their initial level during the remaining of the experimental period. In the *Control* group, BCS score was positively correlated to IGF-I (r=0.41, P<0.001, n=43), leptin (r=0.49, P<0.001, n=43), and T_4 (r=0.35, P<0.05, n=43) and negatively correlated to BHB and urea (r=-0.25 P=0.08 and r=-0.26, P=0.07, respectively, n=43). BCS was positively correlated to IGF-I, and leptin (r= 0.37, and 0.30, P<0.05, n=41) and T4 (r=0.45, P<0.005, n=41) in the *Energy* group.

Milk production and composition

Milk production increased during the first 4 weeks of lactation, and was higher for the *Energy* group (P<0.05; **Table 5.3.1., Fig. 5.3.2A**). In the *Control* group, milk yield was negatively correlated to milk protein %, and plasma IGF-I and leptin (r=-0.47, -0.42, and -0.49, respectively P<0.05, n=26). In the *Control* group, milk production was positively correlated to T4 (r=0.37, P<0.05, n=30). Milk protein and fat percentage decreased as the lactation progressed, and there were no treatment-related differences (P>0.5; **Figs 5.3.2B**, **5.3.2C**). In the *Control* group, milk fat was negatively correlated with cholesterol (r=0.49, P<0.04, n=20); in the Energy group it was positively correlated to urea (r=0.56, P<0.05, n=19) and negatively correlated to insulin (r=-0.43, P=0.06, n=19).

Metabolites and hormones

There was a treatment effect on plasma NEFA levels (P<0.05; **Table 5.3.1**.), and while prepartum concentrations were similar in both groups, postpartum levels were higher in the *Energy* group. Cows in this group had a marked increase in NEFA after calving and showed a marked decrease from the first week postpartum. Cows in the *Control* group did not have a postpartum increase in NEFA concentration, but also showed a decrease during the postpartum period (**Fig. 5.3.3A**).

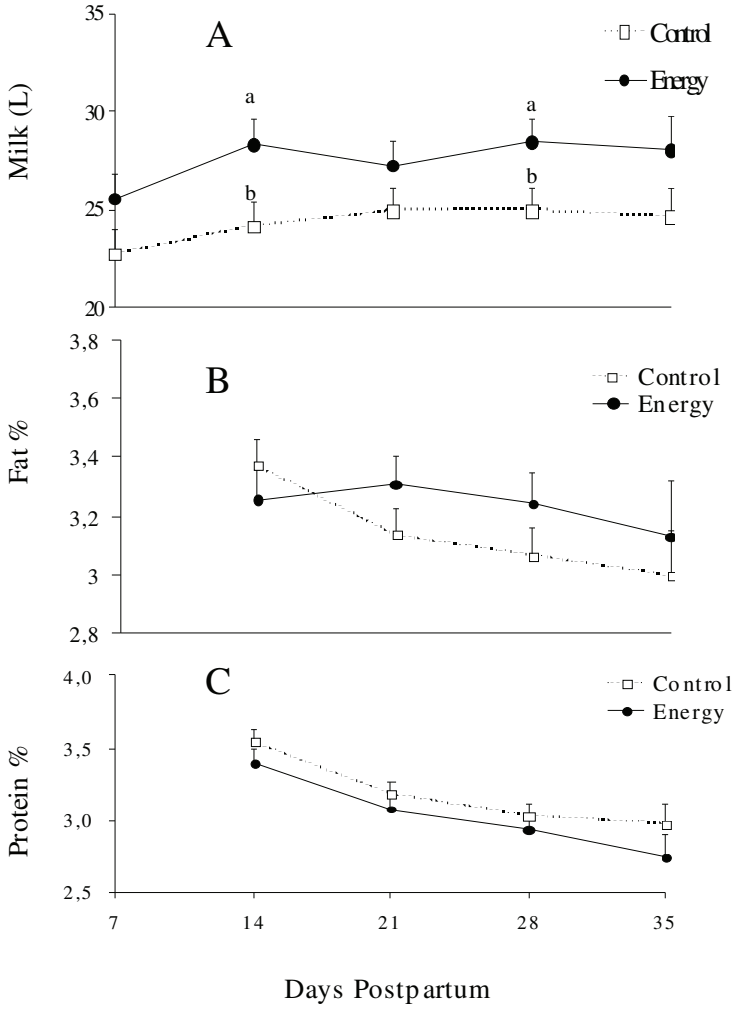


Figure 5.3.2. Milk production (A), milk fat percentage (B) and milk protein percentage (C) in *Control* and *Energy* (prepartum cracked corn addition) groups in dairy cows under pasture system.

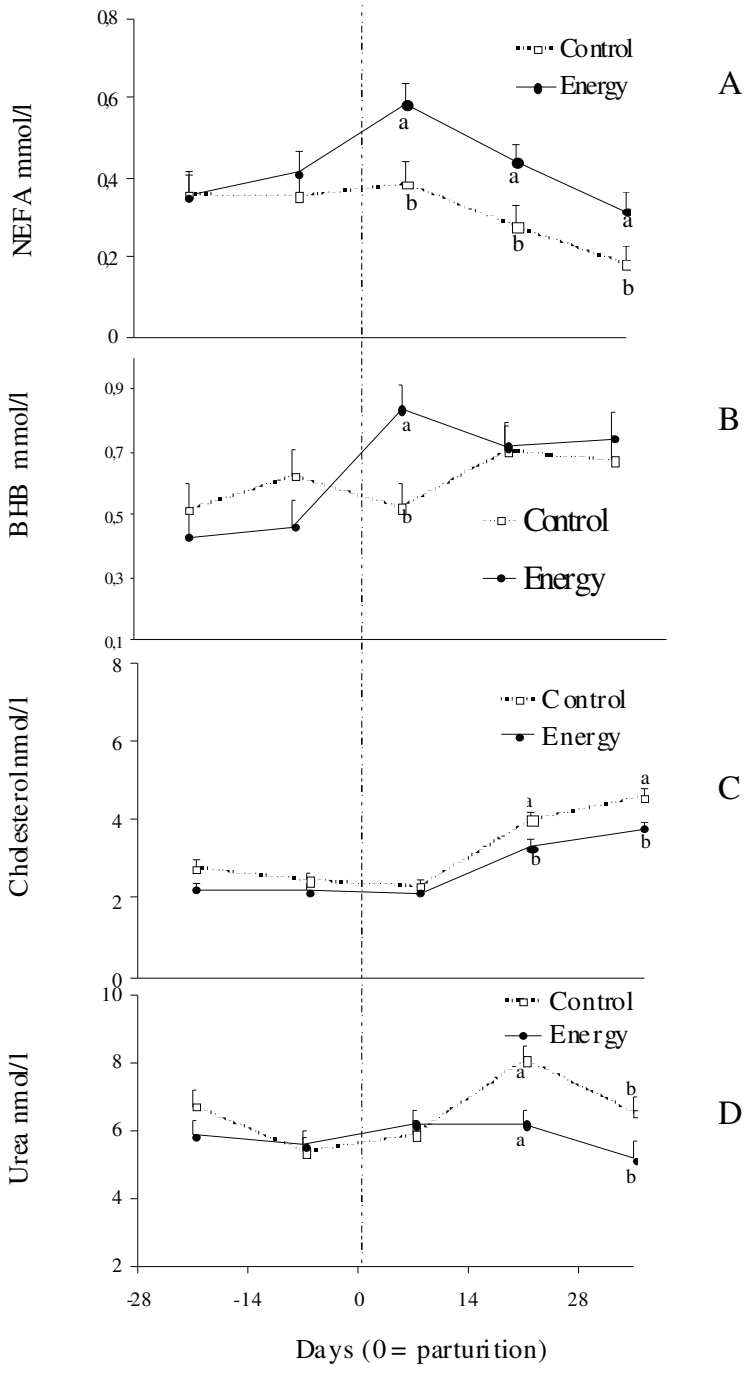


Figure 5.3.3.

Levels of (A) non-esterified fatty acids (NEFA), (B) β-hydroxybutyrate (BHB), (C) cholesterol and (D) urea in *Control* and in *Energy* (prepartum cracked corn addition) groups in dairy cows under grazing conditions.

In the *Energy* group, NEFA was negatively correlated to, T_3 and T_4 (r=-0.30 and -0.58, P<0.05 and P<0.001, n=49) and positively correlated to BHB (r=0.58, P<0.001, n=50). The BHB concentrations were affected by the interaction treatment*week (P<0.001; **Table 5.3.1.).** While *Energy* group showed a significant increase after calving and maintained this level for the remaining period of the trial, the *Control* group maintained lower BHB levels

until the first week postpartum and increased by the second week postpartum. From the third week postpartum BHB levels were similar in both groups (**Fig. 5.3.3B**). In the *Control* group, BHB was positively correlated to T_3 and T_4 (r=0.56 and r=0.57, P<0.001, n=50), while in the *Energy* group BHB was negatively correlated with T_4, IGF-I and leptin (r=-0.60, r=-0.54, and r=-0.46, P<0.001, n=49).

The plasma cholesterol levels were different between the treatment groups (P<0.05). There was a significant effect of the week of sampling but not significant interaction among them (**Table 5.3.1**.). Both groups had constant and similar levels during the prepartum, and rose after calving. Treatment differences were found after the third week postpartum, where the *Control* group had higher levels (P<0.05), and so remained up to the end of the trial (**Fig. 5.3.3C**). In the *Control* group, cholesterol was positively correlated to BHB, urea, T_3, and T_4 (r=0.56, P<0.001, r=0.37, P<0.01, r=0.49, P<0.001, and r=0.29, P=0.06, n=44) and negatively correlated to leptin (r=-0.32, P<0.05, n=44). In the *Energy* group, BHB was positively correlated to milk, and T_3 (r=0.49, P<0.01, n=29, and r=0.38, P<0.01, n=48), and negatively correlated to IGF-I and leptin (r=-0.33, and r=-0.36, P<0.05, n=48).

There was a significant effect of the week of sampling and week*treatment interactions (P<0.01; **Table 5.3.1.)** in plasma urea, that increased markedly in the *Control* group during the postpartum period while in the *Energy* group there was an increase up to the first week postpartum (P<0.05), to return to initial levels at the end of the trial. Urea decreased in both groups from the third week postpartum to the end of the trial (**Fig. 5.3.3D**). In the *Control* group, urea was positively correlated to BHB and cholesterol (r=0.54, and r=0.37, P<0.01, n=50). In the *Energy* group, urea was positively correlated to milk fat percent (r=0.56, P<0.05, n=29), and negatively correlated to leptin (r=-0.28, P<0.05, n=48).

While similar at the beginning of the trial, plasma insulin levels were consistently higher in *Energy* group, and also showed a treatment*week interaction (**Table 5.3.1.**). This treatment difference was due to a marked decrease of plasma insulin level before parturition in the *Control* as compared to the *Energy* group that maintained high levels during the postpartum period (**Fig. 5.3.4A**).

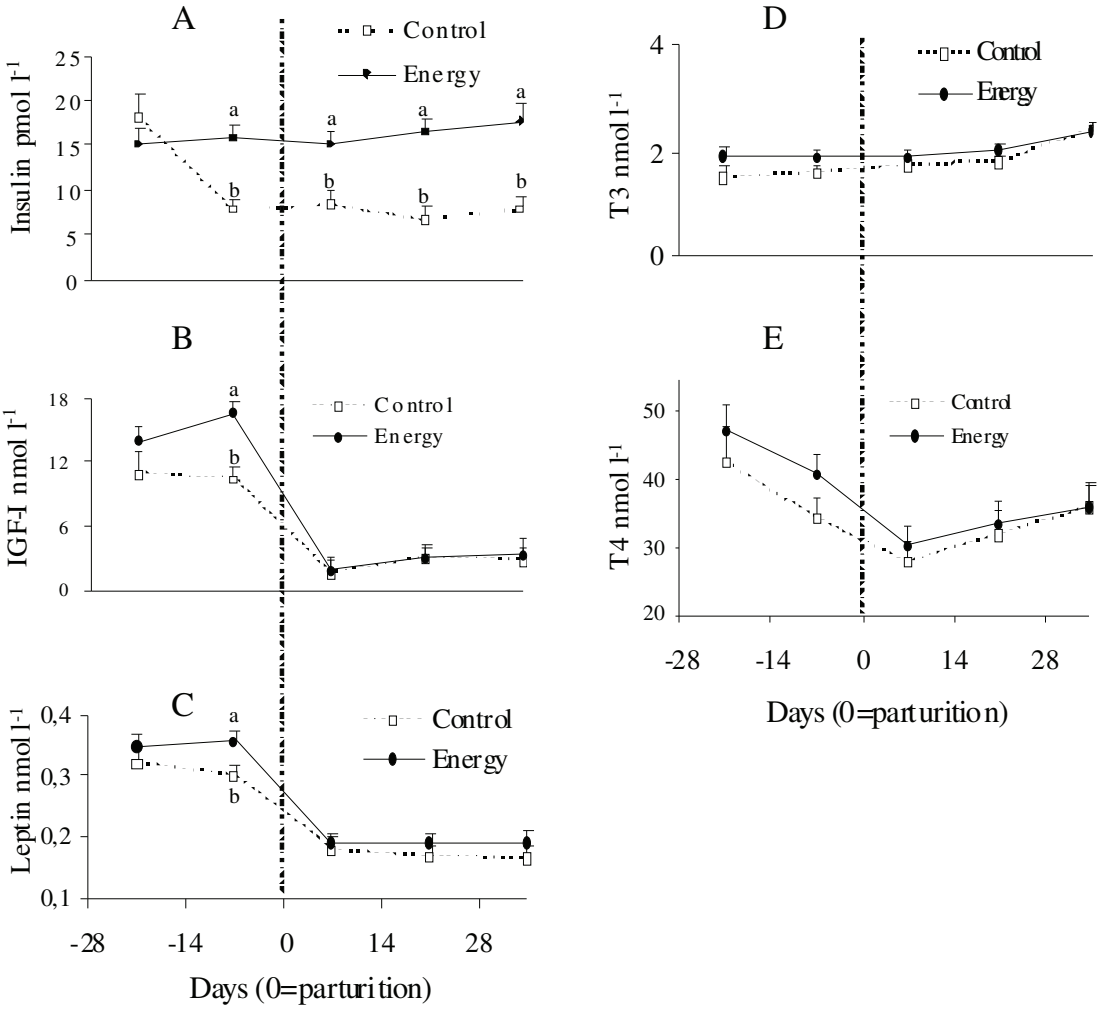


Figure 5.3.4. Concentrations of (A) insulin, (B) IGF-I, (C) leptin, (D) T_3 and (E) T_4 in Control and Energy (cracked corn prepartum supplementation) groups in dairy cows under grazing conditions.

Mean IGF-I levels differed throughout the experimental period, there was also an effect of week of sampling ($P<0.001$) and a significant treatment*week interaction ($P<0.05$) (**Table 5.3.1**.). Treatment difference was due to the higher IGF-I levels during the last week prepartum in the *Energy* group ($P<0.01$, **Fig. 5.3.4B**). However, it decreased in both groups after calving, and those levels remained low throughout the experimental period. In the *Control* group, IGF-I was positively correlated to BCS, leptin, and T_4 ($r=0.41$, $P<0.01$,

r=0.95, and r=0.56, and P<0.001, n=44) and negatively correlated to milk fat percent (r=-0.42, P<0.05, n=29). In the *Energy* group, IGF-I was positively correlated to milk fat percent, BCS, T_4, and leptin (r=0.48, P<0.05, n=19, r=0.36, P<0.05, n=41, r=0.60, P<0.01, n=49, r=0.94, P<0.001, n=49), and negatively correlated to BHB, NEFA and cholesterol (r=-0.54, P<0.001, r=-0.26, P=0.06, r=-0.33, P<0.05, n=49).

Plasma leptin concentration was high during the prepartum period, dropped drastically after calving in both groups, and remained low for the rest of the trial, although it was a not higher in the *Energy* than in the *Control* group (**Table 5.3.1**.). Leptin was higher in the treated cows during the first week prepartum (P<0.05), but levels were similar in both groups during the postpartum period (**Fig. 5.3.4C**). In the *Control* group, leptin was positively correlated to BCS, T_4, and IGF-I (r=0.49, r=0.60, and r=0.94, P<0.001, n=44), and negatively correlated to milk production and cholesterol (r=-0.49, P<0.05, n=26, and r=-0.32, P<005, n=44). In the *Energy* group, leptin was positively correlated to BCS, T_4, and IGF-I (r=0.29, P=0.05, n=41, r=0.59, P<0.001, n=49, and r=0.94, P<0.001, n=49) and negatively correlated to BHB, and urea (r=-0.46, P<0.001, n=49, and r=-0.28, P=0.05, n=48).

The circulating T_3 values were not influenced by treatment (**Table 5.3.1**.), and increased throughout the experimental period (**Fig. 5.3.4D**). There were no treatment differences in the T_4 levels (**Table 5.3.1.),** but decreased in both groups until the first week postpartum, and increased thereafter until the end of the trial. The *Energy* group tend to present higher T_4 levels during the prepartum (P=0.12), but not during the postpartum period (**Fig. 5.3.4E**). T_3 was positively correlated to T_4 (0.34, P<0.05, n=44).

Reproductive parameters

Cows in the *Energy* group ovulated 12 days sooner than the *Control* group (P<0.05). Interval from calving to first ovulation was 25.0±3.7 days for the *Energy* 37.4±3.7 days for the *Control* group (P<0.05). While all the cows in the energy group ovulated before 35 days postpartum, only 60% of those in the control group had the first ovulation before this period (P<0.05).

Discussion

The prepartum energy supplementation resulted in an increase in BCS during the first 2 weeks of treatment and until 1 week prior to calving, probably due to the energy content in the corn grain, which resulted higher estimated energy intake.

Energy supplementation increased by 12% the milk production during the experimental period, which could be due to the better nutrient supply and adaptation to the diet as was offered during the postpartum period. Information regarding the effect of energy supply during prepartum on milk production is conflicting; some studies proved an increase (Ingvartsen and Andersen, 2000; Overton and Waldron, 2004) while others found no effect (Mashek and Beede, 2000; Holtenius et al., 2003; Roche et al., 2005) in milk production. These differences may be attributed to the type of supplement, the way of administration and the quantity, as well as the production traits of the cows used in the different experiments. Another factor susceptible to increase milk production in the *Energy* group is the higher BCS and the resulting higher body fat mobilization (see plasma NEFA at week 1 in **Fig. 5.3.3**.) as also reported by others (Chilliard, 1999; Kokkonen et al., 2005).

The increase in the NEFA levels in the *Energy* group was associated with a drop in the BCS before and after parturition. This could have been due to an effect of the higher body fatness *per se* (Chilliard et al., 2000), and/or to a higher milk production, as also reported by Ingvartsen and Andersen (2000). Since the *Control* group did not show an increase in this metabolite, we suggest that in this group there was not in NEB that is consistent with the lower milk production and loss of BCS. The decrease in NEFA at the third week postpartum in the *Energy* group would be the consequence of marked increase in dry matter intake, which can also be observed in the BCS improvement. In the energy supplemented cows, the elevation of NEFA was paralleled with an increased production of BHB. At this moment the cows in the *Energy* group were in a deeper NEB, probably due to a higher milk production and insufficient dry matter intake. The increase in the cholesterol levels in the postpartum period could be due to the amount of milk production, to the great energetic demands for lactation that could result in an increase of the synthesis of lipoproteins in the liver, but BCS and NEFA profiles suggest that the cholesterol increase is due to a better energy balance as suggested before (Haraszti et al., 1982; Cavestany et al., 2005). The plasma urea content increase in *Control* cows was probably due to a higher protein

catabolism and/or lower protein utilization due to a lower content of glucogenic substrates that forced the cows to use their protein reserves as suggested previously (Jorritsma et al. 2003). These authors found that cows with ruminal flora that not adapted to lactation rations might also face higher plasma urea concentrations due to a mismatch between energy and protein at the level of the rumen.

The most interesting finding in present experiment was that insulin levels remained consistently higher in the *Energy* group through all the experimental period. This suggests that feeding easily fermentable carbohydrates during the prepartum transition period may deliver more glucogenic precursor to the liver and enhance insulin synthesis and/or release. The higher plasma prepartum insulin concentrations with respect to those in the postpartum period in the control group agree with previous works (Holtenius et al., 2003; Meikle et al., 2004; Kokkonen et al., 2005). Furthermore, increased propionate production due to the corn based diet results in an increased secretion of insulin (Brockman and Laarveld, 1986). The higher postpartum NEFA together with higher insulinemia in the *Energy* group could reflect higher insulin resistance due to higher body fatness (Chilliard, 1999), although this is true in relatively obese cows that was not the situation of this study.

In contrast to precalving IGF-I profiles that were higher in *Energy* group, this feeding did not maintain postpartum IGF-I levels as has been previously reported by Lucy (2003) and Roche et al. (2005). After parturition IGF-I decreased despite increasing feed intake (Rhoads et al., 2004). Low plasma IGF-I level in early lactation are generally accepted as a consequence of hypoinsulinemia (Butler, 2003). In this study, however, in the *Energy* group the insulin levels were high in that period. Cows in poor energy status have low circulating concentrations of IGF-I (Pushpakumara et al., 2003). In the *Energy* group despite of increased insulin concentrations during the postpartum period compared to *Controls*, IGF-I levels did not differed. It could be speculated that early lactation cows in NEB are resistant to GH (Chilliard, 1999) and that this decreased plasma IGF-I postpartum is independent of insulinemia. More recently, Butler et al. (2003) reported that the liver is refractory to GH during NEB and this uncoupling of the GH-IGF axis results in diminished plasma concentrations of IGF-I. Mashek and Beede (2000) hypothesized that a nutrition-mediated decline in IGF-I during the peripartum period may prevent a full metabolic adaptation to the nutrient demands of lactation, and consequently reduce milk production.

Higher prepartum levels of plasma leptin in the *Energy* group agree with the results of Holtenius et al. (2003) and Kokkonen et al. (2005) in cows fed confined, and with Roche et al. (2005), who worked with cows under grazing conditions. Leptin acts as an energy reserve signal, secreted by the white adipose tissue (Chilliard et al., 2005) and this is consistent with the BCS increase found in the *Energy* group. Furthermore, the higher energy intake could also act as a short-term stimulus for plasma leptin (Delavaud et al., 2002). The decrease in postpartum leptin levels has also been reported previously (Chilliard et al., 2005). Leptin levels remained low and constant throughout the rest of the experimental period for both groups. This observation is in agreement with data of Holtenius et al. (2003) and Roche et al. (2005), who found that prepartum feeding did not affect blood leptin concentrations postpartum, but not with the results of Kokkonen et al. (2005) who found that prepartum energy supplemented cows had higher postpartum plasma leptin. In agreement with Meikle et al. (2004), the plasma leptin content had a positive correlation with BCS and was a good indicator of level of body fat in peripartum dairy cows.

Plasma T_4 and T_3 levels were the lowest in the early lactation period when production of milk was the highest (Meikle et al,. 2004). In our results, this decrease near calving was found only in the case of T_4, while T_3 remained constant all over the experimental period.

The energy (cracked corn) supplementation on prepartum cows shortened the intervals from parturition to first ovulation. In this study energetic supplementation increased the hormonal levels which have been reported to be related with reproduction in the prepartum period (Spicer and Echtnerkamp, 1995), although in the postpartum period only insulin levels were higher in the *Energy* group. Gong et al. (2002) reported that the diet resulting in a higher insulin concentration reduced the interval from calving to first ovulation. It is likely that increased insulin concentrations promoted the differentiation and maturation of DFs during early lactation, thereby increasing the chance of these DFs ovulating in response to the LH surge (Butler, 2003). Insulin stimulates ovarian P4 and E2 production (Spicer and Echtnerkamp, 1995). We could presume that lower insulin and IGF-I levels in the *Control* group on the week precalving would have affected steroidogenesis and follicle size, and that would be the cause of the longer postpartum acyclic period on that group. Data regarding leptin and reproductive performance are complex and sometimes

contradictory. It has been reported that cows with decreased leptin showed delayed onset of cyclicity on the postpartum (Kadokawa et al., 2000). On the other hand, leptin may play a permissive role, when increased above a critical threshold (0.250 - 0.312 nmol/L according to the review by Chilliard et al., 2005), in the activation of the hypothalamus-pituitary axis and consequent reinitiation of ovarian activity (Meikle et al., 2004). This would allow cows that had higher BCS in the pre- and postpartum and thus, higher leptin plasma levels in the prepartum and/or the postpartum period (Meikle et al., 2004; Kokkonen et al., 2005) to shorten the interval calving to first ovulation.

It was concluded that in lean cows kept under grazing conditions the energetic supplementation administered during the last three weeks precalving had a positive effect on the re-initiation of cyclic ovarian activity, which is consistent with a better energy balance (BCS), higher prepartum levels of insulin, IGF-I and leptin, and higher insulin levels during the early postpartum period.

6. Overview of the new scientific results

The below results are thought to represent remarkable novelty value:

1. Long term hyperketonemia impairs the pancreatic insulin release and the whole-body glucose utilization in Holstein-Friesian cows. Only short term elevations in plasma free fatty acids and BHB may not potentially induce further increase in peripheral tissue insulin resistance in the early lactation *(Exp. 1.)*.
2. Inflammatory diseases like puerperal metritis with intensive release of pro-inflammatory cytokines potentially further depress insulin secretion of the pancreatic ß-cells and the whole body insulin responsiveness in dairy cows, with long term effects on metabolism and reproduction *(Exp. 1.)*.
3. The homeostatic RQUICKI model developed for rapid and easy evaluation of insulin sensitivity in humans, and used previously in healthy cows should be applied only with cautions in dairy cows in different physiological and disease states *(Exp. 1.)*.
4. Feeding a dry propylene glycol preparation absorbed on fumed silica, top dressed on the total mixed ration from d 14 before calving till d 10 after calving had no notable effect on the metabolic profile, insulin sensitivity, on the time of first pp ovulation and on reproductive performance *(Exp. 2.)*.
5. In lean cows kept under grazing conditions the energetic supplementation administered during the last three weeks precalving had a positive effect on the re-initiation of cyclic ovarian activity, which is consistent with a better BCS, higher prepartum levels of insulin, IGF-I and leptin, and higher insulin levels during the early postpartum period *(Exp. 3.)*.

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8. Publications

8.1. Publications related to the present thesis

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

- **Kerestes M.**, Faigl V., Kulcsár V., Balogh O., Földi J., Fébel H., Chilliard Y., Huszenicza G. Periparturient insulin secretion and whole-body insulin responsiveness in dairy cows showing various forms of ketone pattern with or without puerperal metritis. *Dom. Anim. Endocrin*., 2009. 37: 250-261. (last known **IF**: 2,165)

- Cavestany D., Kulcsár M., Crespi D., Chilliard Y., La Manna A., Balogh O., **Keresztes M.**, Delavaud C., Huszenicza G., Meikle A. Effect of prepartum energetic supplementation on productive and reproductive characteristics, and metabolic and hormonal profiles in dairy cows under grazing conditions. *Reprod Dom.Anim*. 2009. 44: 663 – 671. (last known **IF**: 1,526)

- Huszenicza Gy., **Keresztes M**., Balogh O., Faigl Vera, Kátai L., Földi J., Lemoniati, K., Kulcsár M. Periparturient changes of metabolic hormones and their clinical and reproductive relevance in dairy cows. *Magy. Áo. Lapja*, 2008. 130. (Suppl.) 1: 45-51. . (**IF**: 0,089)

- **Kerestes M.**, Faigl V., Kulcsár M., Fébel H., Győrffy A., Gaál T., Husvéth F., Bartha T., Mézes M., Tamási G., Gábor G., Szenci O, Huszenicza G. Effect of pulverized propylene glycol supplementation on periparturient metabolic profile and reproductive performance in Holstein-Friesian cows. *Vet. Med. Cz*., submitted for publication (last known **IF:** 0.659)

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

- **Keresztes M.**, Faigl V, Mézes M, Kulcsár M, Huszenicza Gy. Glükoneogenetikus takarmány-kiegészítők metabolikus és szaporodásbiológiai hatásai tejhasznú szarvasmarhában. Irodalmi áttekintés. *Magy. Áo. Lapja*, accepted for publication (last known **IF**: 0,089)

Poster or oral presentation on international conferences:

- **Keresztes M**., Crespi D., Kulcsár M., Meikle A., La Manna A., Faigl V., Balogh O., Delavaud C., Chilliard Y., Huszenicza Gy., Cavestany D.: Effect of prepartum energetic supplementation on milk yield, metabolism and reproduction in dairy cows under grazing conditions. *Proc. of 24th World Buiatrics Congress, Nice (France), 15-19 Oct.,* 2006, oral presentation

- **Keresztes M**., Faigl V., Gyõrffy A., Langer D., Kulcsár M., Márton A., Gaál T., Fébel H., Bartha T., Tamás G., Szenci O., Huszenicza Gy. The effect of peripartum propylene glycol administration on metabolic and hormonal profile and on

reproductive performances in Holstein-Friesian cows. *Magyar Buiatrikus Társaság XVIII. Nemzetközi Kongresszusa, Siófok, 2007. okt. 10-13,* oral presentation

- **Kerestes M**., Faigl V., Győrffy A., Márton A., Langer D., Kulcsár M., Gaál T., Fébel H., Húsvéth F., Gábor G., Bartha T., Szenci O., Huszenicza G. The effect of periparturient propylene glycol administration on metabolism and on reproductive performance in Holstein-Friesian cows. 11th Annual conference of the European Society of Domestic Animal Reproduction (ESDAR), Celle, Germany. Abstract. *Reprod. Dom. Anim.,* 2007. 42. Suppl. 2. p. 47, poster presentation

- **Kerestes M.,** Faigl V., Kulcsár M., Huszenicza Gy. Insulin secretion in normal, ketonemic and in cows affected by puerperal metritis. 12th Annual conference of the European Society of Domestic Animal Reproduction (ESDAR), Utrecht, Netherland. Abstract. *Reprod. Dom. Anim.,* 2008. 43. Suppl. 5. p103, poster presentation

Academical reports:

- **Kerestes M.,** Kulcsár M, Mircean M, Solti L, Rudas P, Huszenicza Gy. Az ún. poliszacharid-tárolási izombetegség egyes endokrinológiai vonatkozásai lovakban. *MTA akadémiai beszámolók*, 2006.

- **Keresztes M.**, Faigl V, Győrffy A, Dakó Z., Várnai Zs., Kulcsár M., Mézes M., Márton A., Ribiczeiné Szabó P., Gaál T., Fébel H., Bartha T., Tamási G., Husvéth F., Szenci O., Huszenicza Gy. Az ellés körül alkalmazott propilénglikol kiegészítés hatása az energiaháztartásra és az egésztest-inzulinérzékenységre. *MTA akadémiai beszámolók,* 2007

- **Keresztes M.**, Faigl V, Sassi G, Szelényi Zs, Tóth F, Kulcsár M, Fébel H, Mézes M, Tamási G, Gábor Gy, Szenci O, Huszenicza Gy. Többszörösen telítetlen zsírsavakban gazdag védett zsírkészítmény etetésének hatása az ellés utáni energia-háztartásra, tejtermelésre és vemhesülésre holstein-fríz tehenekben. *MTA akadémiai beszámolók*, 2007

- **Keresztes M**., Faigl V, Kulcsár M, Földi J, Chilliard Y, Huszenicza Gy. Az inzulinrezisztencia vizsgálata hyperketonaemiás és puerperalis metritisben szenvedő tehenekben. *MTA akadémiai beszámolók*, 2009

8.2. Candidate's further publications unrelated to this thesis

- 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 összefüggő egyes újabb élettani kérdések és ezek technológiai vonatkozásai. Irodalmi áttekintés. *Magy. Áo. Lapja*, 2005. 127: 586-593. (**IF**: 0,114)

- **Keresztes M**., Faigl V., Márton A., Ihnáth Z., Kulcsár M., Mézes M., Húsvéth F., Huszenicza Gy. A takarmány védett zsírokkal történő kiegészítésének szaporodásbiológiai vonatkozásai kérődzőkben. Irodalmi áttekintés. 1. rész. *Magy. Áo. Lapja*, 2007. 129: 525-530. (**IF**: 0.104)

- **Keresztes M**., Faigl V., Márton A., Ihnáth Z., Kulcsár M., Mézes M., Husvéth F., Huszenicza Gy. A takarmány védett zsírokkal történő kiegészítésének szaporodásbiológiai vonatkozásai kérődzőkben. Irodalmi áttekintés. 2. rész. *Magy. Áo. Lapja*, 2007. 129: 707-712. (**IF**: 0.104)

- Faigl V., **Kerestes M.,** Márton A., Schneider Z., Korvin L., Sándor S, Novotniné D. G., Árnyasi M., Jávor A., Cseh S., Huszenicza Gy.: Melatonin-, ill. fénykiegészítés alapú ciklusindukciós technikák kiskérődzőkben. Élettani vonatkozások és gyakolatialkalmazás. Irodalmi áttekintés. *Magy. Áo. Lapja*, 2007. 129: 219-230.

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- Balogh O., Szepes O., Kovács K., Kulcsár M., Reiczigel J., Alcazar, J. A., **Keresztes M**., Febel H., Bartyik J., Fekete S., Fesus L., Huszenicza Gy: Interrelationships of growth hormone AluI polymorphism, insulin resistance, milk production and reproductive performance in Holstein-Friesian cows. Vet. Med. Cz., 2008. 53: 604-616. (last known **IF:** 0.659)

- Győrffy A., **Kerestes M.**, Faigl V., Frenyó V.L., Kulcsár M., Gaál T., Mézes M., Zsarnovszky A., Huszenicza Gy., Bartha. T.: Glycogenic induction of thyroid hormone conversion and leptin system activation in the liver of postpartum dairy cows. *Acta Vet. Hung*., 2009. 57: 139-146. (last known **IF:** 0.624)

- Faigl V., **Kerestes M**., Kulcsár M., Nagy S., Keresztes Zs., Amiridis, S., Solti L., Huszenicza Gy., Cseh S.: Testicular function and semen characteristics of Awassi rams treated with melatonin out of the breeding season. Acta *Vet. Hung*., 2009. 57: 531-540. (last known **IF:** 0.624)

- Márton A., Faigl V., **Keresztes M**., Kulcsár M., Nagy S., Fébel H., Novotni Dankó G., Magyar K., Husvéth F., Solti L., Cseh S., Huszenicza Gy.: Milk progesterone profiles, blood metabolites, metabolic hormones and pregnancy rates in Awassi ewes treated by gestagen + eCG at the early breeding season. *Vet. Med. Cz.,* 54: 2009 (11): 507–516 (last known **IF:** 0.659)

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