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Metabolic and productive characterisation of multiparous cows grouped for fat-corrected milk yield and milk protein concentration

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Widmung

Allen, die mich bis zu diesem Zeitpunkt unterstützt haben, besonders meinem Mann, meinen Großeltern und meiner Mutter.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACACA	acetyl-CoA carboxylase α
ACADVL	acyl-CoA dehydrogenase, very long chain
ACTB	actin beta
ap	ante partum
AUC	area under the curve
BCS	body condition score
BFT	back-fat-thickness
BHBA	β-hydroxybutyric acid/β-hydroxybutyrate
BW	body weight
cDNA	complementary desoxyribonucleic acid
CF	correction factor
CN	casein
CPT1A	carnitine palmitoyltransferase
Cq	quantitative cycle
CR	clearance rate
CS	citrate synthase
CTSL	cathepsin L
d	day
Da	Dalton
DMI	dry matter intake
EB	energy balance
ECHS1	enoyl CoA hydratase 1
EDTA	ethylenediamine-tetraacetic acid
EIA	enzyme immuno assay
EIF4B	eukaryotic translation initiation factor 4B
EP	European Pharmacopoeia
FPR	milk fat to milk protein ratio
FR	feed restriction
GAPD	glyceraldehyde-3-phosphate dehydrogenase
GLUT	glucose transporter
GPAM	glycerol-3-phosphate acyltransferase
H3F3A	H3 histone family 3A
HEC	hyperinsulinaemic euglycaemic clamp
HGC	hyperglycaemic clamp
HMGCS2	3-hydroxy-3-methylglutaryl-coenzyme A synthase 2
HNF4A	hepatocyte nuclear factor 4A
HOMA-IR	homeostatic model assessment of insulin resistance
HPLC	high performance liquid chromatography
IGF1	insulin-like growth factor 1
INSR	insulin receptor
IR	insulin resistance
ivGTT	intravenous glucose tolerance test
LA	lactalbumin
LD	lactation diet
LfL	Bavarian State Institute of Agriculture, Landesanstalt fuer Landwirtschaft
LG	lactoglobulin
MEC	mammary epithelial cells
MJ	mega joule
mRNA	messenger ribonucleic acid
MS	mass spectrometry
NAD, $NADH_2$	oxidised, reduced nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information

NEFA	non-esterified fatty acids
NE_L	netto energy lactation
PAGE	polyacrylamide gel electrophoresis
PBST	phosphate buffered saline Tween-20
PC	pyruvate carboxylase
PCK1	phosphoenolpyruvate carboxykinase, cytosolic
PCK2	phosphoenolpyruvate carboxykinase, mitochondrial
pp	postpartum
PPARA	peroxisome proliferator activated receptor α
QUICKI	quantitative insulin sensitivity check index
RD	restriction diet
RT	room temperature
RT-qPCR	real time quantitative polymerase chain reaction
SCC	somatic cell count
SDS	sodium dodecyl sulphate
SLC2A	solute carrier family 2
SREBF1	sterol regulatory element binding transcription factor 1
TAT	tyrosine aminotransferase
tBR	total bilirubin
TMB	tetramethylbenzidine
TNFA	tumour necrosis factor α
USP	United States Pharmacopoeia
wk	week

I. INTRODUCTION

Milk protein yield has become the major economic output of the dairy industry. In 2010, 47.5% of collected milk was devoted to protein dependent production of cheese, including cream, curd and pulled curd cheese (Bundesministerium für Ernaehrung, 2011). This demand is reflected by the paying system of dairy industry: in 2011, the dairy industry paid for one kg milk (4.2% milk fat and 3.4% milk protein) 34.5 ± 1.2 €-cent, corrected by 2.70 €-cent for percentage fat and 4.10 €-cent for percentage protein (dairy factory Weihenstephan, Freising; Topagrar, 2011).

Milk protein yield is mainly depending on milk yield (correlation 0.92), but also on milk protein content (correlation 0.10; Teepker and Swalve, 1988). After focussing on efforts to increase milk yield during the last decades, special emphasis of genetic selection is nowadays also devoted to other traits including milk protein content (Lipkin et al., 2008).

Regardless of the economic value of milk protein yield, cows with elevated protein content could have metabolic advantages in comparison to their herd mates with equal protein yield as a result of lower protein content but higher milk yield. The amount of produced milk correlates positively to the lactose yield (0.96; Shahbazkia et al., 2010). Regarding cows with similar milk yield, the energy demand for milk synthesis depends on milk fat and protein content (Kamphues et al., 2004):

$$milk\ energy\ \left[\frac{MJ}{kg}\right] = 0.38 \times fat\% + 0.21 \times protein\% + 1.05.$$

Consequently, energy demand for synthesis of milk protein is nearly half of the energy demand for the same amount of milk fat. Regarding the critical situation of high yielding dairy cows during the first weeks of lactation, when feed intake regularly lags behind energy demand for milk synthesis, it is worthwhile to select cows with high economic and concurrently low energetic output to decrease risks of typical diseases during early lactation like ketosis, hepatic steatosis, dislocation of abomasum and following events (impaired immune function, metritis, mastitis, lameness). However, selecting cows with low milk fat content is not desirable due to concomitant decrease of milk protein concentration (correlation 0.47), as well as selecting for low milk fat yields which would reduce milk yield (correlation 0.77) and protein yield (correlation 0.82; Teepker and Swalve, 1988).

Consequently an increase of the economically valuable milk protein yield should be achieved by the less energy demanding increase of milk protein content with constant or lower milk and fat yields.

In this study, dairy cows were classified into four groups according to milk yield and milk protein content. The aim was to evaluate metabolism, milk productivity and metabolic situation of these four groups during early and mid-lactation. Therefore, cows were submitted to short-term feed restrictions in early (wk 4 pp) and mid-lactation (wk 21 pp) and to intravenous glucose tolerance tests (ivGTTs) two weeks before parturition (d 14 ap), in early (d 20 pp) and mid-lactation (d 127 pp). Furthermore, blood and milk as well as hepatic and muscular tissue samples were collected throughout the trial to investigate the influence of productivity on metabolic adaptations.

II. LITERATURE

1. Milk composition

Producing a nutritive excretion for the nourishment of the offspring is the evolutional advantage of many mammalian species. However, in the following sections the term milk refers to cow's milk.

Milk is a very complex emulsion, containing approximately 3.0 to 5.0% fat mainly organised in fat globules and around 5% carbohydrates, mainly lactose, but also galactose and glucose in the aqueous phase (Schultz, 1974; Cerbulis and Farrell, 1975). Another nutritive ingredient are the milk proteins (around 3.2 to 3.8%), consisting of 20% whey proteins with the major components α-lactalbumin (α-LA) and β-lactoglobulin (β-LG) and of 80% caseins, divided into major subclasses α-, β- and κ-casein (-CN), arranged in micelles (Swaisgood, 1982; Rodriquez et al., 1985). Further constituents are proteolysed fragments ($\gamma_{1\text{-}3}$-CN and proteose peptone, plasmin derived from β-CN), cells (mammary epithelial cells or immune cells like leucocytes), but also urea, amino acids, immunoglobulins, lactoferrin, vitamins, minerals and hormones along with electrolytes and other components (Ng-Kwai-Hang et al., 1984; Shutt and Fell, 1985; Palmano and Elgar, 2002). Hereafter, only the major milk components lactose, fat and protein are further described in detail.

1.1. Lactose

Concentration of lactose is the volume limiting factor for milk synthesis, due to its osmotic activity and its disability to cross the membrane of mammary epithelial cells (MEC; Larson, 1969; Bleck et al., 2009).

The disaccharide lactose consists of glucose and galactose, which is metabolised in MEC from glucose. Lactose is synthesised in the Golgi apparatus of MEC by lactose synthase, which is a complex of α-LA and ubiquitous galactosyltransferase (Larson, 1969; Kuhn et al., 1980). With increasing milk protein content, concentration of lactose synthase increases and concentrations of galactosyltransferase also increase with days of lactation (Bleck et al., 2009). After synthesis, lactose is secreted by exocytose into the mammary alveoli lumen (Shennan and Peaker, 2000). Lactose content in milk of different breeds ranges from 4.66 ± 0.34% in Guernsey, 4.93 ± 0.61% in Holstein to 5.15 ± 0.46% in Brown Swiss cows, but is equally distributed within breeds (Cerbulis and Farrell, 1975).

1.2. Fat

Milk fat provides the main energy source of milk, whereas milk protein and lactose accounts for half the energy density of milk fat (Emery, 1973). Moreover, lipid droplets contain lipohilic vitamins and other biological active substances (Parodi, 1997; Molkentin, 1999).

Fat in milk comprises mainly of triacylglycerols which are synthesised in MEC either *de novo* or from fatty acids extracted from blood (Linzell and Peaker, 1971; Shennan and Peaker, 2000). About 40% of fat mass consists of short-chain and medium-chain fatty acids (4 to 14 carbon atoms) which are mainly synthesised from blood derived precursors acetate and β-hydroxybutyric acid (BHBA) and the remaining long-chain fatty acids are synthesised from blood non-esterified fatty acids (NEFA) and blood triacylglycerol (Linzell and Peaker, 1971). Esterification of fatty acids takes place in the rough endoplasmatic reticulum. Afterwards, lipid droplets migrate to the apex of MEC, where they buckle out the surface of the cell and then are snapped off with an intact membrane of phospholipids around them (Linzell and Peaker, 1971). The proteins of milk fat globule membrane (MFGM) represent 1-4% of total milk protein concentration and have low

nutritional value, but since proteomic studies give new insights, it is obvious, that MFGM proteins are important for cellular processes and defence mechanisms in newborns (Cavaletto et al., 2008).

Concentration of milk fat averages 4.34 ± 0.71%, ranging from Jersey (5.42 ± 0.53%), Holstein (3.73 ± 0.32%) to Milking Shorthorn cows (3.58 ± 0.26%; Cerbulis and Farrell, 1975), whereas in Holstein herds mean fat content is 3.684 ± 0.003% (Ng-Kwai-Hang et al., 1984).

1.3. Protein

Synthesis of milk protein

Precursors for mammary protein synthesis are amino acids extracted from blood or synthesised in MEC. Milk protein synthesis follows the same principles as in other cells: transcription of DNA, translation at the ribosomes of rough endoplasmatic reticulum and posttranslational modifications (Larson, 1969; Shennan and Peaker, 2000). The genes for the caseins are clustered in close proximity on bovine chromosome 6 and are conserved throughout evolution with α_{S1}-, α_{S2}- and β-CN having a common ancestor whereas κ-CN was derived from the fibrinogen gene family (Mercier and Vilotte, 1993). Furthermore, encoding genes for α-LA can be found on chromosome 5 and for β-LG on chromosome 11 (Mercier and Vilotte, 1993).

Some proteins derive from blood and are secreted unchanged into milk by vesicular transport across MECs or by paracellular migration (e.g. BSA, immunoglobulins; Larson and Gillespie, 1957; Shennan and Peaker, 2000). During lactation in healthy cows, the transcellular path predominates whereas in cows suffering inflammation of mammary gland, tight junctions between MEC are becoming leaky and the paracellular way gains importance (Shennan and Peaker, 2000; Hogarth et al., 2004).

1.3.1. Composition of milk protein

Depending on their solubility at pH 4.6 and 20°C, milk proteins are divided into indissoluble caseins and soluble whey proteins (Swaisgood, 1982).

Casein

According to De Marchi et al. (2009), the casein fraction of milk mainly comprises of α_{S1}-, α_{S2}-, β- and κ-CN in concentrations of 31.3, 10.8, 36.9 and 9.3% of total protein. Morris (2002) observed 42.8, 19.4 and 11.7% of total protein for α_S-, β- and κ-CN. All of the caseins bind calcium and this binding capacity is proportional to the phosphate content (Swaisgood, 1992). During migration through the cytoplasm, caseins cluster with calcium and phosphate to micelles (Walstra, 1999).

Individual caseins exhibit varying structures: α_{S1}-CN bears 199 amino acids (23.0 kM), two hydrophobic and one polar group and assembles with itself stepwise. Furthermore α_{S2}-CN consists of 207 amino acids (25.0 kM), can contain a disulfide bond, is the most hydrophilic of the caseins and assembles also stepwise to build polymers (Morris, 2002). Moreover, β-CN consists of 209 amino acids (24.0 kM) and acts as a detergent due to its amphiphilic character because of the highly charged polar N-terminal region and the hydrophobic C-terminal region. Self-assembly of β-CN produces large spherical aggregates (Morris, 2002).

Although it is the least abundant casein, κ-CN has importance in stabilisation of caseins and micelles. After protein synthesis, caseins associate with themselves and also with other caseins to form sub-micelles (diameter around 14 nm). Those sub-micelles bind calcium phosphate during migration in Golgi vesicles through the cytoplasm of MEC, therefore loosing partly their negative charge, hence decreasing their size and, due to stronger interaction with other micelles, rearrange in micelles (Walstra, 1999). Distribution of κ-CN shows different patterns: it can be found as 'hairy' layer at the periphery of micelles, with the hydrophilic portion pointing outside, therefore

providing solubility of micelles in aqueous media and it can also be uniformly distributed within micelles. The higher the κ-CN content at the periphery of micelles, the bigger they grow (92 - 142 nm; Carroll and Farrell, 1983). Composited of 169 amino acids (19.0 kM), it is cleaved by rennet into its hydrophobic portion para-kappa-casein and the hydrophilic portion caseinomacropeptide, therefore causing the micelles to agglutinate (Hallen et al., 2010). Moreover, during heat-induced coagulation, κ-CN interacts with β-LG (Morris, 2002).

Whey protein

Already in ancient times, healthy aspects of whey were known. Nowadays the underlying proteins and bioactive peptides are revealed, which account for effects on satiety and therefore obesity management or influence blood pressure (Barth and Behnke, 1997; Clare and Swaisgood, 2000; D'Amato et al., 2009). Apart from minor constituents like BSA, immunoglobulins and lactoferrin, whey consist mainly of proteins α-LA and β-LG. De Marchi et al. (2009) reported 3.2 and 9.3% of total protein for α-LA and β-LG and according to Morris (2002) α-LA, β-LG, immunoglobulin G and BSA account for 3.1, 13.3, 2.5 and 1.4% of total protein.

The more abundant whey protein β-LG comprises of 178 amino acids (18.3 kM) and binds hydrophobic and amphiphilic molecules like hexane, palmitic acid, vitamin D or retinol (Sawyer, 2003; Farrell Jr et al., 2004). As mentioned before the 123 amino acid containing globular protein α-LA is part of the complex for synthesis of lactose (Kuhn et al., 1980). Furthermore α-LA binds calcium, zinc and other metals due to its classification as calcium metalloprotein (Farrell Jr et al., 2004).

1.3.2. Quantification of milk protein fractions

For separation and quantification of milk protein fractions and for analysis of genetic varieties, methods often apply high performance liquid chromatography (HPLC). In HPLC, protein solutions together with solvents are pumped through a column filled with specific materials like silica beads with or without carbon chains. Proteins attach according to size, ionisation or other properties to these fillings and duration of migration varies for different proteins (Bordin et al., 2001; Bonfatti et al., 2008; Bonizzi et al., 2009). Moreover, HPLC coupled to mass spectrometry (MS) has also been used. Principle of MS is based on a separation by the ratio of mass to charge: samples are vaporized, ionised (e.g. by laser or electrospray), the ions are guided through an electromagnetic field, where a separation according to ratio of mass to charge occurs and ions are detected by an electron multiplier (Galvani et al., 2001; Fröhlich and Arnold, 2006; Boehmer et al., 2010). To obtain single peptides for analysis in MS, protein solutions are subjected to a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) followed by trypsin digestion. In 2D-PAGE, proteins are first separated according to their isoelectric point by isoelectric focussing and then according to their molecular weight by sodium dodecyl sulphate electrophoresis (SDS; O'Farrell, 1975). Despite of the high power of resolution and separation, 2D-PAGE and also SDS-PAGE are very time consuming and have low throughput rates. Goetz et al. (2004) and Wu et al. (2008) showed that miniature electrophoresis on microfluidic chips offers a fast and reliable alternative for conventional SDS-PAGE. On these chips, all steps of traditional gel electrophoresis can be performed: gel preparation, sample loading, separation (reducing or non reducing conditions), staining and destaining and detection. Integration is done automatically with the possibility of manual integration (Agilent 2100 Expert Software).

1.4. Effects on milk and protein composition

Milk is not only a complex secretion, also regulation of lactation and composition of individual components is complex (Svennersten-Sjaunja and Olsson, 2005). Hereafter, some of the possibilities to influence milk and milk protein composition are discussed.

Genetics

From the middle of the 20th century, dairy cows have been selected for milk and fat yield (Schultz, 1974). Since then, new developments in molecularbiology have altered traditional breeding programs. Rolleri et al. (1956) have shown, that different breeds vary in milk protein composition: On the one hand, Holstein cows show lowest total casein, α- and β-CN, on the other hand, cows of the Ayrshire breed have less milk α-CN compared to Brown Swiss, Guernsey and Jersey cows. Nowadays breeders have a deeper understanding of the mechanisms underlying these variations due to comprehension of most of the impacts of genetic polymorphisms on milk protein composition or the detection of quantitative trait loci for protein composition (Martin et al., 2002; Schopen et al., 2009). All these factors together represent a new breeding system according not only to conventional breeding values (e.g. performance of offspring) but also to the genomic breeding value (Hayes et al., 2009). After decades of selecting for milk yield and fat yield, emphasis today shifts towards milk protein yield and concentration (Lipkin et al., 2008).

Lactation

Independent of the genetic equipment, milk composition alters with progressing lactation. During the first days, secretion of mammary gland provides immune components for the newborn. Concentrations of fat, protein, immunoglobulins and lactoferrin are higher in colostrum and several low abundance proteins appear in colostrum which are not found in mature milk (Kehoe et al., 2007; Stelwagen et al., 2009). With ongoing lactation, milk fat, protein and immunoglobulin concentrations decrease and reach levels of mature milk. Protein concentration in Brown Swiss cows shows a nadir at 3.0% around week (wk) 4 postpartum (pp) and increases from wk 8 pp until wk 44 pp (to 4.0%; Schlamberger et al., 2010). Especially in high yielding cows, milk fat concentration is comparative high in early lactation due to the metabolic imbalance caused by enormous acceleration of produced milk yield, concurrent insufficient energy intake and resulting mobilisation of body fat tissue (Grummer et al., 2004; Ingvartsen, 2006).

Additionally with increasing numbers of lactation, dairy cows produce more milk (maximum in 4th or 5th lactation), but concentrations of main components remain relatively constant (Waite et al., 1956; Ng-Kwai-Hang et al., 1984; Ray et al., 1992).

Feeding

Besides genetic and lactational effect, feeding has also a high but complex influence on milk and milk protein composition: adequate amounts of rumen-undegradable protein and energy intake, suitable roughage supply and improved peri-parturient feeding regime avoiding over-conditioning of dry cows optimise milk protein concentration (Sutton, 1989; Jouany, 1994; Santos et al., 1998; Pop et al., 2001; Brun-Lafleur et al., 2010). Moreover, energy-insufficient rations especially in early lactation provoke body fat mobilisation and therefore increasing milk fat concentration and milk fat to milk protein ratios (FPR).

The composition of dietary fat may influence the ratio of short to long fatty acids in milk fat (Palmquist et al., 1993; Sigl et al., 2010). Moreover during restricted pasture allowance, Auldist et al. (2000) found 4% increase of caseins and 9% decrease of whey proteins as well as declining milk yield and fat concentration. Elevation of available protein in small intestine by infusion of proteins resulted only in decrease of β-LG (-4.7%; Mackle et al., 1999).

Management and environment

Moreover, milk composition varies through course of milk removal: fat concentration increases throughout the removal, protein concentration decreases only in residual milk and lactose concentration first increases and decreases again in residual milk (Ontsouka et al., 2003). Length

of dry period and milking frequency also affect milk composition. Madsen et al. and Schlamberger et al. found 0.4% increase of protein concentration at wk 5 pp or respectively throughout 305 day (d) performance in continuously milked cows without drying off at wk 8 ante partum (ap; Madsen et al., 2008; Schlamberger et al., 2010). Furthermore milking frequency in Holstein cows correlates positively to milk yield (0.40) and negatively to milk fat (-0.13) and protein concentration (-0.20; Løvendahl and Chagunda, 2011).

Moreover decreasing temperatures increase protein and casein concentrations in milk and concentrations of all milk proteins increase after a nadir in the 2nd lactation month until end of lactation (Ng-Kwai-Hang et al., 1982; Rodriquez et al., 1985).

Disease

Besides physiological effects, also pathological mechanisms alter milk and protein composition. During infection of mammary gland, milk yield declines resulting in higher concentrations of components and the protein composition is altered towards more whey proteins (especially the blood derived proteins) and less caseins (Munro et al., 1984; Hogarth et al., 2004). These changes are complex and results of studies often contradictory as Seegers et al. (2003) reviewed.

2. Characterisation of metabolic situation in dairy cows

Due to selection programs over the last decades and favouring of high milk output, high yielding dairy cows have to face enormous changes through the gestation-lactation cycles in their productive life. Prior to parturition, metabolism of dairy cows undergoes tremendous alterations, which involve complex feedback and control mechanisms of reproductive hormones (e.g. increase in estrogens, decrease in progesterone), insulin (increase in blood levels, decrease in tissue sensitivity) as well as diminished anabolic with concurrent increased catabolic situation in adipose and muscle tissue (Ingvartsen and Andersen, 2000).

Moreover, during last weeks before parturition, dry matter intake (DMI) decreases, resulting in increasing nutrient and energy deficit (Drackley, 1999; Ingvartsen and Andersen, 2000; Grummer et al., 2004). Onset of lactation enhances this deficit by direction of glucose to mammary gland due to up-regulation of messenger ribonucleic acid (mRNA) encoding for insulin independent glucose transporters (Bell and Bauman, 1997; Komatsu et al., 2005). To cope with this metabolic imbalance, dairy cows show sophisticated regulation mechanisms: increase of body fat mobilisation and hepatic oxidation of fatty acids, increasing gluconeogenesis and ketogenesis in liver as well as diminished utilisation of glucose by muscle and adipose tissue (Trenkle, 1981; Loor et al., 2005; Nafikov and Beitz, 2007; McCarthy et al., 2010). As a sign of this catabolic situation, specific alterations can be found in blood parameters of cows. Blood glucose decreases and stays on low levels and NEFA increase due to enhanced mobilisation of body fat tissue. The product of hepatic fatty acid oxidation acetyl-CoA is not metabolised in the citric acid cycle because of glucose deficiency and therefore induces ketogenesis (Zammit, 1983).

As a function of body fat mobilisation and β-oxidation of fatty acids, blood levels of cholesterol increase from wk 4 ap until wk 13 pp (Graber et al., 2010). Loor et al. (2007) show that during restricted feeding and ketosis, mRNA encoding for enzymes of cholesterol synthesis are decreased. Only 20% of milk cholesterol concentrations are produced in mammary gland, the greater part is produced in liver and transported via blood lipoproteins to mammary gland (Long et al., 1980). Furthermore total bilirubin (tBR) is a hepatic marker for function and integrity of liver cells with extent of hyperbilirubinaemia depending on location of impaired cell function and integrity of cell membranes (Gopinath and Ford, 1972).

Nevertheless, milk yield is increasing steeply with maximum around 5th week pp, whereas DMI

intake has its maximum later on, around 10th week pp (Schröder and Staufenbiel, 2006). During this critical timeframe of lactation in cows, several production diseases can occur: ketosis, displacement of abomasum, infections of uterus or mammary gland, hepatic steatosis and reproductive disorders like delayed ovulation, acyclic oestrus or ovarian cysts (Ingvartsen, 2006; LeBlanc, 2010).

Later on in lactation, during mid and late lactation, energy output (for maintenance, milk production, if pregnant for growth of embryo) is covered or exceeded through DMI and energy intake so that body storages can be refilled. Nevertheless, excessive gain of body fat reserves has to be avoided due to the higher risk of obese cows to develop severe problems during parturition or to develop fatty liver syndrome after parturition (Ingvartsen, 2006; Roche et al., 2009).

Feed restriction (FR) is an appropriate tool for enhancing energy deficit in early lactation. Furthermore milk and blood parameters are altered by FR during early or mid-lactation. Diverging results can be found in studies: a 49% FR for three weeks in mid-lactation resulted in declining milk yield (by -10%) and milk protein content (-5%; Gross et al., 2011), whereas 25% FR reduced milk yield by 12% and milk protein concentration from 3.36% to 3.09% (Guinard-Flament et al., 2007). A 51% FR over 5 days during mid-lactation provoked 22% reduction in milk yield and had no effect on milk composition (Velez and Donkin, 2005).

3. Hepatic gene expression

Several enzymes play important roles in liver metabolism of cows. Hereafter functions of those individual proteins, of which abundances of encoding mRNA were measured, are discussed.

Lipid metabolism

Glycerol-3-phosphate acyltransferase (encoding gene *GPAM*) catalyses the initial step of triacylglycerol synthesis from acyl-CoA (Roy et al., 2006) and acetyl-CoA carboxylase α (encoding gene *ACACA*) is the rate limiting enzyme for *de novo* synthesis of long chain fatty acids in liver and adipose tissue (Mao et al., 2001).

In β-oxidation of fatty acids, carnitine palmitoyltransferase 1A (encoding gene *CPT1A*) is involved as transport enzyme for long chain fatty acids across the mitochondrial membrane by binding to carnitine (van der Leij et al., 2000). Furthermore, acyl-CoA dehydrogenase very long chain (encoding gene *ACADVL*) catalyses the first step of β-oxidation of long chain fatty acids in liver (van Dorland et al., 2009) and enoyl CoA hydratase 1 (encoding gene *ECHS1*) reversibly hydrates unsaturated fatty acyl-CoA derivatives to hydroxy-acyl-CoA compounds (Furuta et al., 1980).

Furthermore, several transcription factors are involved in regulation of fatty acid metabolism in liver: sterol regulatory element binding transcription factor 1 (encoding gene *SREBF1*) up regulates *GPAM* transcription, peroxisome proliferator activated receptor α (*PPARA*) promotes overall fatty acid oxidation and hepatocyte nuclear factor 4A (*HNF4A*) enhances fatty acid oxidation as well as gluconeogenesis (Loor et al., 2005). Eukaryotic translation initiation factor 4B (*EIF4B*) is involved in lipid oxidation due to diminished nutrient availability (Gingras et al., 2001).

During transition period, hepatic mRNAs encoding for these enzymes show higher (*CPT1A*, *ACADVL*, *ECHS1*, *PPARA*, *HNF4A*, *EIF4B*) or lower (*GPAM*, *ACACA*, *SREBF1*) abundances respective to function in lipid catabolism or anabolism (Loor et al., 2005; 2006; 2007).

Protein metabolism

In protein catabolism, tyrosine aminotransferase (*TAT*) catalyses the first step of tyrosine depletion

(Johnson et al., 1973; Dietrich, 1992) and cathepsin L (*CTSL*) being a lysosomal cysteine proteinase is also involved (Stearns et al., 1990; Becker et al., 2010).

Carbohydrate metabolism

During gluconeogenesis, the enzyme pyruvate carboxylase (*PC*) catalyses the irreversible carboxylation of pyruvate to oxaloacetate and cytosolic phosphoenolpyruvate carboxykinase (*PCK1*) as well as mitochondrial form (*PCK2*) metabolise oxaloacetate further to phosphoenolpyruvate and carbon dioxide (Aschenbach et al., 2010). Increased activity of these enzymes could be observed by Greenfield et al. (2000) during early lactation, whereas FR induces only *PC* (Stangassinger and Sallmann, 2004; Velez and Donkin, 2005).

Furthermore, the facilitated glucose transporter member 2 (GLUT2 or solute carrier family 2, member 2; encoding gene *SLC2A2*) enables passive glucose transport into hepatic cytoplasm and can also be found in kidney and small intestine (Zhao et al., 1993; Zhao and Keating, 2007). Insulin receptor (*INSR*), part of tyrosine kinase receptor family, is a glycoprotein in cell membranes that binds insulin, which is essential for promoting glucose utilisation, glycogen and fat synthesis and for diminishing catabolism of fat. After three weeks of 49% FR, abundance of *INSR* mRNA is increased in restricted compared to control cows and in hepatic tissue of cows with fatty liver compared to healthy cows, less mRNA is found (Liu et al., 2010; Gross et al., 2011).

Acetyl-CoA from fatty acid oxidation is metabolised by 3-hydroxy-3-methylglutaryl-coenzyme A synthase 2 (*HMGCS2*) with acetoacetyl-CoA to HMG-CoA, which is an intermediate in cholesterol synthesis and ketogenesis. If oxaloacetate from gluconeogenesis is diminished, HMG-CoA is further metabolised to ketones (van Dorland et al., 2009). In fasting rats, increased activity of hepatic *HMGCS2* is found by Hegardt (1999).

As already mentioned, transcription factor *HNF4A* modulates fatty acid oxidation and furthermore gluconeogenesis by activation of *PCK1* transcription (Loor et al., 2005).

Common hepatic metabolism

Citrate synthase (*CS*) condensates acetyl-CoA and oxaloacetate to citrate, thereby representing the first and pace-making step in citric acid cycle (van Dorland et al., 2009) and high acyl-CoA concentrations from fatty acid oxidation lead to inhibited activity resulting redirection to ketone body synthesis (Ballard et al., 1968).

The cytokine tumour necrosis factor α is involved in systemic inflammation, stimulates the acute phase reaction and *TNFA* mRNA is up regulated by *ad libitum* feeding before parturition (Loor et al., 2006). Furthermore, hepatic mRNA of *TNFA* is up regulated after parturition and is positively correlated with mobilisation of body fat (Loor et al., 2005).

Moreover, mRNA encoding for insulin-like growth factor I (*IGF1*) produces a protein similar to insulin in molecular structure and function. In humans, it exerts its main function in growth and development of different tissues (Bonefeld and Møller, 2011). During lactation in cows, concentrations of IGF1 are lower compared to dry period and lower in cows compared to bulls or calves (Ronge and Blum, 1989).

4. Intravenous glucose tolerance test

4.1. Principles of glucose metabolism in ruminants

In lactating cows, 72 g of glucose are needed to synthesise 1 L milk (Davis and Collier, 1985). Contrary to monogastric animals, in ruminants the ingested carbohydrates are mainly metabolised by ruminal microbes and provided as short chain fatty acids (propionic, butyric and acetic acid).

These are immediately absorbed and metabolised in the liver, whereas only 10% of carbohydrates can be absorbed as glucose in small intestine (Sutton, 1971; Baird et al., 1980). Already during absorption by epithelium, butyrate is metabolised to BHBA (Stangassinger and Giesecke, 1986; Bell and Bauman, 1997). Feeding influences proportions of occurring volatile fatty acids, e.g. feeding less roughage decreases acetic acid and increases the others (Sutton, 1971).

Gluconeogenesis provides almost all required glucose in cows and is regulated by insulin, growth hormone and glucagon (Aschenbach et al., 2010). Substrates are propionic acid as major substrate, lactate, glycerol and amino acids (Stangassinger and Sallmann, 2004; Nafikov and Beitz, 2007). In contrast to monogastric animals, rate of gluconeogenesis is higher after feeding (Young, 1977). Furthermore, gluconeogenesis prevents excess of volatile fatty acids in blood with its negative effects on appetite or acid-base balance (Aschenbach et al., 2010; Stangassinger, 2010).

Glucose derived from gluconeogenesis is distributed to heart and further on to cells of the body. Transport of glucose into body cells is mediated by facilitated transport systems, called solute carrier family 2 (genes *SLC2A* and proteins GLUT), by sodium-dependent transporter systems, the sodium/glucose cotransporters (SGLT) and by the myoinositol transporter 1 (HMIT1; Zhao and Keating, 2007). According to sequence similarities, GLUT1 to GLUT13 are divided in three classes: class 1 containing GLUT1-4, class 2 with the fructose transporter GLUT5, GLUT7, 9 and 11, and class 3 (GLUT6, 8, 10, 12 and HMIT1; Joost and Thorens, 2001).

The glucose transporters have different tissue specificities: GLUT1 appears in human placenta, brain, blood-tissue barriers, in bovine mammary gland and at low levels in bovine erythrocytes, adipose and muscle tissue, GLUT2 in liver, pancreatic β-cells, renal tubular cells and at basolateral membrane of small intestine, GLUT3 in human brain and neurons and at low levels in bovine mammary gland, skeletal muscle and duodenum, GLUT4 in all insulin-sensitive tissues like muscle, heart and adipose tissue, GLUT5 at apical membranes of small intestine, in muscle and adipose tissue, in liver and kidney of lactating cows and at low levels in mammary gland and GLUT7 in liver as microsomal glucose transporter (Gould and Holman, 1993; Zhao et al., 1993; Hocquette and Abe, 2000). Furthermore, regarding their controllability by insulin, GLUT4 is called insulin-sensitive and GLUT1, 2, 3 as well as GLUT5 are insulin-insensitive, ensuring the basal requirements of cells (Hocquette and Abe, 2000). In lactation, high levels of insulin-insensitive transporters can be found in mammary gland whereas during dry period, they are observed mainly in adipose and muscle tissue (Komatsu et al., 2005; Zhao and Keating, 2007).

4.2. Hormonal regulation of glucose homeostasis

In cows, blood glucose is lower (2.5 to 3.3 mmol/L) compared to monogastric animals or calves (3.9 to 6.6 mmol/l; Klinik für Wiederkäuer, 2012). The level of circulating glucose is complex regulated, assuring homeostasis and homeorhesis (Bauman and Currie, 1980; Stangassinger, 2010).

Insulin

Synthesis of insulin occurs in β-islets of pancreas by formation of preproinsulin with acidic A chain, basic B chain and connecting (C-) peptide. After cleavage of amino acids and formation of disulphide bond between A and B chain, proinsulin emerges. Insulin consists after removing of C-peptide of the A chain with 21 amino acids and B chain with 30 amino acids, conjugated by two disulfide bonds between 7^{th} amino acid of A and B chain and between 20^{th} amino acid of A chain and 19^{th} amino acid of B chain. It is stored in granules and very similar amongst mammalians, human insulin differs in only three amino acids from the bovine one (Hsu and Crump, 1989; Hayirli, 2006).

Intracellular calcium increases after glucose or in ruminants volatile fatty acid stimulation and

leads to increased production of ATP. Afterwards ATP-sensitive potassium channel closes and following cellular depolarisation is the final signal for fusion of insulin granules with plasma membrane (Mineo et al., 1990; Hou et al., 2009). Release of insulin is enhanced by nutrient availability (e.g. glucose, arginine, lysine, long chain fatty acids, calcium), gastrointestinal hormones like glucagon or parasympathetic stimuli and diminished by fasting or exercise, gastrointestinal hormones like somatostatin, sympathetic stimuli and prostaglandin $F_{2\alpha}$ (Hayirli, 2006). Insulin secretion after infusion of glucose is biphasic: storages in pancreatic β-cells are cleared within 10 to 20 minutes and if glucose stimulation lasts, synthesis of insulin is started and immediately secreted (60 - 120 minutes; Hove, 1978). Furthermore in dairy cows, insulin levels in blood are lowest in early lactation (Stangassinger, 2006) and increase with progression of lactation (peak lactation 18.5 µU/ml, mid-lactation 19.9 µU/ml; Bonczek et al., 1988) and is lower in high yielding cows (4.96 µU/ml in high yielding cows during first 50 days after parturition and 7.44 µU/ml in low yielding cows; Gong et al., 2002).

Insulin enhances glucose uptake in peripheral tissues, resulting in enhanced lipogenesis and protein anabolism, stimulates synthesis of glycogen in liver and muscle and inhibits hepatic gluconeogenesis (McDowell, 1983; Hayirli, 2006). After acting on receptors, insulin is released from receptors or depleted by cells of liver and kidney. Degradation of insulin involves endocytosis of the insulin-receptor complex, cleavage by insulin-degrading enzyme (proteolysis), protein disulfide isomerase (formerly glutathione insulin transhydrogenase, cleavage of disulfide bonds) and acidic proteinases in lysosomes (Duckworth et al., 1998).

Glucagon

The 29 amino acids containing peptide hormone glucagon is synthesised in α-cells of Langerhans-islets in pancreas via preglucagon and preproglucagon (Hsu and Crump, 1989; Hayirli, 2006). Release of glucagon is stimulated by low blood glucose levels, but also by postprandial high levels of propionate and butyrate, mainly to avoid insulin-induced hypoglycaemia (Brockman, 1978). Glucagon stimulates glycogenolysis in liver and gluconeogenesis from proprionate, amino acids and lactate (McDowell, 1983; Donkin and Armentano, 1995; Hayirli, 2006) and shows weak positive influence on lipolysis (Brockman, 1979). Furthermore, glucagon decreases milk protein concentration and yield and alters milk protein composition towards more glycosylated κ- and α_{S2}-CN and less α_{S2}-CN and α-LA without altering milk yield and other components (Bobe et al., 2003; 2009). Rather than absolute concentration, the ratio of insulin to glucagon influences glucose homoeostasis (McDowell, 1983). After a nadir around d 50 pp, ratio of insulin to glucagon increases with progressing lactation and absolute concentrations of glucagon are higher in early lactation compared to later stages (Herbein et al., 1985; Stangassinger, 2011).

When glucagon binds to its hepatic receptor, it is degraded partly by membrane-associated proteinases and partly after endocytosis in endosomes or lysosomes (Authier and Desbuquois, 1991).

Growth hormone (somatotropin)

The peptide hormone somatotropin is synthesised, stored and pulsatile secreted by somatotrophic cells of anterior pituitary gland, stimulated by growth hormone-releasing factor and inhibited by somatostatin. In contrast to insulin, the 191 amino acid containing hormone shows similarity amongst cows and pigs (90%), but not amongst these animals and humans (35%), even though their receptors are similar to human ones (pig 89%, cow 76% receptor similarity; Buonomo and Baile, 1990; Etherton and Bauman, 1998).

Somatotropin exerts various effects during growth and lactation: enhanced protein synthesis in muscles, enhanced blood flow and secretory activity of mammary gland and its cells, diminished

glucose uptake, lipogenesis and translocation of GLUT4 in adipose tissue and increased hepatic gluconeogenesis. By elevating glucose levels, somatotropin increases milk quantity after peak yield without altering composition (McDowell, 1983; Etherton and Bauman, 1998). Therefore, one molecule of somatotropin dimerises two of its receptors and induces a signalling cascade. Subsequently, somatotropin is degraded by endocytosis and cleaved by proteinases (Etherton and Bauman, 1998; van Kerkhof et al., 2000).

If ruminants are not fed continuously, somatotropin level decreases with every feeding, stays low for several hours and then increases again until next feeding due to stretch receptors in cranial rumen (McDowell, 1983). With proceeding lactation, somatotropin concentration increases but its ratio to insulin decreases during early lactation, directing amino acids to hepatic gluconeogenesis (Stangassinger, 2006). In feed deprivation, levels of growth hormone increase from 2 ng/mL in fed cows to 4 ng/mL at the second day of feed deprivation (Samuelsson et al., 1996).

Somatostatin

The peptide hormone somatostatin is synthesised in δ-islets of pancreas and in posterior pituitary and is highly conserved within vertebrates (Buonomo and Baile, 1990; Hayirli, 2006). Somatostatin occurs in two forms, one with 14 and the other with 28 amino acids, and is found in central nervous system, pancreas and intestine (Buonomo and Baile, 1990). It inhibits secretion of somatotropin, insulin and glucagon and is secreted with increasing blood levels of glucose, amino and fatty acids (Brockman and Greer, 1980; Brockman and Halvorson, 1981). Elevation of somatostatin levels in blood with concurrent insulin infusions increases insulin effects on tissues and therefore glucose clearance (Rose et al., 1997).

Glucocorticoids

The corticosteroids are produced in the cortex of adrenal glands. In the *Zona fasciculata* glucocorticoids such as hydrocortisone are produced, in the *Zona glomerulosa* mineralocorticoids like aldosterone and in the *Zona reticularis* androgens. Synthesis is under control of neuroendocrine hormones of hypothalamus and pituitary gland.

As a response to decreased blood glucose levels, hydrocortisone stimulates insulin secretion, protein catabolism, decreases milk yield and therefore increases gluconeogenesis (Baird, 1981; McDowell, 1983). Furthermore, Exton (1979) showed that glucocorticoids regulate substrate supply to and modulate pathways of hepatic gluconeogenesis (increase of PCK activity) and exert permissive effects on lipolytic and glycogenolytic action of catecholamines and on stimulation of gluconeogenesis by glucagon and epinephrine.

Levels of glucocorticoids increase during starvation, exercise (Exton, 1979; McDowell, 1983) or after temperature or behavioural stress (Trenkle, 1978; McDowell, 1983) and are involved in long term regulation of energy supply (Trenkle, 1981).

Glucocorticoid administration in supraphysiological doses induces hyperglycaemia, hyperinsulinaemia and increases NEFA levels, concurrently decreases insulin-stimulated glucose uptake in muscle cells (insulin resistance, IR) and diminishes hepatic glycogen synthesis (Guillaume-Gentil et al., 1993). This IR is mediated by reduced translocation of GLUT4 in muscle cells (Weinstein et al., 1995). Furthermore, dexamethasone activates a posttranslational degradation mechanism, resulting in decreased GLUT2 abundance with no effect on *SLC2A2* mRNA abundance and reduced half live of the glucose transporter, leading to inhibition of glucose-induced insulin secretion and elevated insulin levels in pancreatic β-cells (Gremlich et al., 1997).

Catecholamines

In adrenal medulla synthesised catecholamines adrenaline and noradrenaline are produced from tyrosine which is converted to dopamine, further to noradrenaline and finally to adrenaline which is stored in chromaffin granules. After release into blood, half time is short and the molecules are degraded by methylation or deamination (Molinoff and Axelrod, 1971). Synthesis and release are regulated by acetylcholine from sympathetic nervous system and glucocorticoids (Edwards and Jones, 1993). Due to various receptors (α_1, α_2, β_1, β_2, β_3) in different tissues, catecholamines exert different effects. Adrenaline shows far higher influence on glucose homeostasis than noradrenaline, it inhibits insulin secretion, promotes glycogenolysis and lipolysis, stimulates glucagon secretion and increases gluconeogenesis leading to increasing blood glucose levels (McDowell, 1983). Under normal conditions, catecholamines are more effective at nerve endings as compared with effects on glucose homeostasis (Exton, 1979). However in stress situations (fight or flight), which can be simulated by infusions of adrenaline, catecholamines show glycogenolytic and lipolytic effects resulting in suppressed insulin secretion or lowering of its effect. Moreover, they promote gluconeogenesis directly and by enhanced glucagon secretion and after end of infusion, insulin levels and glucose utilisation increase steeply (McDowell, 1983). Furthermore, adrenaline distributes glucogenic precursors such as alanin, glycerol and lactate from peripheral tissues to liver and antagonises effect of insulin (Stevenson et al., 1991; Capaldo et al., 1992).

4.3. Insulin resistance

According to Kahn (1978) IR describes the situation of normal levels of insulin producing only a diminished biological response based on either diminished insulin sensitivity or diminished maximal effect of insulin (insulin responsiveness of tissue) or based on both mechanisms. During diminished insulin responsiveness, no biological response can be achieved even with high insulin levels due to alterations at receptor or post-receptor levels, whereas during decreased insulin sensitivity, only dose-response curve is shifted to the right.

Therefore, IR can have various reasons: at pre-receptor level diminished insulin production and/or increased depletion, at receptor level decreased number of INSR and binding affinity and at post-receptor level impaired signalling pathways and translocation of GLUT (Hayirli, 2006).

During late pregnancy and early lactation in dairy cows, glucose is distributed to foetus or mammary gland (Baird, 1981; Bell, 1995; Bell and Bauman, 1997). In this time period dairy cows experience a more or less severe but physiological IR (Stangassinger, 2006).

Nevertheless, no differences can be observed in expression of insulin responsive glucose transporter mRNA (*SLC2A4*) in adipose tissue of lactating and dry cows. Furthermore resistin, an inhibitor of adipocyte differentiation, glucose tolerance and promoter of IR, shows higher levels in adipose tissue and lower levels in mammary gland tissue of lactating cows than in dry cows, suggesting a contribution to inhibition of GLUT4 translocation during lactation (Komatsu et al., 2003).

4.4. Methods for determination of insulin response

For determination of insulin response and IR, various tests derived from human medicine are also conducted in cows: intravenous (and in humans also oral) glucose tolerance tests (ivGTT; McCann and Reimers, 1985b; Bickhardt et al., 1989; Bigner et al., 1996; Chagas et al., 2009), insulin tolerance tests (McCann and Reimers, 1985a; Ohtsuka et al., 2006; Oikawa and Oetzel, 2006; Kerestes et al., 2009), hyperglycaemic (HGC; Sano et al., 1993; Blum et al., 1999; Holtenius et al., 2000) or hyperinsulinaemic euglycaemic clamp (HEC; Dunshea et al., 1995; Andersen et al., 2002).

Furthermore, model estimations can be applied such as the homeostatic model assessment of insulin resistance (HOMA-IR) or the quantitative insulin sensitivity check index (QUICKI) in humans (Radziuk, 2000; Muniyappa et al., 2008), as well as the revised QUICKI, which can also be applied in cows (Holtenius and Holtenius, 2007; Kerestes et al., 2009; Stengarde et al., 2010).

Clamp techniques

The clamp techniques are the gold standard procedures for determination of insulin sensitivity *in vivo* (Katz et al., 2000; Wallace and Matthews, 2002). However they demand more technical equipment and are more time consuming compared to tolerance tests.

During hyperglycaemic clamps (HGCs), blood glucose levels are elevated by adjusting intravenous glucose infusion rates until blood glucose levels are clamped on a hyperglycaemic plateau (steady state) within 60 minutes (2.78 mmol/l above preinfusion values; Sano et al., 1993). Because of the constant blood glucose, infusion rate reflects endogenous insulin secretion whereas infused glucose is metabolised. Therefore function of pancreatic β-cells can be determined by HGC (Sano et al., 1991; Holtenius et al., 2000).

During hyperinsulinaemic euglycaemic clamp (HEC), two infusions are administered: one with insulin, elevating blood level and clamping it on a hyperinsulinaemic level. Infusion of 1 µg insulin per kg BW per hour resulted in fourfold increase of blood insulin levels (Griinari et al., 1997; Mackle et al., 1999). The second infusion provides glucose to avoid hypoglycaemia and maintain normoglycaemic glucose levels. At the time point when steady state is achieved, glucose infusion rates equals glucose uptake by body cells and therefore reflects insulin responsiveness of tissues. For measurement of peripheral IR (at receptor or post-receptor level), HEC is the most appropriate test in human and veterinary medicine (Holtenius et al., 2000; Muniyappa et al., 2008).

Glucose and insulin tolerance test

During ivGTT, insulin sensitivity is indirectly measured (Stangassinger, 2006). A bolus injection of glucose (100 mg per kg BW; Cummins and Sartin, 1987; Roche et al., 2008; 300 mg per kg BW; Grünberg et al., 2011) provokes insulin release from pancreas. Repeated measurements of glucose and insulin levels are done before the injection for basal values and in short intervals for around 120 minutes after injection. These values give information on maximum increase of insulin, half-live and turnover rate of glucose and area under the curve (AUC) of insulin and glucose (Palmquist and Moser, 1981; Opsomer et al., 1999; Murphy et al., 2000; Roche et al., 2008). Glucose tolerance defines the efficiency of homeostatic mechanisms (e.g. cellular uptake, gluconeogenesis, excretion) to restore basal glucose levels (Radziuk, 2000).

Due to lactation and insulin independent partitioning of glucose to mammary gland (Bell and Bauman, 1997), this method provides only marginal insight into insulin responsiveness of peripheral tissue in lactating cows. Nevertheless, advantage of ivGTT is the easy implementation and its non-invasive character, especially in studies with large test numbers.

Instead of glucose a bolus injection of insulin is administered during insulin tolerance tests and decrease of blood glucose levels is measured (Muniyappa et al., 2008). Insulin tolerance tests consume less time compared to ivGTT, blood samples are taken for 15 minutes after injection every 2 minutes. Afterwards, glucose is injected intravenously to avoid hypoglycaemia. Insulin resistance is calculated by logarithmic glucose concentrations (Wallace and Matthews, 2002).

Model estimations

To avoid the time and material consuming clamps and tolerance tests, various calculation models can be applied based on blood levels of glucose, insulin and NEFA. According to Kusenda (2010),

the correlations of HEC to various indices range from 0.44 for HOMA-IR to 0.61 for RQUICKI in lactating cows.

According to Radziuk (2000), HOMA-IR is calculated with fasted basal blood glucose levels and mean of thrice sampled basal insulin concentrations:

$$HOMA - IR = \frac{basal\ glucose\ \left[\frac{mmol}{L}\right] \times basal\ insulin\ \left[\frac{\mu U}{mL}\right]}{22.5} \times 0.5$$

The higher HOMA-IR, the higher is IR and the lower is insulin sensitivity. The denominator of 22.5 derives from the product of normal fasting blood glucose (4.5 mmol/L) and insulin (5 µU/mL) in healthy humans (Muniyappa et al., 2008). For using this index in other subjects, basal levels of the respective animal have to be considered. Furthermore in evaluation of IR, the decal logarithm of HOMA-IR provides better correlations compared to HOMA-IR in humans (Muniyappa et al., 2008).

Holtenius and Holtenius (2007) modified the QUICKI according to Katz et al. (2000) with levels of blood NEFA and evaluated its validity in lactating dairy cows:

$$QUICKI = \left[\lg\left(basal\ insulin\ \frac{\mu U}{mL}\right) + \lg\left(basal\ glucose\ \frac{mg}{dL}\right)\right]^{-1}$$

$$RQUICKI = \left[\lg\left(basal\ insulin\ \frac{\mu U}{mL}\right) + \lg\left(basal\ glucose\ \frac{mg}{dL}\right) + \lg\left(NEFA\ \frac{mmol}{L}\right)\right]^{-1}$$

The lower this index, the lower is insulin sensitivity and the higher IR. The RQUICKI is not influenced by week of lactation and shows good correlation to insulin sensitivity during the first 15 weeks of lactation (Hayirli, 2006; Holtenius and Holtenius, 2007).

5. Hydrocortisone and β-hydroxybutyric acid in milk

Hydrocortisone

As shown before, hydrocortisone is involved in gluconeogenesis by directing amino acids to liver and blood levels increase during fasting or stress, controlled by pituitary adrenocorticotropic hormone and corticotrophin-releasing hormone from hypothalamus.

Measurements of blood levels are inappropriate due to pulsatile secretion of hydrocortisone and elevation during blood sampling procedure (Bitman et al., 1990; Lefcourt et al., 1993). In milk, the situation of hydrocortisone levels in blood is reflected (Bremel and Gangwer, 1978). Milk hydrocortisone is associated with aqueous phase, not with milk fat like other steroid hormones (Butler and Des Bordes, 1980; van der Kolk, 1990). Acute stress situations can only be detected in milk via elevated hydrocortisone levels, if the acute stressor happens within four hours prior to milking (Gwazdauskas et al., 1977; Fox et al., 1981; Verkerk et al., 1998).

Therefore, measurement of hydrocortisone in milk of dairy cows is an appropriate method to determine extent of chronic stress due to automatic milking systems (Abeni et al., 2005), restricted lying behaviour (Fisher et al., 2002) or postpartal hypocalcaemia with downer-cow syndrome (Horst and Jorgensen, 1982; Waage et al., 1984; Forslund et al., 2010).

According to Breves et al. (1980), blood glucose levels are positively (0.18) and blood ketone body levels are negatively (-0.31) correlated to blood hydrocortisone levels. In ketotic cows, hydrocortisone blood levels are lower (9.6 nmol/L) compared to cows with mastitis (23.3 nmol/L) or recumbent animals (96.7 nmol/l; Forslund et al., 2010) and milk levels are higher in early lactation (1.63 nmol/L) compared to later stages (0.69 nmol/l; Schwalm and Tucker, 1978).

Especially in high yielding cows, increased pituitary activity with increased reactivity to corticotrophin-releasing hormone as well as reduced adrenocortical reactivity to adrenocorticotropic hormone is observed (Beerda et al., 2004). Furthermore, milk hydrocortisone levels are negatively correlated to milk protein content (Fukasawa et al., 2008) and milk fat concentration (Schwalm and Tucker, 1978).

β-hydroxybutyric acid

In milk, BHBA is secreted if levels in blood exceed metabolising capacity of cells due to enhanced ketogenesis in liver during metabolic imbalance and excessive mobilisation of body fat. Besides loss of appetite, decreasing milk yield, loss of body condition and excitation in the nervous form of ketosis, ketolactia is one of the signs for clinical and without other signs for subclinical ketosis. This disease occurs in early lactation and risk for ketosis increases with number of lactation. Furthermore, cows with ketosis show hyperketonaemia, hypoglycaemia, increased levels of NEFA as well as fatty liver and loss of liver glycogen (Baird, 1982).

In fasted cows, alimentary ketogenesis (production of BHBA in rumen epithelium) decreases until cessation at 3rd day and hepatic ketogenesis increases (Heitmann et al., 1987). Moreover, milk levels of BHBA are influenced by milking interval and sampling time point during milking (Nielsen et al., 2005b). Due to correlation of blood and milk levels of BHBA (0.66; Enjalbert et al., 2001), threshold values for detecting ketosis in cows can be evaluated with BHBA tests in milk (over 0.6 mmol/l; Nielsen et al., 2005a).

Most of tests are provided as semi quantitative tests in form of strips or powder, most relying on the principle of Rothera test, a reaction of nitroprussid with acetone derived from acetoacetate from BHBA metabolism. Positive Rothera test results in different shades of violet, the more acetone, the darker violet appears.

For quantitative measurements, flow injection analysis (Marstorp et al., 1983) or enzymatic test systems are necessary (Stein and Bässler, 1968). According to Bergmeyer and Bernt (1965), BHBA is metabolised by 3-hydroxybutyrate-dehydrogenase to acetoacetate:

$$hydroxybutyric\ acid + NAD^+ \xrightarrow{3-HBDH} acetoacetate + NADH + H^+$$

$$NADH + H^+ + INT \xrightarrow{NAD-dehydrogenase} NAD^+ + tetrazolium\ chloride$$

(3-HBDH: 3-hydroxybutyrate-dehydrogenase, INT: iodonitrotetrazoliumchlorid)

Afterwards, tetrazolium chloride can be photometrical determined at 492 nm (Willibald, 2011).

III. MATERIALS AND METHODS

1. Animal experiment

1.1. Experimental design and experimental animals

The animal welfare committee of the government of Upper Bavaria, Germany approved this study (AZ 55.2-1-54-2531-110-09) and federal guidelines were followed throughout the experimental period. From August 2009 to January 2011, the animal trial was conducted at the research farm Veitshof of the Technische Universitaet Muenchen in Freising, Germany. Multiparous Holstein-Friesian cows (n = 26), which had been raised at a saxonian dairy farm (AgroProdukt Leubsdorf GmbH, Leubsdorf, Germany), were transported in groups of 4 to 6 animals each to the research farm approximately 4 weeks prior to expected parturition. Cows were selected according to their milk yield and milk protein concentration during lactation in Saxony. According to performance in early lactation (d 23 to 25 pp) at Veitshof, cows were then assigned to four groups: high FCM yield and high milk protein concentration (MP-cows), low FCM yield and low milk protein concentration (mp-cows), high FCM yield and low milk protein concentration (Mp-cows) and low FCM yield and high milk protein concentration (mP-cows). Health status was determined daily and disease was defined as necessary veterinary intervention. In this study retained placenta, ketosis, lameness and mastitis occurred.

The cows were housed at the research farm Veitshof, together with its dairy herd (70 Brown Swiss cows) in a freestall barn with rubber-coated slatted floors and cubicles bedded with straw powder. Prior to parturition, they were moved to a single calving box bedded with barley straw. One day pp they were reintegrated into the herd. From arrival on, cows conceived lactation diet (LD), containing 60% corn silage, 23% grass silage, 4% hay, 12% concentrates and 1% mineral mix (Ingredients: 14% calcium, 10% sodium, 5% phosphor, 5% magnesium; Josera, Kleinheubach, Germany; table 1). The partly mixed ration, calculated for a basis milk yield of 22 kg/d, was delivered once daily at 0700 h and intended to offer *ad libitum* intake (residual feed >5%). Additional concentrates, composited of 18.4% corn gluten, 13.8% turnips molasses chips, 10.0% wheat, 10.0% triticale, 10.0% rape cake, 8.8% maize, 6.0% malt germ, 5% grain distillation residual (ProtiGrain), 5% rape extraction grist, 5% rumen protected rape extraction grist, 3.3% palm corn cake, 2.8% soy extraction grist, 1.0% sodium bicarbonate, 0.99% calcium bicarbonate and 0.40% plant oil (palm coconut) and containing 7 mega joule netto energy lactation (MJ NE_L/kg; Raiffeisen Kraftfutterwerke Sued, Wuerzburg, Germany) were fed in automated feeding stations. Cows received 2 kg of concentrates after parturition and amounts increased by 0.3 kg daily. From d 14 to 100 pp, 6 kg of concentrates were fed, thereafter amounts declined by 0.03 kg per day until end of experiment (d 155 pp). Additional concentrates were fed depending on day of lactation to eliminate effects of performance-related feeding. Nutritional values and composition of the partial-mixed ration were determined by enhanced Weender-analysis performed at the Bavarian State Institute of Agriculture (Landesanstalt fuer Landwirtschaft, LfL), Zentrallabor Grub (Poing, Germany). Cows had free access to fresh drinking water at all times. Milking was conducted in a 2 × 2 tandem milking parlour (GEA WestfaliaSurge GmbH, Boenen, Germany) twice daily at 0420 and 1540 h.

Table 1: Components and nutritional values of lactation diet (LD).

Components, %	LD	Nutritional values, % of dry matter	LD	energetic value, MJ/kg dry matter	LD
corn silage	60.0	**crude ash**	6.3	**metabolisable energy**	11.2
grass silage	23.0	**crude protein**	16.7	**NE_L**	6.8
hay	4.0	**crude fibre**	17.2		
concentrates	12.0	**crude fat**	3.4		
mineral mix	1.0	**non-fibre carbohydrates**	56.4		
straw	0.0	**neutral detergent fibre**	37.4		
dry matter	45.2	**acid detergent fibre**	22.4		
		available crude protein	15.7		
		ruminal nitrogen balance	0.1		

1.2. Body weight, body condition and back-fat-thickness

Every two weeks body weight (BW), body condition score (BCS) and back-fat-thickness (BFT) were measured (figure 1). Weighing was conducted using weighing elements underneath the claw stand (FX1, Texas Trading, Windach, Germany). Estimation of BCS was performed according to a five point scale (1 = meagre, 5 = obese) subdivided in quarter points (Edmonson et al., 1989). Thickness of subcutaneous fat was measured via ultrasound with a 7.5 megahertz probe (Universal Ultrasound, Sonovet 2000, Kretztechnik AG, Tiefenbach, Austria) in an area one handbreadth cranial of ischial tuberosity (Schröder and Staufenbiel, 2006), including skin thickness of 6 mm. The same persons conducted measurements of BCS and BFT at all times.

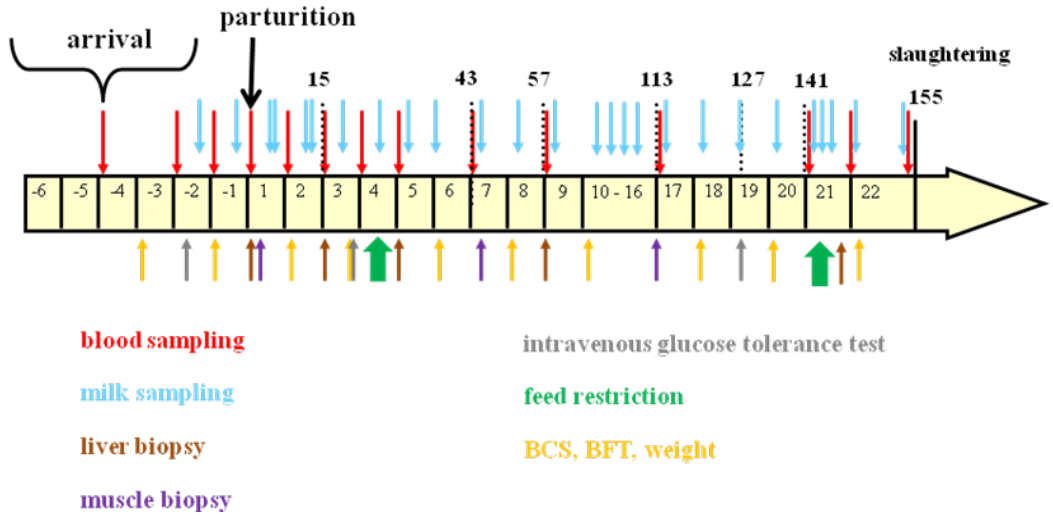

Figure 1: experimental design

Coloured arrows show sampling time points during study. Numbers on pale yellow arrow indicate weeks, numbers above coloured arrows indicate days relative to parturition.

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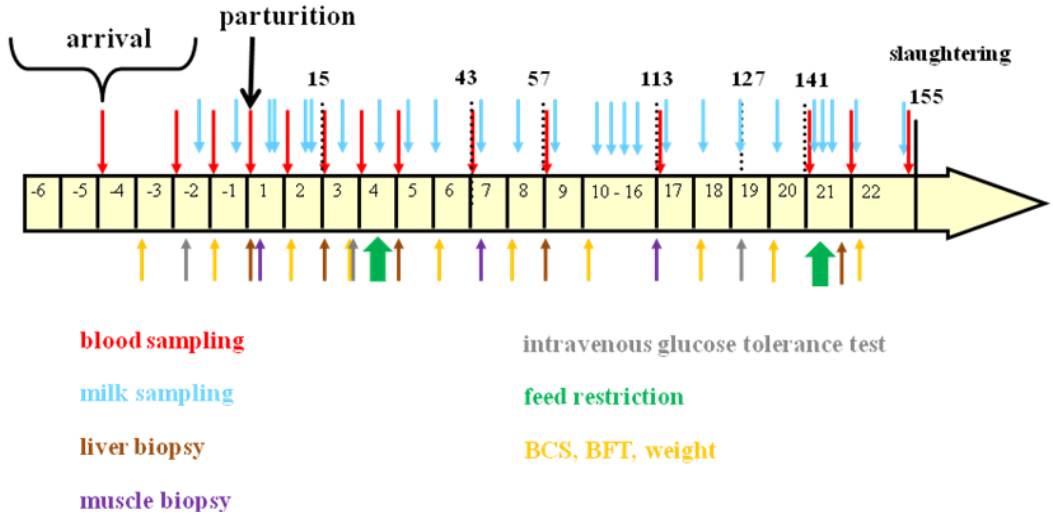

Figure 1: experimental design

Coloured arrows show sampling time points during study. Numbers on pale yellow arrow indicate weeks, numbers above coloured arrows indicate days relative to parturition.

1.3. Collection of milk samples and measurement of milk composition

Milk yield was recorded with electronic milk meters (Metatron P21, GEA WestfaliaSurge GmbH, Boenen, Germany). Approximately 500 mL of milk were obtained as proportional subsamples of total milk during each morning and evening milking depending on total amount of milk and milk flow rate. Milk yield data were stored electronically (DairyPlan C21, GEA WestfaliaSurge GmbH, Boenen, Germany). Milk samples for analysis of milk components and protein fractions were taken on d 1 to 10, 12, 15, 17, 20 to 22, 32, 36, 43, 50, 57, 64, 71, 78, 85, 92, 99, 106, 113, 120, 127, 134, 137 and 155 pp. To obtain a representative sample, aliquots of morning and evening milk were composited according to the morning and evening milk yield and 5 aliquots (one with 50 mL and four with 11 mL) were stored at -20°C until analysis of progesterone, hydrocortisone, BHBA and protein fractions. From d 39 pp on, additional milk samples (11 mL) for analysis of progesterone were taken three days after weekly milk sample, resulting in two milk samples per week for progesterone monitoring. These additional milk samples were also stored at -20°C until analysis. For analysis of milk fat, protein, lactose and urea concentration as well as somatic cell count (SCC) and pH, milk samples were stored with acidiol as preserving agent at 4°C until analysis (maximum seven days) in the laboratories of Milchpruefring Bayern e.V. (Wolnzach, Germany). Analysis of total protein, fat, lactose, urea and pH were done by infrared-spectrophotometry (MilkoScan-FT-6000, VOSS GmbH, Rellingen, Germany). Measurement of SCC was conducted by fluorescence-optical counting (Fossomatic-FC, FOSS GmbH, Rellingen, Germany).

1.4. 100 day performance in previous and current lactation

Performances of milk yield, milk protein yield and milk fat yield in Leubsdorf during previous lactation were estimated from at least three milk yield recordings and calculated at LKV Sachsen (Lichtenwalde, Germany). At Veitshof, milk yield, milk fat yield as well as milk protein yield were summarised from weekly means multiplied by number of days, meaning by 7. Afterwards, milk fat yield and protein yield were divided by milk yield, to achieve milk fat and protein concentrations.

1.5. Collection of blood samples and measurement of metabolites

Blood samples were taken one and two weeks before expected parturition (d 7 and 14 ap) and on first day of wk 1, 2, 3, 4, 7, 9, 17 and 23 (d 1, 8, 15, 22, 43, 57, 113 and 155 pp; figure 1). Jugular veins were punctured after milking and before feeding (0645 h). Two vacuum tubes (9 mL, Vacuette, Greiner Bio-One, Kremsmuenster, Austria) were used for collection of blood per sampling time, one with anticoagulation factor ethylenediamine-tetraacetic acid (EDTA) for blood plasma and the other with clotting assistance for blood serum. Plasma tubes were directly cooled, serum tubes allowed to coagulate at room temperature (RT; maximum 1 h). Afterwards, plasma and serum were separated by centrifugation (2,000 × g, 15 min, 4°C) and three aliquots each (1.5 mL) were stored at -20°C until analysis. Measurement of serum parameters was conducted at Tieraerztliche Hochschule (Hannover, Germany) with an automated clinical chemistry analyzer (ABX Pentra 400, Horiba, Montpellier, France), including daily calibrations and quality controls. Glucose concentrations were determined by hexokinase method (coefficient of variation, CV = 2.3%) and NEFA concentrations by colorimetric enzymatic reactions (CV = 6.2%; both Hoffmann La-Roche, Basel, Switzerland). Measurement of BHBA concentrations was done by spectrophotometric enzymatic analysis (CV = 7.1%; Sigma-Aldrich Diagnostics, Munich, Germany). Serum tBR concentrations were determined with the reaction of Jendrassik and Grof (1938) and serum cholesterol concentrations with an enzymatic colorimetric test (Sigma-Aldrich, Munich, Germany).

1.6. Tissue collection

Hepatic tissue

Liver biopsies were obtained after milking and before feeding (0650 h). Hepatic tissue was sampled at day of parturition within 24 hours after calving (d 1 pp) and at d 15 and 57 pp. On the right side of the cow, an area of 15 cm × 15 cm was shaved, washed and degreased with 70% ethanol prior to disinfection with iodine solution (Vet-Sept®, Albrecht GmbH, Aulendorf, Germany). Skin, subcutaneous and intercostal muscle tissue was desensitized with local anaesthetic (7 mL procaine hydrochloride, Procasel®, Selectavet, Weyarn, Germany). At the intersection of the 11th intercostal space with an imaginary line running from the tuber coxae to the shoulder joint (Pearson and Craig, 1980), a small incision was made through the skin to admit the trocar (12 Gauge, 2.7 mm) for the blind percutaneous needle biopsy (Bard®Magnum™, Covington, USA). Approximately 200 mg of liver tissue were obtained and subdivided. One part was deep-frozen in liquid nitrogen and afterwards stored at -80°C. The other aliquot was transferred into RNA stabilisation solution (1 mL RNAlater®, Applied Biosystems, Darmstadt, Germany), incubated over-night at 4°C and stored at -80°C until mRNA extraction.

Muscle tissue

Muscle biopsies were performed after milking and before feeding (0650 h) at day of parturition within 24 hours after calving (d 1 pp) and at d 43 and 113 pp. An area above the intervertebral space of 3rd and 4th caudal vertebra and an area of 20 cm × 20 cm on the caudal upper distal ischial tuberosity, alternating with every biopsy on the right or the left, were washed, shaved and degreased with 70% ethanol prior to disinfection with iodine solution (Vet-Sept®, Albrecht GmbH, Aulendorf, Germany). An epidural anaesthesia (5 mL) was applied between 3rd and 4th caudal vertebra and subsequently skin of upper leg was desensitized (7 mL, both procaine hydrochloride, Procasel®, Selectavet, Weyarn, Germany). To obtain best possible sterile conditions, hands of surgeon were thoroughly washed, disinfected with ethanol and sterile gloves (Vasco OP Protect®, B. Braun Melsungen AG, Melsungen, Germany) worn throughout surgery. An incision of 2-3 cm through the skin was made one handbreadth distal of ischial tuberosity. Subcutaneous tissue was cut until semitendinous muscle became visible. Two pea-sized samples (approximately 600 mg) of semitendinous muscle were removed using surgical scissors. Samples were cut free of visible connective tissue. One aliquot was immediately deep-frozen in liquid nitrogen and stored at -80°C, another aliquot was transferred into RNA stabilization solution (1 mL RNAlater®, Applied Biosystems, Darmstadt, Germany), incubated over-night at 4°C and stored at -80°C. Muscle tissue was sutured continuously with absorbable multifilamentous suture (Surgicryl® PGA, USP 0/EP 3.5, SMI, St. Vith, Belgium) with a hemicyclic circular needle. Subcutaneous tissue and skin were adapted with single sutures, using non-absorbable multifilamentous suture (Dermafil® Green, USP 5/EP 7, SMI, St. Vith, Belgium) and three-eighths outward cutting needle. A sterile bandage was applied to the biopsied area and was renewed every three days. Furthermore, to prevent bacterial inflammations, cows subcutaneously received 1 mg ceftiofur per kg body weight (Excenel RTU®, Pfizer GmbH, Berlin, Germany) at day of biopsy and at the following day. After 10 days, the skin stitches were removed.

1.7. Feed restrictions in early and mid-lactation

Cows were subjected to a metabolic challenge in early lactation (d 26 to 28 pp) and in mid-lactation (d 141 to 143 pp). From d 23 until d 31 pp and from d 138 until d 146 pp, cows were moved to a tie-stall with eye contact to the herd. They had free access to water. In the first three days (d 23 to 25 pp and d 138 to 140 pp), cows were fed *ad libitum* with LD and additional concentrates (6 kg in FR_1 and 4.5 kg in FR_2) in separated feeding troughs. From d 26 to d 28 pp (FR_1) and d 141 to d 143 pp (FR_2) cows received a restriction diet (RD) containing 56.4% corn

silage, 21.6% grass silage, 3.8% hay, 11.3% concentrates, 0.9% mineral mix and 6.0% straw (table 2) and received no additional concentrates. Fresh feed was mixed daily and cows were fed half of their daily allotment of RD at 0700 h and at 1700 h, respectively. The following three days (d 29 to 31 pp and d 144 to 146 pp, respectively) they were fed again with LD *ad libitum* and 6 or 4.5 kg of additional concentrates. The amount of feed offered and refused was weighed and recorded daily for calculation of DMI.

Table 2: Components and nutritional values of restriction diet (RD).

Components, %	RD	Nutritional values, % of dry matter	RD	energetic value, MJ/kg dry matter	RD
corn silage	56.4	**crude ash**	6.3	**metabolisable energy**	11.0
grass silage	21.6	**crude protein**	15.9	**NE_L**	6.6
hay	3.8	**crude fibre**	18.7		
concentrates	11.3	**crude fat**	3.2		
mineral mix	0.9	**non-fibre carbohydrates**	55.7		
straw	6.0	**neutral detergent fibre**	39.9		
dry matter	47.6	**acid detergent fibre**	23.8		
		available crude protein	15.2		
		ruminal nitrogen balance	-0.2		

Milk samples were collected every day according to the procedure as described before. Blood samples were collected before FR_1 at d 26 pp, each day from d 27 to 29 pp (FR_1) and after FR at d 32 pp. In FR_2, blood samples were collected in the morning before FR at d 141 pp, each day from d 142 to 144 pp and after FR at d 147 pp. Biopsies of hepatic tissue were taken as described before after morning milking at d 29 (FR_1) and 144 pp (FR_2).

1.8. Intravenous glucose tolerance tests

All cows were subjected to an ivGTT 14 days before expected parturition and at d 20 and 127 pp. Cows were moved to a tie-stall with eye contact to the herd at the day prior to ivGTT after evening milking (1800 h). Thereafter they received only hay and drinking water to avoid effects of concentrate feeding on blood glucose levels. After morning milking on test-day, cows were weighed and clinically examined. Only healthy animals were allowed to enter the test. A 10 cm × 10 cm area in the central right jugular sulcus was washed, shaved, degreased with 70% ethanol and disinfected with iodine solution (Vet-Sept®, Albrecht GmbH, Aulendorf, Germany). Afterwards, animals were catheterised into the right jugular vein, using a 12 G (2.7 mm) Braunuele® (length 80 mm, B. Braun Melsungen AG, Melsungen, Germany). The intravenous catheter was fixed with a non-absorbable multifilamentous suture (Dermafil® Green, USP 5/EP 7, SMI, St. Vith, Belgium) stitched singly through the skin. A 50 cm elongation (internal diameter 2.5 mm, volume 2.5 mL, Ho-Med GmbH, Vienna, Austria) with a three-way cock (Discofix®, B. Braun Melsungen AG, Melsungen, Germany) was filled with 0.9% saline solution (B. Braun Melsungen AG, Melsungen, Germany) and applied to the catheter. After assurance of patency, an elastic protective bandage (Bellissimo, Dachau, Germany) provided support to the elongation (Figure 2).

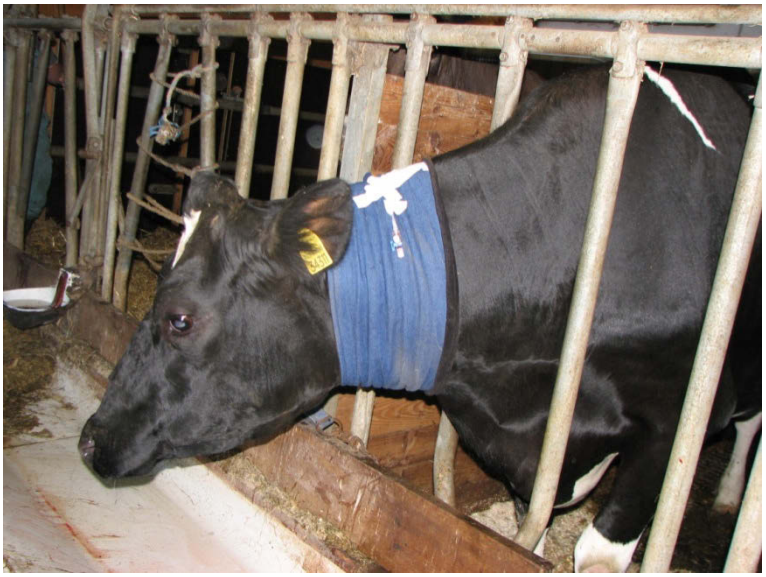

Figure 2: Cow with elastic bandage during ivGTT.

After 30 minutes without handling, blood glucose was measured and blood samples were taken for serum and plasma preparation at 20, 15 and 10 minutes and immediately before infusion of glucose solution. Cows received 1 g D-Glucose per kg metabolic body weight ($BW^{0.75}$) over 4 minutes as 40% D-Glucose solution (B. Braun Melsungen AG, Melsungen, Germany). Afterwards, 50 mL of saline were infused to prevent contamination of blood samples by glucose solution. Each blood sampling procedure began with removal of 5 mL saline and blood and ended with administration of 10 mL saline to maintain patency. Blood samples (1 mL) for glucose measurements were obtained 2, 4, 6, 8, 10, 12, 14, 20, 25, 30, 40, 45, 50, 60, 75, 90, 105 and 120 min after infusion (figure 3).

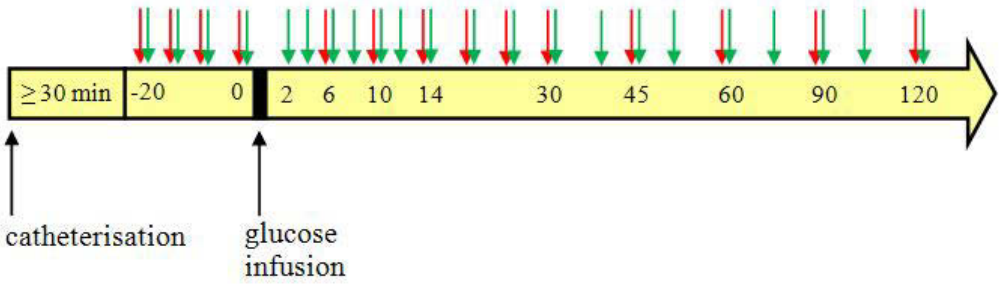

Figure 3: sampling time points during ivGTT

Time is given in minutes relative to glucose infusion. Duration of glucose infusion was 4 minutes. Red arrows show sampling for blood serum and plasma, green arrows show glucose determination.

Blood glucose was determined without delay applying the glucose-dehydrogenase-method with glucometer Contour® (Bayer Vital GmbH, Leverkusen, Germany):

$$glucose + NAD \xrightarrow{glucose-dehydrogenase} gluconolactone + NADH_2$$

At time points 6, 10, 14, 20, 25, 30, 45, 60, 90 and 120 minutes post infusion, additional samples

for blood plasma and serum separation were collected in two 10 mL tubes (Sarstedt, Nuembrecht, Germany). Tubes contained anticoagulation factor (200 µL EDTA) or beads and clotting assistance to obtain blood plasma or serum, respectively. Plasma tubes were immediately placed on ice, serum tubes were stored at RT for coagulation (maximum 1 h) and then stored at 4°C. Afterwards, plasma and serum were separated by centrifugation (2,000 × g, 15 min, 4°C) and three aliquots (1.5 mL) of each were stored at -20°C until analysis. After end of measurements and blood collection, stitch and catheter were removed and the cow was released into the herd.

Analysis of insulin in blood plasma was conducted at the endocrinological laboratory of Tieraerztliche Hochschule Hannover (Hannover, Germany), using an insulin radioimmunoassay (IM3210, Immunotech, Beckman Coulter, Brea, USA). The intra-assay CV was 7.6% and the inter-assay CV 10.7%. Values below 3.2 µU/mL were not clearly distinguished, so for further calculations, they were defined as 2.0 µU/mL.

Model estimations of insulin resistance were calculated as follows:

$$HOMA - IR = \frac{basal\ glucose\ \left[\frac{mmol}{L}\right] \times basal\ insulin\ \left[\frac{\mu U}{mL}\right]}{22.5} \times 0.5$$

$$QUICKI = \left[\log\left(basal\ insulin\ \frac{\mu U}{mL}\right) + \log\left(basal\ glucose\ \frac{mg}{dL}\right)\right]^{-1}$$

1.9. Slaughter and tissue collection

Cows were slaughtered around d 155 pp and at ovarian cycle d 12. All cows were synchronised by implanting the intravaginal progesterone device CIDR® (Pfizer GmbH, Berlin, Germany) three weeks prior to slaughtering. Progesterone device was removed two weeks before slaughtering, and 2 mL sodium-cloprostenol were applied intramuscularly (Estrumate®, Intervet Deutschland GmbH, Unterschleißheim, Germany). At d 155 pp, cows were brought to the slaughterhouse of LfL in Grub, Germany (veterinary control number BY-ES 101). According to animal welfare guidelines, animals were numbed with a captive bolt stunner and afterwards exsanguinated. Approximately 5 g of each of the following tissues were obtained: skin, mammary gland, tongue, lungs, heart, liver, spleen, kidney, adrenal gland, muscle, fatty tissue, rumen, abomasum, small intestine, caecum, colon, mesenteric lymph node, cerebrum and pituitary. Tissues were divided in four parts, three were deep-frozen in liquid nitrogen and stored at -80°C and the fourth one was incubated over-night in 1 mL RNA stabilization solution (RNAlater®, Applied Biosystems, Darmstadt, Germany) and afterwards stored at -80°C until mRNA extraction.

2. Analytical methods

2.1. Extraction, transcription and real time qPCR of hepatic mRNA

Liver tissue (50 mg) stored in RNAlater was added to tubes containing ceramic beads (400 mg, Matrix-Green, MP Biomedicals Europe, Illkirch, France). Messenger RNA was extracted using peqGOLD TriFast® (Peqlab, Erlangen, Germany) in a one-step liquid-phase extraction according to the manufacturer`s protocol. TriFast (1 mL) containing phenol and guanidine thiocyanate was added to liver tissue and beads. The mixture was homogenised two times for 20 seconds (FastPrep®-24, 4 m/s, MP Biomedicals Europe, Illkirch, France). Between the homogenisation steps, sample tubes were placed on ice for 30 seconds. Incubation at RT (5 min) allowed the dissociation of nucleotide-complexes. Chloroform (200 µL, Carl Roth GmbH & Co KG, Karlsruhe, Germany) was added and tubes were shaken thoroughly (Vortex mixer classic, Velp scientifica srl, Usmate, Italy), followed by another incubation (10 min) at RT. Afterwards, tubes were centrifugated (15 min, 4°C, 12,000 × g, Centrifuge 5415 R, Eppendorf AG, Hamburg,

Germany), resulting in different phases within the tube: at the ground tissue rests and ceramic beads, in the middle DNA in red phenol-chloroform-phase, a small white interphase with proteins and DNA and on top RNA in achromatic aqueous solution. To precipitate the RNA, only the upper phase was transferred into new tubes and 2-propanol (500 µL, Scharlau, Barcelona, Spain) was added. Subsequently, tubes were mixed and incubated on ice (15 min). After centrifugation (10 min, 4°C, 12,000 × g) the precipitated RNA was attached to the bottom of the tube. Tubes were placed on ice, the supernatant was removed and the pellet was washed twice in 1 mL of 75% ethanol (Merck KGaA, Darmstadt, Germany). Afterwards, the RNA pellet was dried and then dissolved in sterile RNase-free water (50 µL, Quiagen, Hilden, Germany). RNA quality and quantity were determined by spectrophotometry (BioPhotometer, Eppendorf, Hamburg). Reverse transcription to complementary desoxyribonucleic acid (cDNA) was conducted with constant amounts of 1 µg RNA and with the following reverse transcription master mix: 12 µL 5 × Buffer (Promega, Mannheim, Germany), 3 µL Random Hexamer Primers (50 mM; Invitrogen, Carlsbad, USA), 3 µL dNTP Mix (10 mM; Fermentas, St. Leon-Rot, Germany) and 200U of MMLV-H- reverse transcriptase (Moloney Murine Leukemia Virus Reverse Transcriptase, RNAse H Minus, Promega, Regensburg, Germany). According to the manufacturer, reaction was carried out in a 60-µL volume using a polymerase chain reaction (PCR) thermocycler (Biometra, Goettingen, Germany). Successive incubations at 21°C for 10 min and 48°C for 50 min, finishing with enzyme inactivation at 90°C for 2 min were performed. Reverse transcription products were stored at -20°C.

Prior to real time quantitative PCR (RT-qPCR), gene sequences of candidate genes were obtained from the gene bank of the National Centre for Biotechnology Information (NCBI) for primer design. Exon-spanning primer sequences were designed using NCBI primer tool except for those previously published for *HNF4A* (Loor et al., 2005), *PPARA* and *PPARG* (Sigl et al., 2010), and *SREBF1* (van Dorland et al., 2009). Synthesis of primers was done at Eurofins MWG (Ebersberg, Germany). Primer sequences, accession numbers and product lengths for each gene are listed in Table 3.

Table 3: Primer sequences, accession numbers and product lengths.

Function	Gene	Sequence 5‘-3‘	GenBank accession no.	Length [bp]
Reference genes	*ACTB* for	AACTCCATCATGAAG TGTGAC	AY141970	202
	ACTB rev	GATCCACATCTGCTG GAAGG		
	GAPD for	GTCTTCACTACCATG GAGAAGG	U85042	197
	GAPD rev	TCATGGATGACCTTG GCCAG		
	H3F3A for	ACTTGCTACAAAAGC CGCTC	BT025472	183
	H3F3A rev	ACTTGCCTCCTGCAA AGCAC		
Protein metabolism	*CTSL* for	CACTGGTGCTCTTGA AGGACA	BC102312	177
	CTSL rev	TAAGATTCCTCTGAG TCCAGGC		
	TAT for	ACCCTTGTGGGTCAG TGTTC	BT021798	165
	TAT rev	ACAGGATGGGGACTT TGCTG		
Carbo- hydrate metabolism	*PC* for	ATCTCCTACACGGGT GACGT	NM_177 946	214
	PC rev	TGTCGTGGGTGTGGA		

Function	Gene	Sequence 5‘-3‘	GenBank accession no.	Length [bp]
	PCK1 for	TGTGCA TTTGGCGTCGCTCCG GGAAC	AY 145503	244
	PCK1 rev	GGCACTGGCTGGCTG GAGTG		
	PCK2 for	TACGAGGCCTTCAAC TGGCGT	XM_58 3200	365
	PCK2 rev	AGATCCAAGGCGCCT TCCTTA		
Glucose transport	*SLC2A2* for	GGACCTTGGTTTTGG CTGTC	BC149324	275
	SLC2A2 rev	CACAGACAGGGACCA GAACA		
Hormone receptor	*INSR* for	CCAACTGCTCAGTCA TCGAA	XM_002688832	164
	INSR rev	GTTGGGGAACAAGTC CTTCA		
Keto-genesis	*HMGCS2* for	CGCCCGGCGTCCCGT TTAAA	NM_001045883	294
	HMGCS2 rev	GGACCCGCCACACTT TCGGTC		
Translation	*EIF4B* for	CCACGCCGGGACATG GATCG	NM_001035028	164
	EIF4B rev	TCATAGCGGTCCCCG CCTCC		
Trans-cription regulation	*HNF4A* for	GCATGGCCAAGATCG ACAA	AY318752	73
	HNF4A rev	TGGGCATGAGGTGCT TCAC		
	PPARA for	GGATGTCCCATAACG CGATTCG	BT020756	235
	PPARA rev	TCGTGGATGACGAAA GGCGG		
	SREBF1 for	CCAGCTGACAGCTCC ATTGA	NM_001113302	67
	SREBF1 rev	TGCGCGCCACAAGGA		
	PPARG for	CTCCAAGAGTACCAA AGTGCAATC	NM_181024	198
	PPARG rev	CCGGAAGAAACCCTT GCATC		
Final metabolism	*CS* for	TGGACATGATGTATG GTGG	BC114138	217
	CS rev	AGCCAAGATACCTGT TCCTC		
Anabolism	*IGF1* for	CATCCTCCTCGCATCT CTTC	NM_001077828	239
	IGF1 rev	CTCCAGCCTCCTCAG ATCAC		

ACACA = acetyl-CoA carboxylase α; *ACADVL* = acetyl-CoA dehydrogenase, very long chain; *ACTB* = actin beta; *CPT1A* = carnitinepalmitoyltransferase; *CS* = citrate synthase; *CTSL* = cathepsin L; *ECHS1* = enoyl CoA hydratase 1; *EIF4B* = eukaryotic translation initiation factor 4B; *GAPD* = glyceraldehyde-3-phosphate dehydrogenase; *GPAM* = glycerol-3-phosphate acyltransferase; *H3F3A* = H3 histone family 3A; *HMGCS2* = 3-hydroxy-3-methylglutary-coenzyme A synthase 2; *HNF4A* = hepatocyte nuclear factor-4A; *IGF1* = insulin-like growth factor 1; *INSR* = insulin receptor; *PC* = pyruvate carboxylase; *PCK1* = phosphoenolpyruvate carboxylase, cytosolic; *PCK2* = phosphoenolpyruvate carboxylase, mitochondrial; *PPARA* = peroxisome proliferator activated receptor-α; *PPARG* = peroxisome proliferator activated receptor-γ; *SLC2A2* = facilitated glucose

transporter, member 2; *SREBF1* = sterol regulatory element binding transcription factor 1; *TAT* = tyrosine aminotransferase

MESA Green qPCR MasterMix plus for SYBR® Assay w/fluorescein (Eurogentec, Cologne, Germany) was used and a standard protocol recommended by the manufacturer was followed to perform RT-qPCR. All components were mixed in the reaction wells of semi-skirted twin.tec PCR plate 96 (Eppendorf, Hamburg, Germany). The mastermix contained 7.5 µL 2 × MESA Green qPCR MasterMix, 1.5 µL forward primer (10 pmol/µL), 1.5 µL reverse primer (10 pmol/µL), and 3.0 µL RNase free water. Per well, 13.5 µL mastermix and 1.5 µL cDNA were added. The plate was sealed, placed in the iQ5 Cycler (Bio-Rad, Munich, Germany), and the following PCR protocol was started: denaturating step (95°C, 5 min), cycling program (95°C, 3 s; primer specific annealing temperature, 60 s) and melting curve analysis.

Genes were selected as reference genes using GenEx Pro Software Version 5.2.7.44 (MultiD Analyses, Gothenburg, Sweden). The mean of the three selected housekeeping genes actin beta (*ACTB*), glyceraldehyde-3-phosphate acyltransferase (*GAPD*) and H3 histone family 3A (*H3F3A*) was calculated for the reference gene index and used for normalisation. Quantitative cycles (Cq) were calculated by Bio-Rad iQ5 Optical System Software Version 2.1 with the analysis mode 'PCR base line substracted curve fit'.

The ΔCq-values were calculated as $\Delta Cq = Cq_{target\ gene} - \text{mean } Cq_{refence\ genes}$ and subtracted from the arbitrary value of 15 (15 - ΔCq) to avoid negative digits and to allow a relative comparison between two time points. High ΔCq-values show high transcript abundance and increase of one ΔCq represents two-fold increase of mRNA transcripts (Livak and Schmittgen, 2001).

Data of genes from all animals and at all time points were excluded from statistical analysis, if more than 5 amplifications in one of the groups failed. Furthermore, data of animals at one time point were excluded, if more than 5 RT-qPCR runs of different genes did not work.

2.2. Analysis of major proteins in skim milk samples

Protein fractions in skim milk were analysed at first day of wk 2, 3, 4, 6, 7, 8, 9, 10, 12, 14, 16, 17, 18, 20 and 22 pp, during FR at d 25, 28, 31, 140, 143 and 146 pp and at days of ivGTT (d 20 and 127 pp; figure 4).

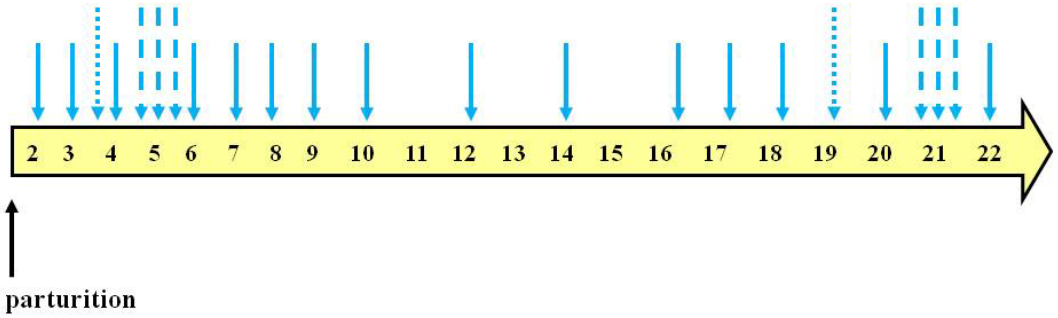

Figure 4: sampling time points for protein fractions

Arrows indicate day of sampling relative to parturition. Dotted arrows show days of ivGTT, dashed arrows the last day before FR, last day of FR and three days after FR.

Analyses of α-LA, β-LG, α-, β-, and κ-CN were conducted using a microfluidic electrophoresis (Agilent 2100 Bioanalyzer system, Agilent Technologies, Waldbronn, Germany). The provided Protein 80 kit (Agilent Technologies, Waldbronn, Germany) contained chips and all reagents. For

measurement necessary reagents were gel matrix, dye concentrate, sample buffer with upper (95 kDa) and lower (1.6 kDa) marker and molecular mass ladder. According to the manufacturer's protocol, gel-dye mix was prepared by spin filtration (2,500 × g, 15 minutes) of 650 µL gel matrix and addition of 25 µL dye concentrate. Destaining solution was obtained by solely spin filtration of gel matrix (650 µL). A reducing denaturing solution was prepared by addition of 1 mol dithiothreitol solution (7 µL, 3.5%) to 200 µL sample buffer. After thawing of milk samples (37°C, 20 min), skim milk was obtained by centrifugation (3,000 × g, 4°C, 15 minutes) and diluted in deionised water (1:20). Protein mix (200 µg/mL of each α-LA, β-LG, α-, β-, κ-CN; all Sigma-Aldrich Chemie GmbH, Munich, Germany), milk samples and ladder were prepared according to the manual: ladder (6 µL) or sample with denaturing solution (4 µL and 2 µL, respectively) were placed in a 0.5 mL tube and heated (95°C, 5 minutes). After cooling, tubes were shortly centrifuged and 84 µL of deionised water were added to receive total volume of 90 µL. For purposes of variance calculation and to ensure comparability between chips, the protein mix was measured on every chip.

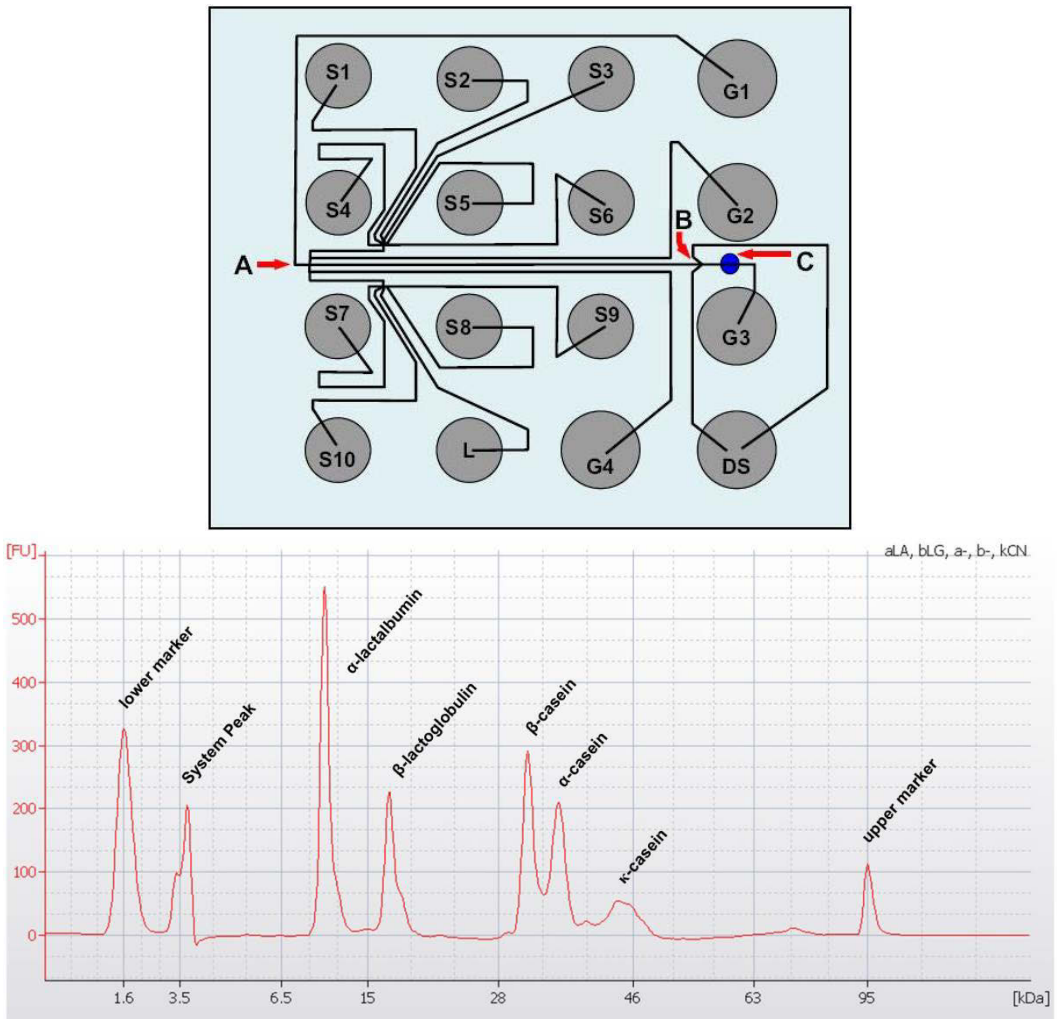

Figure 5: Layout of wells and channels of typical microfluidic chip in upper part (adopted from Anema and Lloyd, 1999) and electropherogram of standard proteins in lower part.

G1-G4 denotes wells to be filled with 12 µL gel-dye mix, DS well with 12 µL destaining solution, L well with 6 µL of prepared ladder, S1-S9 wells for 6 µL of prepared samples and S10 well for 6 µL of prepared protein mix. Arrows highlight the points of separation (A), destaining (B) and detection (C).

Figure 5 (Anema and Lloyd, 1999) shows the layout of a microfluidic chip and the electropherogram of the standard proteins. The chip was primed by pushing 12 µL gel-dye mix from well G1 into the channels of the chip with air pressure produced by a syringe. Afterwards the wells were filled with either gel-dye mix (12 µL, G2 – G4), destaining solution (12 µL, DS), prepared ladder (6 µL, L) or prepared samples (6 µL, S1 – S9) respectively prepared protein mix (S10). The points of separation (A), destaining (B) and detection (C) are highlighted with arrows.

After the chip was loaded into the Bioanalyzer and the electrodes were inserted into each well by closing the lid, measurement was started immediately. Electrophoresis and simultaneous automatic integration took approximately 30 minutes. For standardisation of peak area and migration time, the upper and lower markers were used as internal standards. For standardisation of molecular mass the molecular mass of ladder proteins were used. If required, automatic integration could be corrected manually by using Agilent 2100 Expert software. The chips were discarded after completed runs and electrodes were cleaned after every run with the provided cleaning chip and fresh deionised water.

2.3. Enzyme immuno assay of progesterone in skim milk

Beginning d 8 pp, ovarian activity was monitored by twice weekly measurements of progesterone in skim milk with a competitive enzyme immuno assay (EIA) described by Meyer et al. (1986) with modifications. Day of first ovulation was defined as three days before progesterone concentrations were for the first time greater or equal 0.5 ng/mL.

Microtiterplates (F96 MikroWell Plates Maxisorb, Nunc, Thermo Fisher Scientific, Langenselbold, Germany) were coated with 1 µg/well affinity purified antibody against rat IgG raised in goats (Physiology Weihenstephan, Freising, Germany) and residual binding sites blocked with 200 µL/well 0.1% BSA in PBST (phosphate buffered saline with Tween-20), containing 7.12 g/L $Na_2HPO_4 \times 2\ H_2O$, 8.5 g/L NaCl (both Merck KGaA, Darmstadt, Germany), 1 g/L BSA (Serva GmbH, Heidelberg, Germany), deionised water and HCl until pH 7.5 (Merck KGaA, Darmstadt, Germany). Microtiterplates were stored at -20°C until day of measurement. Monoclonal anti-progesterone antibodies produced in rat (Sigma-Aldrich Chemie GmbH, Munich, Germany) were used as second antibodies, diluted 1:3000 in PBST. Horseradish peroxidase (Roche Applied Science, Mannheim, Germany) was coupled to Progesterone-3CMO (Steraloids, Newport, USA) and diluted 1:3000 in PBST. Standard samples (0.2, 0.4, 0.8, 1.6, 3.2, 6.3 and 12.5 ng/mL) and control samples (0.5, 1.0 and 2.0 ng/mL) were obtained by spiking progesterone-free (around d 10 pp) skim milk with considered concentrations of progesterone (Sigma-Aldrich Chemie GmbH, Munich, Germany).

Milk samples were thawed (37°C, 20 min) and skimmed by centrifugation (3,000 × g, 4°C, 15 minutes). Competitive binding reaction was performed with duplicate 20 µL skim milk samples, standard or control samples and 100 µL of each enzyme-bound progesterone and anti-progesterone-antibody per well during 2 hours at RT and darkness. Afterwards, plates were washed and 20 minutes at RT in darkness incubated with 150 µL/well substrate, containing equally substrate A (1 g/L $CH_6N_2O_3$, 18 g/L $Na_2HPO_4 \times 2\ H_2O$, 10.3 g/L $C_6H_8O_7 \times\ H_2O$, pH 5) and substrate B (500 mg/L tetramethylbenzidine (TMB), 40 mL/L DMSO, 10.3 g/L $C_6H_8O_7 \times H_2O$, pH 2.4). TMB and DMSO were provided from Sigma-Aldrich Chemie GmbH, Munich, Germany and all other chemicals from Merck KGaA, Darmstadt, Germany. Horseradish peroxidase cleaves hydrogen peroxide in OH-radicals, which react with amino-group of TMB, resulting in blue TMB-cations. Enzymatic reaction was stopped with 50 µL/well 2 M H_2SO_4, followed by protonation of TMP-radical and therefore colour change to yellow. Extinction was photometric measured at 450 nm (Sunrise microplate reader) and standard curves as well as

concentrations of progesterone in skim milk were calculated with Magellan data analysis software (both Tecan Group Ltd, Maennedorf, Switzerland). Variance between assays was 13.5%, within the assays 5.7%. Values within the physiological range of 0.2 to 3.0 ng/mL were distinguishly quantified.

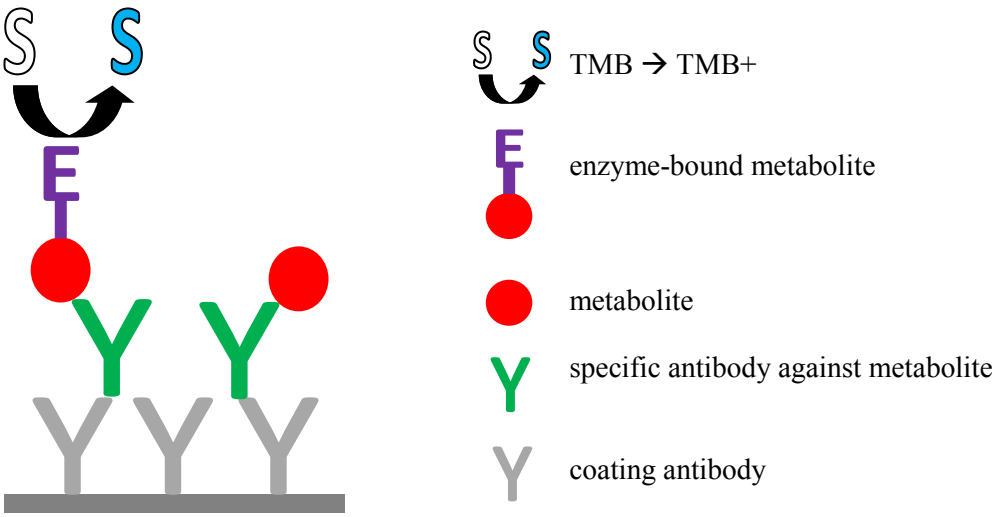

Figure 6: competitive EIA with second antibody-technique.

2.4. Enzyme immuno assay of hydrocortisone in skim milk

Measurement of hydrocortisone in skim milk was performed by a competitive EIA (Sauerwein et al., 1991) with additional modifications of Physiology Weihenstephan. Coating antibody was raised in goats against rabbit IgG (Sigma-Aldrich GmbH, Munich, Germany) and second antibody (C_1Pool_2 Weihenstephan) in rabbits against hydrocortisone-21-hemisuccinate-BSA (Sigma-Aldrich GmbH, Munich, Germany). After affinity purification, antibodies were solved in ammonium sulphate. Like described before, 96-well-microtiterplates were activated with 1 µg/well of polyclonal antibody against rabbit IgG and residual binding sites were blocked with BSA in PBST. Microtiterplates were stored at -20°C until use. Competitive agent was hydrocortisone-21-glucuronide (Steraloids, Newport, USA) labelled with horseradish peroxidase (1:12000, Roche Applied Science, Mannheim, Germany).

Skim milk obtained by centrifugation (3,000 × g, 4°C, 15 minutes) of bulk tank milk was used to create hydrocortisone standards and control samples. Endogenous hydrocortisone was removed by treatment with activated charcoal. Therefore 0.7 mg charcoal per 100 mL skim milk were added and shaken horizontally (30 min, 1500 min^{-1}). After centrifugation (3,000 × g, 4°C, 15 min), desired amounts of hydrocortisone (0.1 to 34.5 nmol/L and 1.0, 2.0, 5.0 nmol/L; Sigma-Aldrich Chemie GmbH, Munich, Germany) were added to skim milk.

Experimental milk samples were thawed (37°C, 20 min) and skimmed by centrifugation (3,000 × g, 4°C, 15 min). Duplicate skim milk samples or standards (10 µL each) were pipetted to respective coated EIA wells and enzyme-conjugated hydrocortisone (100 µL, diluted 1:12,000) and specific antibody C_1Pool_2 (100 µL, diluted 1:90,000) were added. After over-night incubation (4°C), microtiterplates were washed with PBST. Above described substrate solution (150 µL) was added. After incubation in the dark (RT, 40 min), reaction was stopped by addition of sulphuric acid (50 µL). Extinction was determined photometrically at 450 nm (Sunrise microplate reader) and standard curves as well as concentrations of hydrocortisone in skim milk samples were

calculated with Magellan data analysis software (both Tecan Group Ltd, Maennedorf, Switzerland).

Interassay variance was 12.6% and intraassay variance 6.7%. Values within the range of 1.0 to 10.0 nmol/L were well distinguished and quantified.

2.5. Analysis of β-hydroxybutyrate in skim milk

BHBA was measured in skim milk samples at the day before, the last day of and three days after FRs: d 25, 28, 31, 140, 143 and 146 pp (Willibald, 2011) using a BHBA test kit (R-Biopharm AG, Darmstadt, Germany). To create a standard curve, skim milk was treated with activated charcoal and BHBA (R-Biopharm AG, Darmstadt, Germany) added in concentrations of 0.0, 6.5, 13.0, 19.5, 26.0, 39.0, 52.0 and 78.0 mg/L. By addition of 39.0, 52.0 and 78.0 mg/L BHBA control samples for purposes of variance calculation were created. Frozen experimental milk samples were thawed in a water bath (37°C, 20 min) and skimmed by centrifugation (3,000 × g, 4°C, 15 min). To eliminate protein-derived adulterations of measurements, Carrez-precipitation of proteins was carried out with 250 µL each of Carrez-solution I (zinc sulphate, Sigma Aldrich Chemie GmbH, Munich, Germany) and II (potassium ferrocyanide, Merck KGaA, Darmstadt, Germany) to skim milk (2 mL). Sodium hydroxide was added until pH 7.5 to 8.5 was reached. After filtration, BHBA was measured with a photometric test system on 96-well microtiterplates. Chemicals were provided with BHBA test kit. In every well were pipetted: 60 µL solution 1 (potassium phosphate/triethanolamine buffer, Triton X-100), 20 µL solution 2 (diaphorase and nicotinamide adenine dinucleotide, NAD), 20 µL solution 3 (iodnitrotetrazolium chloride), 10 µL sample and 190 µL water. Reaction was initiated when 5 µL of 3-hydroxybutyrate-dehydrogenase (solution 4) were added. After incubation (RT, 20 min), extinction was measured at 492 nm (Sunrise microplate reader) and BHBA concentration in standard samples as well as in experimental samples were calculated with Magellan data analysis software (both Tecan Group Ltd, Männedorf, Switzerland). Variance within the assay was 12.2% and between assays 36.6%.

Therefore, all samples were multiplied with a correction factor (CF) derived from measured concentrations and known concentrations of control samples:

$$CF = \left(\frac{meas.\ BHBA\ (control\ 39)}{39} + \frac{meas.\ BHBA\ (control\ 52)}{52} + \frac{meas.\ BHBA\ (control\ 78)}{78} \right) \times \frac{1}{3}$$

3. Statistical analysis

Statistical analysis was conducted using REML in the MIXED procedure in SAS (SAS SAS, 2002). Standard model for a repeated measurements experiment is:

$$Y_{ijk} = \mu + group_i + d_k + \text{cow}_j(group_i) + (group_i \times d_k) + e_{ijk}$$

Y_{ijk}: parameter of cow j in group i at time k.
μ: overall mean
$group_i$: fixed effect of group i
d_k: fixed effect of day in lactation k
$\text{cow}_j(group_i)$: random effect of cow j in group i
$group_i \times d_k$: fixed interaction effect of group i with time k
e_{ijk}: random error at time k on cow j in group i

As measurements of different animals were independent, covariance structure referred to variances at different time points and to correlation between measurements of the same animal. This

correlation consists firstly of the fact that two measurements of the same animal are correlated just because it is the same animal (variation between animals, RANDOM statement) and secondly it consists of covariation within the same animal (measurements close in time tend to be more highly correlated than those far apart, REPEATED statement). For every parameter, three covariance structures were evaluated: unstructured, compound symmetry or autoregressive order one. Used was the covariance structure that was closest to zero in the Akaike information criterion or Sawa's Bayesian information criterion (Littell et al., 1998). Day of lactation and group as well as their interaction (day × group) were used as fixed effects, whereas cow within treatment was the random effect. Furthermore, cow was the repeated subject. PDIFF function was used to determine the differences between treatments. Data was considered to differ significantly at $P < 0.05$.
Specific calculations or statistic calculations differing from above mentioned approach are explained below.

3.1. Milk parameters during experimental period

Energy balance (EB) was calculated using the formula $EB = (DMI\ diet \times NE_L\ diet) + (DMI\ concentrates\ \times\ NE_L concentrates) - (0.293 \times body\ weight^{0.75} - [(0.38 \times milk\ fat\ concentration) - (0.21 \times milk\ protein\ concentration) + 0.95] \times milk\ yield$ as described by Kamphues et al. (2004). ECM was calculated with the formula $ECM = (milk\ yield\ \times 0.327) + (fat\ yield\ \times 12.86) + (protein\ yield \times 7.65)$ and FCM with $FCM = (milk\ yield\ \times 0.4) + (fat\ yield\ \times 15)$.

Prior to statistical analysis, repeated end point measurements (daily milk yield, milk composition and hydrocortisone) were pooled to weekly means. Days 21, 23 to 31, 127 and 138 to 146 pp were excluded from calculation of weekly means because FR and ivGTT were conducted at these days. For better illustration, milk parameters were subsumed to 6 time periods: d 1 to 22 pp (period 1), d 33 to 56 pp (period 2), d 57 to 84 pp (period 3), d 85 to 112 pp (period 4), d 113 to 134 pp (period 5) and d 148 to 155 pp (period 6). Calculation of statistical differences were performed using REML in the MIXED procedure in SAS. To conserve the effect of different intervals between measurements, SAS received the first day of each period as day of interest (e.g. 1 for period 1 or 85 for period 4).

3.2. Feed restrictions in early and mid-lactation

Repeated end point measurements of the last day before, the last day during and the last day after restricted feeding were compared within groups for milk parameters including hydrocortisone and β-hydroxybutyrate (d 25, 28 and 31 pp as well as d 140, 143 and 146 pp), blood parameters (d 26, 29 and 32 pp as well as d 141, 144 and 147 pp) and for feed intake and EB (d 25, 28 and 31 pp as well as d 140, 143 and 146 pp) using REML in the mixed procedure in SAS, like mentioned above. Differences between groups within each FR and differences between similar days of early and mid-lactation FR were evaluated correspondingly.

3.3. Intravenous glucose tolerance tests

Area under the curve for insulin (AUC_I) and glucose (AUC_G) from -20 to 120 minutes after infusion were calculated with the trapezoid-rule:

$$AUC \approx \sum_{i_1=-20}^{i_n=120} \left(t_{i_2} - t_{i_1}\right) \times \left(C_{i_1} + C_{i_2}\right) \times \frac{1}{2}$$

t: minute of sampling
C: concentration of glucose [mmol/L] or insulin [µU/mL]
i: sampling time point

Values for basal insulin and basal glucose were obtained by calculating arithmetic means of the four measurements before glucose infusion (-20, -15, -10 and immediately before infusion). Afterwards, basal AUC_I and AUC_G ($bAUC_I$ and $bAUC_G$) were calculated as $bAUC \approx basal\ insulin$ or $glucose \times 140$, assuming that the base is a rectangle with height of basal insulin or glucose concentrations and length of 140 minutes. Then net AUC_I and AUC_G ($nAUC_I$ and $nAUC_G$) were calculated by subtraction of $bAUC_I$ from AUC_I and $bAUC_G$ from AUC_G, respectively.

According to Kerestes et al. (2009), clearance of glucose was calculated using the formula $CR = \frac{\ln glucose_{i_5} - \ln glucose_{i_{60}}}{60-5} \times 100$. Consistent with Radziuk (2000), homeostatic model assessment of insulin was calculated as $HOMA - IR = \frac{basal\ glucose \times basal\ insulin}{22.5}$.

Statistical differences were calculated using REML in the MIXED procedure in SAS, like mentioned before.

3.4. Major milk proteins in skim milk

Data was provided by the software as percentage amount of total protein concentration. Due to technical reasons, the absolute amount of individual proteins were constantly over- or underestimated, so all following calculations were conducted with the percentage amount of protein concentration in the sample. To obtain comparable results between chips, every protein fraction was corrected by multiplication with a correction factor on the basis of the results of the respective proteins in the protein mix sample. Correction factor (CF) was calculated as $CF = \frac{20\%}{\%\ of\ protein\ fraction\ in\ protein\ mix}$. After correction, percentage amount of each protein fraction within the sum of all protein fractions was determined. Results were subsumed to six time periods like done before with milk parameters. Differences between groups and time points were estimated with the above described model in SAS.

IV. RESULTS

1. Animal experiment

1.1. Cows and classification

Approximately four weeks before parturition 26 cows were transported from a farm in Saxony to the research farm Veitshof. One cow died during the course of parturition due to severe calving difficulties caused by an oversized calf. Another cow had to be euthanized shortly after parturition due to downer-cow-syndrome and slipping injuries. Additionally, all data of one animal had to be omitted because of recurring inflammations which were caused by a perforating foreign body in the reticulum.

Classification of remaining 23 cows was done based on mean FCM yield and mean milk protein content during d 23, 24 and 25 pp and resulted in four groups: 6 cows with high FCM (52.66 ± 2.91 kg/d) and high protein content (3.28 ± 0.07%; MP-cows), 5 cows showing low FCM (40.49 ± 1.15 kg/d) and low protein content (2.84 ± 0.06%; mp-cows), 7 cows with high FCM (48.98 ± 2.12 kg/d) and low protein content (2.90 ± 0.06%; Mp-cows) and 5 cows showing low FCM (39.08 ± 0.60 kg/d) and high protein content (3.40 ± 0.05%; mP-cows; table 4). Furthermore MP- and Mp-cows showed higher FCM compared to mp- and mP-cows ($P < 0.01$), whereas mp- and Mp-cows showed lower milk protein content compared to MP- and mP-cows ($P < 0.001$).

Parity amongst all groups was comparable (MP-cows 2.8 ± 0.3, mp-cows 2.4 ± 0.2, Mp-cows 2.3 ± 0.3, and mP-cows 2.8 ± 0.4; $P = 0.35$).

Table 4: Parameters of classification (mean FCM yield, kg/d and mean milk protein content, %) of 23 cows during d 23, 24 and 25 pp.

cow	parity	group	milk FCM, kg/d	mean FCM, kg/d	milk protein content, %	mean protein content, %
14024 57758	4	MP	58.73	52.66 ± 2.91[a]	3.07	3.28 ± 0.07[a]
14027 34346	3	MP	51.04		3.06	
14027 34439	3	MP	60.16		3.50	
14030 03463	3	MP	55.78		3.42	
14030 03870	2	MP	40.97		3.29	
14031 15582	2	MP	49.25		3.33	
14026 25564	3	mp	44.25	40.49 ± 1.15[b]	3.02	2.84 ± 0.06[b]
14027 34303	3	mp	37.70		2.70	
14031 15366	2	mp	38.64		2.86	
14032 20073	2	mp	41.59		2.73	
14032 20330	2	mp	40.24		2.89	
14026 25242	4	Mp	54.13	48.98 ± 2.12[a]	3.00	2.90 ± 0.06[b]
14028 63689	2	Mp	46.72		3.00	
14030 03642	2	Mp	55.40		2.71	
14030 03827	2	Mp	41.13		2.95	
14030 03863	2	Mp	44.10		3.02	
14031 15265	2	Mp	54.23		2.95	
14031 15625	2	Mp	47.17		2.67	

cow	parity	group	milk FCM, kg/d	mean FCM, kg/d	milk protein content, %	mean protein content, %
14027 34230	4	mP	40.32	39.08 ± 0.60[b]	3.28	3.40 ± 0.05[a]
14027 34311	3	mP	40.47		3.51	
14027 34460	3	mP	38.70		3.36	
14031 15263	2	mP	38.74		3.53	
14031 15662	2	mP	37.19		3.31	

[abcd]Means with alphabetic superscripts indicate differences between groups (P < 0.05)

1.2. Mean milk and blood parameters during 22 weeks of lactation

Data of mean milk parameters during 22 weeks of lactation and mean blood serum parameters during 2 weeks before until 22 weeks after parturition of the four groups can be found in table 5.

Table 5: Mean milk and blood serum parameters of the four groups during 155 days of lactation.

	MP	mp	Mp	mP
milk parameters, mean wk 1 to 22 pp				
yield, kg/d	37.2 ± 0.55[a]	34.7 ± 0.62[b]	41.7 ± 0.52[c]	30.4 ± 0.44[d]
FCM, kg/d	41.7 ± 0.74[a]	35.6 ± 0.74[b]	42.8 ± 0.56[a]	34.0 ± 0.50[b]
ECM, kg/d	44.7 ± 0.73[a]	38.4 ± 0.76[b]	45.9 ± 0.54[a]	37.2 ± 0.49[b]
protein content, %	3.46 ± 0.06[a]	3.22 ± 0.05[b]	3.15 ± 0.04[b]	3.75 ± 0.05[c]
protein yield, g/d	1,256 ± 15[a]	1,101 ± 22[b]	1,295 ± 14[a]	1,124 ± 13[b]
fat content, %	4.89 ± 0.10[a]	4.19 ± 0.10[b]	4.22 ± 0.08[b]	4.79 ± 0.09[a]
fat yield, g/d	1,799 ± 36[a]	1,445 ± 38[b]	1,741 ± 31[a]	1,452 ± 26[b]
lactose content, %	4.74 ± 0.02	4.69 ± 0.02	4.77 ± 0.01	4.81 ± 0.02
lactose yield, g/d	1,774 ± 31[a]	1,637 ± 35[ab]	1,993 ± 27[c]	1,469 ± 23[b]
FPR	1.43 ± 0.03[a]	1.32 ± 0.02[b]	1.36 ± 0.02[ab]	1.29 ± 0.02[b]
SCC, × 1,000/mL	64.9 ± 9.6[ab]	148.7 ± 53.4[ab]	64.5 ± 13.3[a]	482.8 ± 96.4[b]
pH	6.63 ± 0.01[a]	6.58 ± 0.01[b]	6.61 ± 0.01[ab]	6.63 ± 0.01[a]
urea, mg/L	201.1 ± 3.8	197.8 ± 5.1	201.5 ± 4.6	202.4 ± 4.4
hydrocortisone, nmol/L	3.80 ± 0.13	3.32 ± 0.14	3.71 ± 0.12	3.66 ± 0.20
blood serum parameters, mean wk -2 to 22 pp				
glucose, mmol/L	3.99 ± 0.07	3.91 ± 0.16	3.74 ± 0.07	3.98 ± 0.06
NEFA, µmol/L	562 ± 54	478 ± 60	525 ± 60	470 ± 74
BHBA, mmol/L	0.54 ± 0.04	0.65 ± 0.06	0.53 ± 0.04	0.49 ± 0.04
cholesterol, mmol/L	3.49 ± 0.23	3.18 ± 0.17	3.80 ± 0.22	3.19 ± 0.18
tBR, µmol/L	5.15 ± 0.40	4.77 ± 0.43	4.69 ± 0.35	4.61 ± 0.40

[abcd]Means with alphabetic superscripts indicate differences between groups (P < 0.05)

Mean ECM and FCM were higher in cows grouped for high FCM (MP- and Mp-cows) than in those with low (mp- and mP-cows; P < 0.05, table 5).

Mean milk fat content was highest in MP- (4.89 ± 0.10%) and mP-cows (4.79 ± 0.09%) compared to mp- (4.19 ± 0.10%; P < 0.01) and Mp-cows (4.22 ± 0.08%; P < 0.01, table 5).

Milk protein yield and milk fat yield were higher in cows with high milk yield (MP- and Mp-cows) than in those with low milk yield (mp- and mP-cows; P < 0.05, table 5).

In milk lactose content, there was no difference observed between groups (table 5). Highest milk lactose yield was shown by Mp-cows (1,993 ± 27 g/d; $P < 0.05$). Furthermore, milk lactose yield of MP-cows (1,774 ± 31 g/d) was similar to mp-cows (1,637 ± 35 g/d; $P = 0.19$) but higher than in mP-cows (1,469 ± 23 g/d; $P < 0.01$). No difference could be found in milk lactose yield of mp- and mP-cows ($P = 0.16$).

FPR was highest in MP-cows (1.43 ± 0.03) compared to mp- (1.32 ± 0.02; $P < 0.05$) and mP-cows (1.29 ± 0.02; $P < 0.01$, table 5). Moreover, Mp-cows (1.36 ± 0.02) had similar FPR compared to mp- ($P = 0.45$) and mP-cows ($P = 0.21$). SCC was highest in mP-cows (482.8 ± 96.4 × 1,000/mL) compared to Mp-cows (64.5 ± 13.3 × 1,000/mL; $P < 0.05$, table 5), whereas no difference could be observed between those two groups and MP- (64.9 ± 9.6 × 1,000/mL) or mp-cows (148.7 ± 53.4 × 1,000/mL).

Milk pH was lower in mp-cows (6.58 ± 0.01) than in MP- (6.63 ± 0.01; $P < 0.05$) and mP-cows (6.63 ± 0.01; $P < 0.05$, table 5). Mp-cows were similar in milk pH to MP- and mP-cows ($P > 0.30$).

No differences could be observed in milk urea, milk hydrocortisone contents and concentrations of blood serum glucose, NEFA, BHBA, cholesterol and tBR (table 5).

1.3. Course of milk parameters during 22 weeks of lactation

Milk yield

Milk yield increased during period 1 and decreased slowly from period 2 to period 5 (figure 7). During period 1, Mp-cows (39.4 ± 1.38 kg/d) had highest milk yield compared to MP- (33.1 ± 1.66 kg/d; $P < 0.01$), mp- (32.3 ± 1.73 kg/d; $P < 0.01$) and mP-cows (28.4 ± 1.43 kg/d; $P < 0.001$; table 6). Furthermore during period 2, Mp-cows (46.1 ± 0.82 kg/d) showed higher milk yield compared to mp- (38.0 ± 1.08 kg/d; $P < 0.01$) and mP-cows (34.3 ± 0.68 kg/d; $P < 0.001$). Moreover, MP-cows (42.1 ± 0.65 kg/d) had higher milk yield than mP-cows (34.3 ± 0.68 kg/d; $P < 0.01$). Milk yield in period 3 was still highest in Mp-cows (45.3 ± 0.79 kg/d) compared to MP- (40.7 ± 0.70 kg/d; $P < 0.05$), mp- (37.5 ± 1.09 kg/d; $P < 0.01$) and mP-cows (32.7 ± 0.49 kg/d; $P < 0.001$). Moreover, MP-cows showed higher milk yield than mP-cows ($P < 0.01$).

Throughout period 4, 5 and 6, mP-cows showed lowest milk yields (30.3 ± 0.46, 27.8 ± 0.71 and 22.9 ± 3.00 kg/d) compared to MP- (37.9 ± 0.79, 32.8 ± 1.06 and 32.6 ± 1.47 kg/d; $P < 0.05$) and Mp-cows (42.2 ± 0.72, 37.8 ± 1.17 and 36.7 ± 1.56 kg/d; $P < 0.001$). Furthermore, milk yield of Mp-cows was higher compared to mp-cows (34.5 ± 1.08, 32.5 ± 1.25 and 28.4 ± 3.45 kg/d in period 4, 5, and 6; $P < 0.01$). Additionally in period 5, milk yield of Mp-cows was higher compared to MP-cows ($P < 0.05$).

In all cows, highest milk yield was obtained in period 2 (42.1 ± 0.65, 38.0 ± 1.08, 46.1 ± 0.82 and 34.3 ± 0.68 kg/d for MP-, mp-, Mp- and mP-cows; $P < 0.001$). Furthermore, milk yield decreased from period 3 to 5 in MP-cows by 7.9 kg (to 32.8 ± 1.06 kg/d; $P < 0.01$) and in Mp-cows from period 3 to 6 by 8.6 kg (to 36.7 ± 1.56 kg/d; $P < 0.01$). Additionally, declines were found from period 3 to 4 by 3 kg in mp-cows ($P < 0.01$) and by 2.4 kg in mP-cows ($P < 0.05$) to 34.5 ± 1.08 and 30.3 ± 0.46 kg/d. From period 5 to 6, milk yield decreased further in mp-cows by 4.1 kg (to 28.4 ± 3.45; $P < 0.01$) and by 4.9 kg in mP-cows (to 22.9 ± 3.00 kg/d; $P < 0.01$).

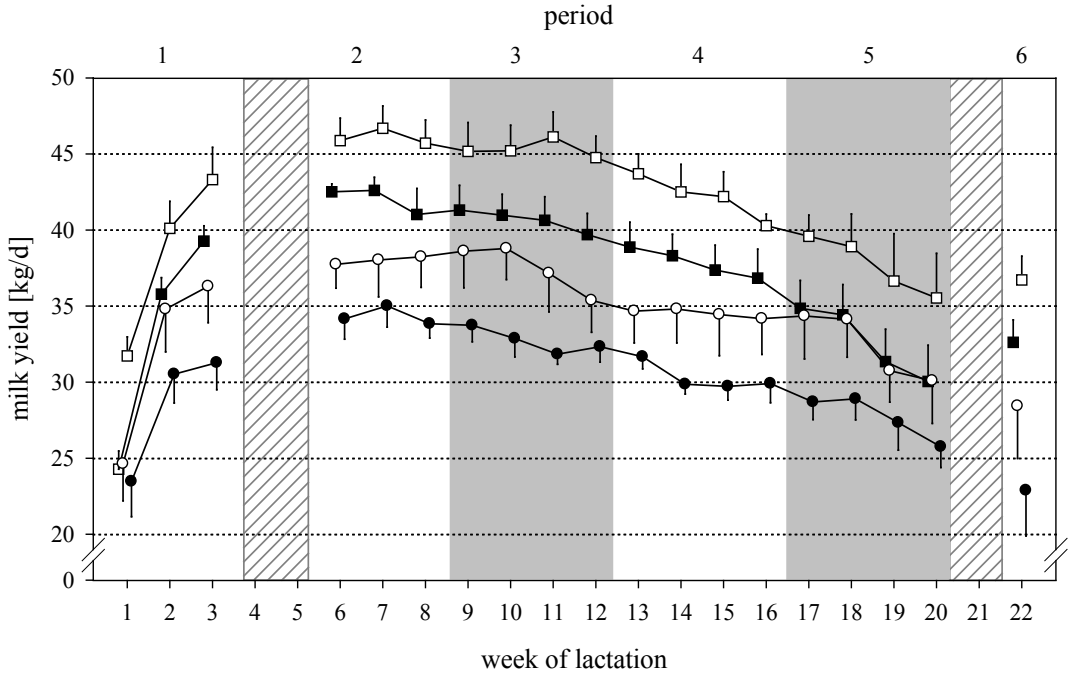

Figure 7: Milk yield (kg/d) during first 22 weeks of lactation

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.41$. Differences between periods and between groups can be found in table 6.

FCM

After increase of FCM in period 1, cows showed slowly decreasing yields in periods 2 until 5. During this time, high yielding cows (MP- and Mp-cows) were clearly distinguishable from low yielding cows (mp- and mP-cows, figure 8).

During period 1, Mp-cows showed highest FCM (47.0 ± 1.19 kg/d) compared to mp- (38.7 ± 1.94 kg/d; $P < 0.01$) and mP-cows (35.5 ± 1.78 kg/d; $P < 0.001$; table 6). Moreover, higher FCM was observed in MP- (43.5 ± 1.61 kg/d) compared to mP-cows ($P < 0.01$). During periods 2, 3 and 4, MP- (48.7 ± 1.58, 44.2 ± 0.90 and 40.9 ± 0.70 kg/d) and Mp-cows (47.5 ± 1.28, 45.1 ± 0.83 and 40.2 ± 0.90 kg/d) showed higher FCM yields compared to mp- (40.1 ± 1.61, 36.3 ± 1.47 and 33.0 ± 1.38 kg/d; $P < 0.01$, only in period 3 $P < 0.05$) and mP-cows (36.8 ± 1.06, 35.0 ± 0.69 and 33.0 ± 0.56 kg/d; $P < 0.01$). In period 5, FCM was higher in Mp- (37.7 ± 1.07 kg/d) compared to mP-cows ($P < 0.05$). During period 6, MP- (34.1 ± 1.53 kg/d) and Mp-cows (36.9 ± 1.61 kg/d) showed higher FCM compared to mP-cows (25.6 ± 3.42 kg/d; $P < 0.05$) and higher FCM was observed in Mp- compared to mp-cows (29.4 ± 3.65 kg/d; $P < 0.05$).

In MP-cows, FCM increased from period 1 to period 2 by 5.2 kg ($P < 0.001$) and decreased afterwards to period 3 by 4.5 kg ($P < 0.001$) and from period 4 to period 5 by 5.0 kg ($P < 0.001$). Furthermore, declining FCM was observed in mp-cows from period 2 through period 4 by total 7.1 kg ($P < 0.05$). In Mp-cows, FCM decreased from period 2 until period 5 (-9.8 kg; $P < 0.05$) and solely decrease in mP-cows was observed between period 5 and 6 (-6.0 kg; $P < 0.01$).

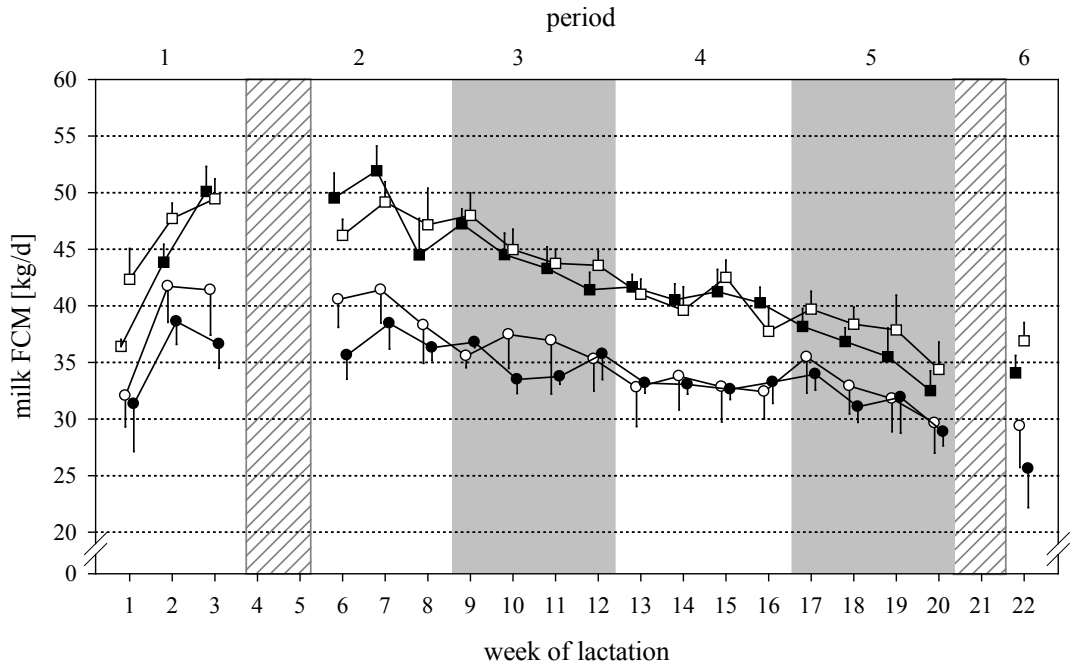

Figure 8: FCM (kg/d) during first 22 weeks of lactation

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.01$ and time × group $P = 0.07$. Differences between periods and between groups can be found in table 6.

ECM

Course of ECM was similar to FCM, during period 1 yield increased and afterwards declined until period 5. High and low yielding cows were clearly distinguishable. During period 1, ECM of Mp-cows (50.3 ± 1.07 kg/d) was higher than ECM of low yielding cows ($P < 0.01$; table 6, figure 9). Furthermore MP-cows (46.5 ± 1.53 kg/d) showed higher ECM compared to mP-cows (38.6 ± 1.73 kg/d; $P < 0.01$). ECM of high yielding cows was higher during period 2 to 4 compared to that of low yielding cows ($P < 0.05$). In period 5, Mp-cows (40.8 ± 1.12 kg/d) showed higher ECM compared to mP-cows (34.9 ± 0.90 kg/d; $P < 0.05$). Moreover, mP-cows (28.7 ± 3.67 kg/d) showed in period 6 lower ECM than MP- (37.1 ± 1.33 kg/d; $P < 0.05$) and Mp-cows (40.0 ± 1.71 kg/d; $P < 0.01$). Additionally, ECM of mp-cows (32.0 ± 4.07 kg/d) was lower than in Mp-cows ($P < 0.05$).

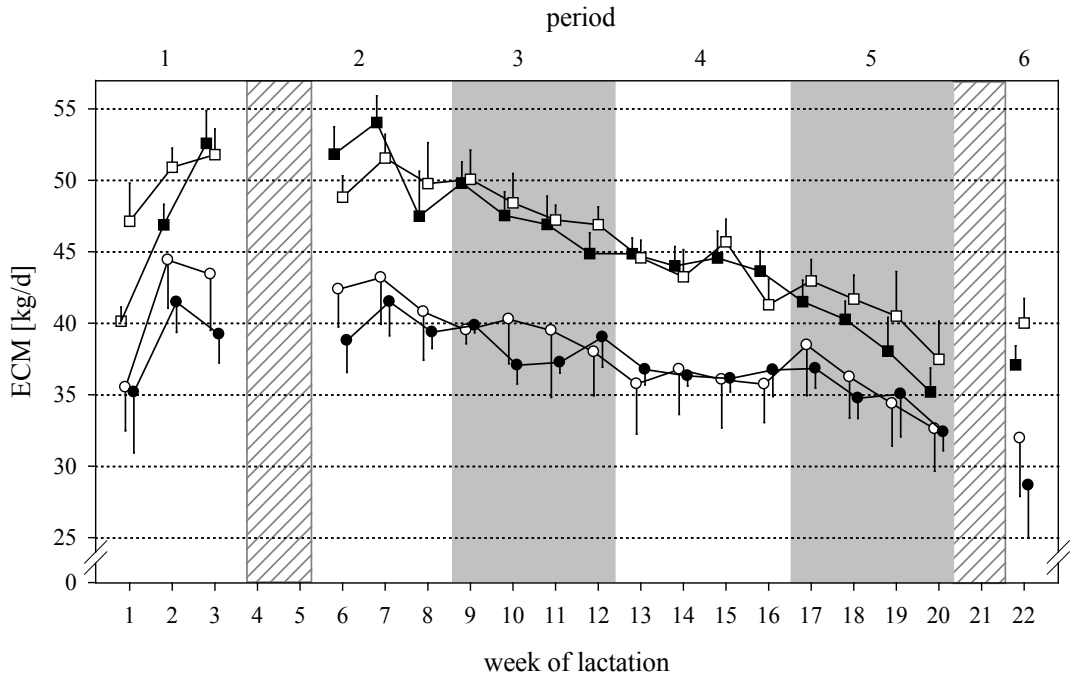

Figure 9: Energy-corrected milk yield (kg/d) during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.01$ and time × group $P = 0.08$. Differences between periods and between groups can be found in table 6.

Milk protein content

Milk protein content showed highest levels during wk 1 pp, decreased until wk 3 pp and increased slowly from wk 6 on. Throughout all periods milk protein content in Mp-cows was lower compared to MP- ($P < 0.05$) and mP-cows ($P < 0.01$; table 6, figure 10). Furthermore, mp-cows had lower milk protein contents compared to MP- ($P < 0.01$) and mP-cows ($P < 0.01$). In period 2, milk protein concentration of mP-cows was higher in comparison to mp-cows (3.38 ± 0.05 and 2.86 ± 0.05% for mP- and mp-cows; $P < 0.001$). Throughout periods 3 to 6, mP-cows showed higher milk protein concentrations than MP- ($P < 0.05$) and mp-cows ($P < 0.001$). In all cows, milk protein concentration was lower in period 2 than in period 1 (3.13 ± 0.06, 2.86 ± 0.05, 2.85 ± 0.02 and 3.38 ± 0.05% for MP-, mp-, Mp- and mP-cows; $P < 0.001$). Afterwards, milk protein content increased with only significant difference in MP-cows between period 3 and 4 (increase by 0.19% to 3.40 ± 0.05%).

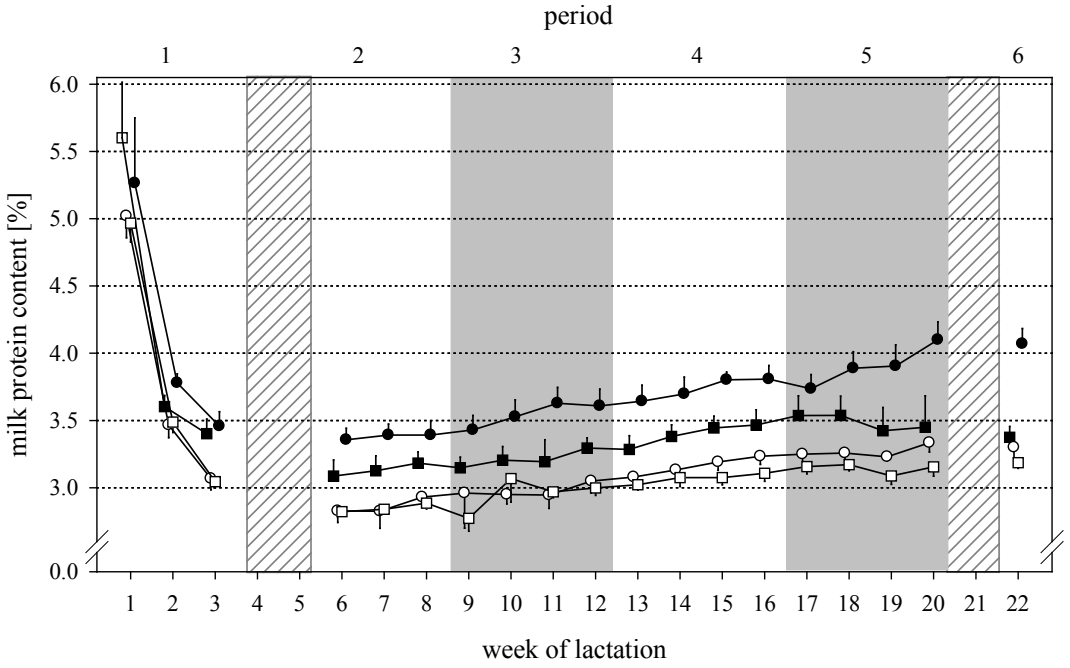

Figure 10: Milk protein content (%) during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.24$. Differences between periods and between groups can be found in table 6.

Milk protein yield

Milk protein yield stabilised on a higher level in high yielding cows and a lower level in low yielding cows. During period 1, milk protein yield was higher in Mp-cows (1,394 ± 34 g/d) compared to mP- (1,127 ± 48 g/d; $P < 0.01$) and mp-cows (1,129 ± 44 g/d; $P < 0.01$; table 6; figure 11). In period 2, mp-cows (1,093 ± 46 g/d) showed lower milk protein yield than MP- (1,318 ± 33 g/d; $P < 0.01$) and Mp-cows (1,313 ± 24 g/d; $P = 0.01$). Throughout period 3 and 6 milk protein yield was higher in Mp-cows (1,338 ± 32 and 1,170 ± 55 g/d) than in mp- (1,148 ± 58 and 943 ± 132 g/d; $P < 0.05$) and mP-cows (1,157 ± 18 and 924 ± 103 g/d; $P < 0.05$). Furthermore, mp-cows (1,094 ± 41 g/d) showed lower milk protein yield during period 4 than MP- (1,279 ± 21 g/d; $P < 0.05$) and Mp-cows (1,292 ± 18 g/d; $P < 0.05$).

Milk protein yield decreased in Mp-cows until period 2 by 81 g ($P < 0.05$) and from period 4 to 6 by 122 g ($P < 0.05$) to 1,313 ± 24 and 1,170 ± 55 g/d, respectively. MP-cows showed single decrease from period 4 to 5 by 148 g to 1,131 ± 27 g/d ($P < 0.01$). From period 5 to 6, milk protein yield declined in mp-cows by 119 g to 943 ± 132 g/d ($P < 0.01$) and in mP-cows by 152 g to 924 ± 103 g/d ($P < 0.01$).

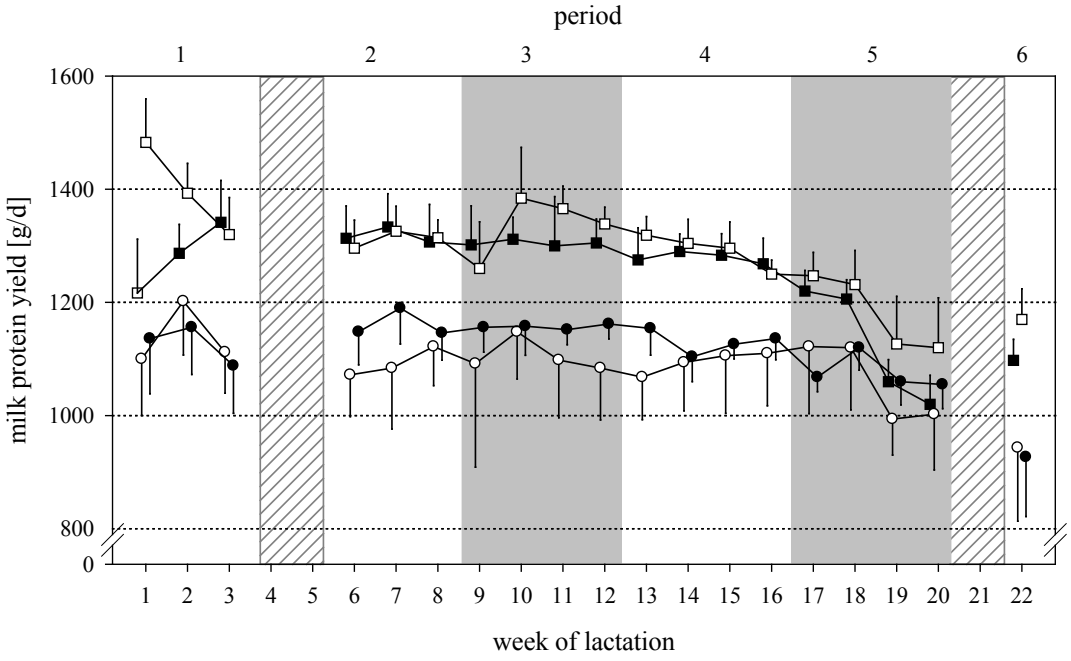

Figure 11: Milk protein yield (g/d) during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.05$ and time × group $P = 0.43$. Differences between periods and between groups can be found in table 6.

Milk fat content

Milk fat content was highest in wk 1 pp, decreased throughout period 2 and levelled higher in high protein cows than in low protein cows. During period 1, milk fat content was higher in MP-cows (6.23 ± 0.26%) than in mp- (5.42 ± 0.30%; $P < 0.05$) and Mp-cows (5.42 ± 0.22%; $P < 0.05$; table 6, figure 12). Further on in period 2, MP-cows (5.03 ± 0.18%) showed higher fat content than Mp-cows (4.21 ± 0.15; $P < 0.05$). High protein cows showed during period 4 higher contents of milk fat compared to low protein cows (4.57 ± 0.10, 4.62 ± 0.10, 3.67 ± 0.10 and 3.70 ± 0.12% for MP-, mP-, mp- and Mp-cows; $P < 0.05$). In period 5, Mp-cows (3.94 ± 0.12%) had lower milk fat content compared to MP- (4.68 ± 0.12%; $P < 0.05$) and mP-cows (4.84 ± 0.18%; $P < 0.01$). Furthermore, milk fat concentration was lower in mp-cows (3.95 ± 0.11%) in comparison to mP-cows ($P < 0.05$).

Moreover, all cows had lower milk fat concentrations in period 2 compared to period 1 ($P < 0.001$). Subsequently, MP- and mp-cows showed further decrease by 0.66 and 0.55% to 4.37 ± 0.09 and 3.79 ± 0.16% in period 3. Thereafter, milk fat content stabilized at a similar level throughout the remaining study period.

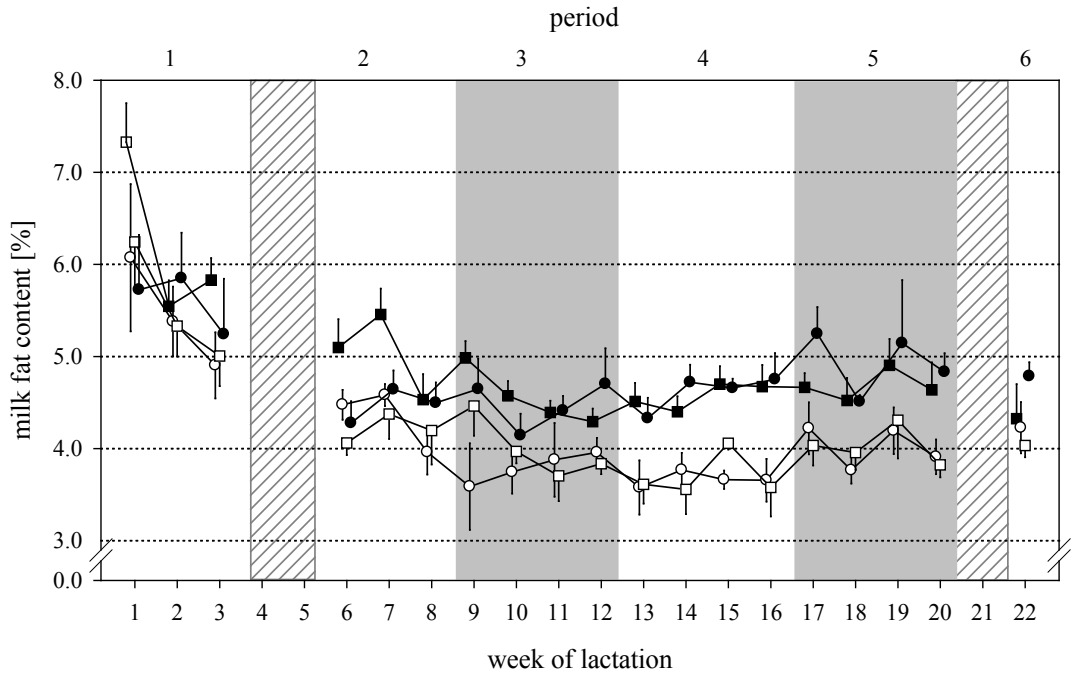

Figure 12: Milk fat content (%) during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.05$ and time × group $P = 0.53$. Differences between periods and between groups can be found in table 6.

Milk fat yield

Milk fat yield showed highest amounts during period 1 and 2 and declined afterwards. All cows showed similar milk fat yields. During period 1 to 3, milk fat yield was higher in high yielding cows (MP- and Mp-cows) compared to low yielding cows (mp- and mP-cows; $P < 0.05$; table 6, figure 13). In period 4, MP-cows (1,720 ± 33 g/d) showed higher milk fat yield than mp- (1,277 ± 65 g/d; $P < 0.01$) and mP-cows (1,394 ± 31 g/d; $P < 0.05$). Furthermore, milk fat yield in Mp-cows (1,558 ± 51 g/d) was higher than in mp-cows ($P < 0.05$). No differences were observed between cows in period 5, whereas milk fat yield in period 6 was higher in Mp- (1,481 ± 73 g/d) than in mP-cows (1,096 ± 149 g/d; $P < 0.05$).

In Mp-cows, milk fat yield declined from period 1 to 4 by 526 g (2,084 ± 62 to 1,558 ± 51 g/d; $P < 0.05$). Furthermore, milk fat yield decreased in MP- and mp-cows from period 2 to 3 by 265 and 237 g (to 1,858 ± 46 g/d in MP-cows and to 1,422 ± 79 g/d in mp-cows; $P < 0.01$). Additionally in MP-cows, milk fat yield declined from period 4 to 5 by 202 g (to 1,518 ± 41 g/d; $P < 0.01$). Moreover, milk fat yield decreased in mP-cows solely from period 5 to 6 by 268 g (to 1,096 ± 149 g/d; $P < 0.05$).

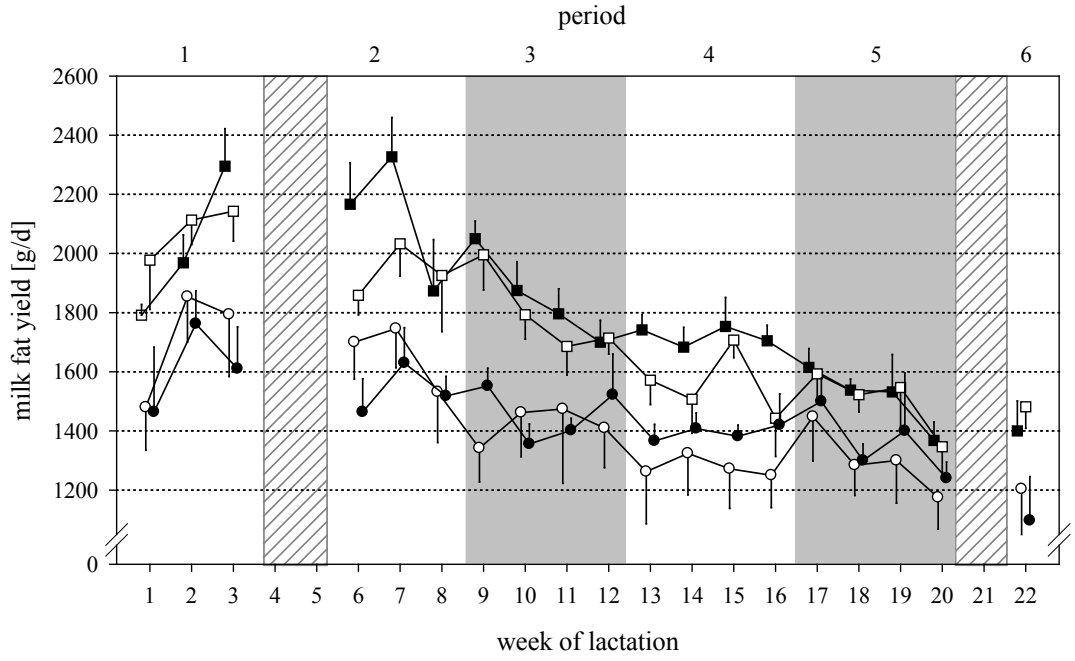

Figure 13: Milk fat yield (g/d) during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.01$ and time × group $P = 0.07$. Differences between periods and between groups can be found in table 6.

Other milk parameters

Concentrations of milk hydrocortisone (figure 14, table 6) showed no distinguishable differences. In wk 2 pp, milk hydrocortisone concentration in one of five mP-cows was outstanding high (18.69 nmol/L) and in wk 19 pp, two out of five MP-cows showed similar high levels (16.63 and 12.50 nmol/L). This resulted in significant difference during period 5, in which MP-cows (4.67 ± 0.31 nmol/L) showed higher levels compared to mp-cows (3.12 ± 0.19 nmol/L; $P < 0.05$). Furthermore, milk hydrocortisone increased in MP-cows from period 4 (3.74 ± 0.20 nmol/L) to period 5 by 0.93 nmol/L ($P < 0.05$) and in mP-cows from period 5 to 6 by 1.39 nmol/L (to 5.00 nmol/L; $P < 0.05$).

Differences between groups and between time periods in milk lactose content and yield, FPR, SCC, pH and milk urea can be found in table 6.

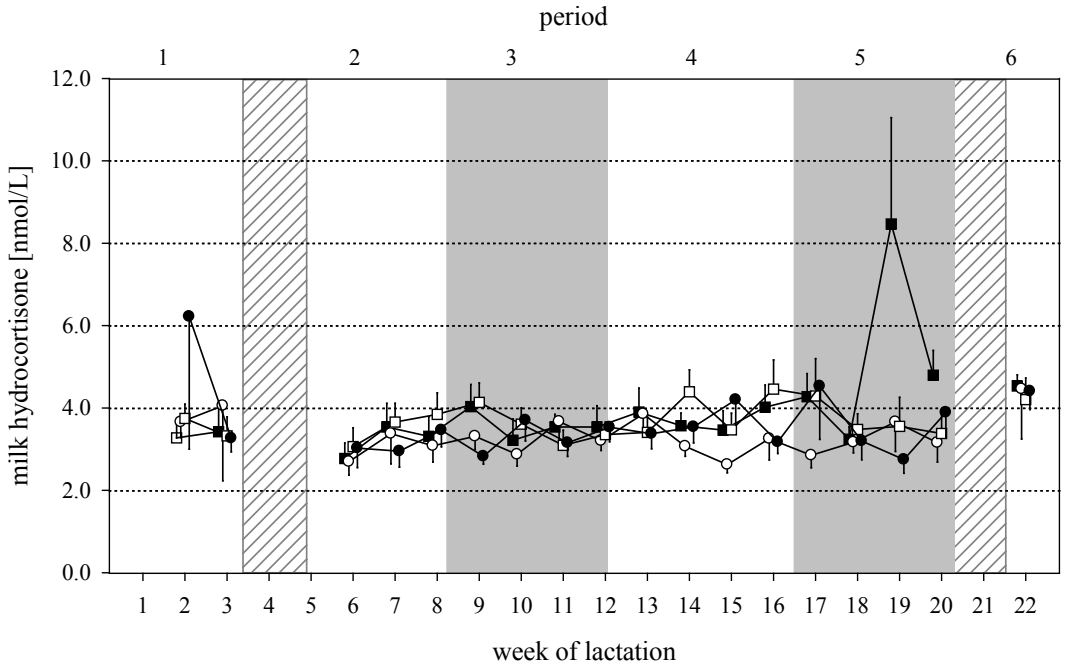

Figure 14: Milk hydrocortisone (nmol/L) during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.05$, group $P = 0.57$ and time × group $P = 0.51$. Differences between periods and between groups can be found in table 6.

Table 6: Mean milk parameters of 6 time periods during 155 days of lactation.

	MP	mp	Mp	mP
milk yield, kg/d				
period 1	33.1 ± 1.66^{a}	32.3 ± 1.73^{a}	39.4 ± 1.38^{b}	28.4 ± 1.43^{a}
period 2	42.1 ± 0.65^{ab}*	38.0 ± 1.08^{ac}*	46.1 ± 0.82^{b}*	34.3 ± 0.68^{c}*
period 3	40.7 ± 0.70^{a}	37.5 ± 1.09^{ac}	45.3 ± 0.79^{b}	32.7 ± 0.49^{c}
period 4	37.9 ± 0.79^{ab}*	34.5 ± 1.08^{bc}*	42.2 ± 0.72^{a}*	30.3 ± 0.46^{c}*
period 5	32.8 ± 1.06^{a}*	32.5 ± 1.25^{ab}	37.8 ± 1.17^{c}*	27.8 ± 0.71^{b}
period 6	32.6 ± 1.47^{ab}	28.4 ± 3.45^{ac}*	36.7 ± 1.56^{b}*	22.9 ± 3.00^{c}*
FCM, kg/d				
period 1	43.5 ± 1.61^{ab}	38.7 ± 1.94^{ac}	47.0 ± 1.19^{b}	35.5 ± 1.78^{c}
period 2	48.7 ± 1.58^{a}*	40.1 ± 1.61^{b}	47.5 ± 1.28^{a}	36.8 ± 1.06^{b}
period 3	44.2 ± 0.90^{a}*	36.3 ± 1.47^{b}*	45.1 ± 0.83^{a}*	35.0 ± 0.69^{b}
period 4	40.9 ± 0.70^{a}	33.0 ± 1.38^{b}*	40.2 ± 0.90^{a}*	33.0 ± 0.56^{b}
period 5	35.9 ± 0.96^{ab}*	32.6 ± 1.38^{ab}	37.7 ± 1.07^{b}*	31.6 ± 0.95^{a}
period 6	34.1 ± 1.53^{ab}	29.4 ± 3.65^{bc}	36.9 ± 1.61^{a}	25.6 ± 3.42^{c}*
ECM, kg/d				
period 1	46.5 ± 1.53^{ab}	41.3 ± 1.91^{ac}	50.3 ± 1.07^{b}	38.6 ± 1.73^{c}
period 2	51.1 ± 1.45^{a}*	42.1 ± 1.68^{b}	50.1 ± 1.17^{a}	39.9 ± 1.11^{b}
period 3	47.3 ± 0.85^{a}*	39.3 ± 1.50^{b}	48.2 ± 0.81^{a}	38.3 ± 0.66^{b}
period 4	44.3 ± 0.69^{a}	36.1 ± 1.47^{b}*	43.7 ± 0.83^{a}*	36.5 ± 0.56^{b}
period 5	38.9 ± 0.96^{ab}*	35.6 ± 1.51^{ab}	40.8 ± 1.12^{a}*	34.9 ± 0.90^{b}
period 6	37.1 ± 1.33^{ab}	32.0 ± 4.07^{ac}*	40.0 ± 1.71^{b}	28.7 ± 3.67^{c}*

	MP	mp	Mp	mP
milk protein content, %				
period 1	4.20 ± 0.28[a]	3.75 ± 0.22[b]	3.73 ± 0.18[b]	4.17 ± 0.26[a]
period 2	3.13 ± 0.06[ab]*	2.86 ± 0.05[ac]*	2.85 ± 0.02[c]*	3.38 ± 0.05[b]*
period 3	3.21 ± 0.05[a]	3.04 ± 0.07[ab]	2.95 ± 0.05[b]	3.55 ± 0.06[c]
period 4	3.40 ± 0.05[a]*	3.16 ± 0.03[ab]	3.07 ± 0.03[b]	3.74 ± 0.05[c]
period 5	3.49 ± 0.08[a]	3.26 ± 0.04[ab]	3.14 ± 0.03[b]	3.90 ± 0.07[c]
period 6	3.37 ± 0.08[a]	3.30 ± 0.08[ab]	3.19 ± 0.04[b]	4.07 ± 0.11[c]
milk protein yield, g/d				
period 1	1,281 ± 43[ab]	1,129 ± 44[a]	1,394 ± 34[b]	1,127 ± 48[a]
period 2	1,318 ± 33[a]	1,093 ± 46[b]	1,313 ± 24[a]*	1,161 ± 31[ab]
period 3	1,304 ± 29[ab]	1,148 ± 58[a]	1,338 ± 32[b]	1,157 ± 18[a]
period 4	1,279 ± 21[a]	1,094 ± 41[b]	1,292 ± 18[a]	1,130 ± 18[ab]
period 5	1,131 ± 27*	1,062 ± 48	1,185 ± 34*	1,076 ± 18
period 6	1,098 ± 36[ab]	943 ± 132[a]*	1,170 ± 55[b]*	924 ± 103[a]*
milk fat content, %				
period 1	6.23 ± 0.26[a]	5.42 ± 0.30[b]	5.42 ± 0.22[b]	5.61 ± 0.31[ab]
period 2	5.03 ± 0.18[a]*	4.34 ± 0.12[ab]*	4.21 ± 0.15[b]*	4.47 ± 0.13[ab]*
period 3	4.37 ± 0.09*	3.79 ± 0.16*	3.99 ± 0.12	4.48 ± 0.14
period 4	4.57 ± 0.10[a]	3.67 ± 0.10[b]	3.70 ± 0.12[b]	4.62 ± 0.10[a]
period 5	4.68 ± 0.12[ab]	3.95 ± 0.11[ac]	3.94 ± 0.12[c]	4.84 ± 0.18[b]
period 6	4.48 ± 0.37	4.26 ± 0.28	4.05 ± 0.13	4.77 ± 0.15
milk fat yield, g/d				
period 1	2,018 ± 71[a]	1,718 ± 94[b]	2,084 ± 62[a]	1,610 ± 93[b]
period 2	2,123 ± 92[a]	1,659 ± 81[b]	1,939 ± 74[a]*	1,538 ± 57[b]
period 3	1,858 ± 46[a]*	1,422 ± 79[b]*	1,797 ± 49[a]*	1,459 ± 43[b]
period 4	1,720 ± 33[a]	1,277 ± 65[b]	1,558 ± 51[ac]*	1,394 ± 31[bc]
period 5	1,518 ± 41*	1,308 ± 63	1,506 ± 49	1,364 ± 53
period 6	1,401 ± 100[ab]	1,202 ± 156[ab]	1,481 ± 73[a]	1,096 ± 149[b]*
milk lactose content, %				
period 1	4.50 ± 0.09	4.50 ± 0.07	4.62 ± 0.04	4.65 ± 0.09
period 2	4.82 ± 0.03*	4.76 ± 0.04*	4.79 ± 0.02*	4.89 ± 0.03*
period 3	4.79 ± 0.03	4.75 ± 0.05	4.82 ± 0.02	4.87 ± 0.03
period 4	4.78 ± 0.03	4.72 ± 0.04	4.79 ± 0.02	4.82 ± 0.03
period 5	4.75 ± 0.05	4.71 ± 0.04	4.80 ± 0.02	4.79 ± 0.04
period 6	4.82 ± 0.04	4.61 ± 0.16	4.74 ± 0.03	4.75 ± 0.11
milk lactose yield, g/d				
period 1	1,524 ± 95[a]	1,480 ± 95[a]	1,832 ± 77[b]	1,352 ± 80[a]
period 2	2,028 ± 40[ab]*	1,812 ± 65[bc]*	2,211 ± 44[a]*	1,678 ± 31[c]*
period 3	1,951 ± 42[ab]	1,786 ± 67[bc]	2,182 ± 38[a]	1,592 ± 24[c]
period 4	1,814 ± 45[ab]*	1,638 ± 65[ac]*	2,021 ± 34[b]*	1,460 ± 22[c]*
period 5	1,561 ± 59[a]*	1,532 ± 68[a]	1,813 ± 57[b]*	1,330 ± 34[a]
period 6	1,571 ± 60[ab]	1,317 ± 201[ac]*	1,740 ± 68[b]*	1,080 ± 123[c]*

	MP	mp	Mp	mP
FPR				
period 1	1.58 ± 0.06	1.52 ± 0.06	1.53 ± 0.06	1.44 ± 0.11
period 2	1.62 ± 0.08[a]	1.52 ± 0.05[ab]	1.48 ± 0.06[ab]	1.32 ± 0.03[b]
period 3	1.42 ± 0.04*	1.27 ± 0.06*	1.37 ± 0.06	1.26 ± 0.04
period 4	1.35 ± 0.03[a]	1.16 ± 0.03[b]	1.21 ± 0.04[b]*	1.24 ± 0.03[ab]
period 5	1.35 ± 0.04	1.24 ± 0.03	1.28 ± 0.04	1.27 ± 0.04
period 6	1.28 ± 0.11	1.28 ± 0.06	1.27 ± 0.05	1.18 ± 0.04
SCC, × 1,000/mL				
period 1	153.0 ± 46.2[ab]	244.6 ± 100.7[ab]	82.6 ± 19.0[a]	571.4 ± 240.7[b]
period 2	51.9 ± 15.7[ab]	54.5 ± 9.9[ab]	23.4 ± 3.1[a]	459.0 ± 279.5[b]
period 3	37.3 ± 6.7	73.5 ± 18.6	19.3 ± 1.6	408.0 ± 230.1
period 4	45.8 ± 9.8[ab]	59.8 ± 11.3[ab]	21.8 ± 2.2[a]	460.9 ± 184.4[b]
period 5	62.2 ± 15.7	410.8 ± 236.3*	167.0 ± 59.4	601.0 ± 211.3
period 6	34.3 ± 8.0[a]	50.2 ± 16.9[ab]	88.8 ± 42.3[ab]	232.8 ± 152.5[b]
pH				
period 1	6.53 ± 0.05[ab]	6.48 ± 0.05[a]	6.57 ± 0.03[b]	6.56 ± 0.05[b]
period 2	6.69 ± 0.01[a]*	6.61 ± 0.01[b]*	6.65 ± 0.01[b]*	6.67 ± 0.01[b]*
period 3	6.65 ± 0.03	6.61 ± 0.01	6.63 ± 0.01	6.66 ± 0.01
period 4	6.64 ± 0.02	6.59 ± 0.01	6.61 ± 0.01	6.64 ± 0.01
period 5	6.62 ± 0.02	6.58 ± 0.01	6.61 ± 0.01	6.62 ± 0.02
period 6	6.63 ± 0.02	6.56 ± 0.01	6.61 ± 0.02	6.62 ± 0.03
milk urea, mg/L				
period 1	186.6 ± 9.7	173.4 ± 13.3	164.0 ± 7.9	186.6 ± 15.1
period 2	194.9 ± 10.3	190.5 ± 10.5	182.5 ± 11.3	185.1 ± 7.7
period 3	188.4 ± 6.7	191.9 ± 9.2	196.8 ± 10.5	201.7 ± 10.5
period 4	211.9 ± 9.0	217.0 ± 12.2	226.6 ± 7.8*	216.1 ± 6.3
period 5	217.3 ± 6.3	206.6 ± 11.9	218.0 ± 8.7	212.1 ± 8.3
period 6	221.1 ± 15.7[ab]	175.7 ± 29.5[a]	240.8 ± 23.3[b]*	224.5 ± 37.4[ab]
milk hydrocortisone, nmol/L				
period 1	3.36 ± 0.32	3.79 ± 0.66	3.60 ± 0.29	4.91 ± 2.22
period 2	3.16 ± 0.37	3.02 ± 0.40	3.48 ± 0.48	3.15 ± 0.40
period 3	3.58 ± 0.39	3.26 ± 0.23	3.55 ± 0.39	3.30 ± 0.24
period 4	3.74 ± 0.20	3.20 ± 0.25	3.93 ± 0.41	3.57 ± 0.23
period 5	4.67 ± 0.31[a]*	3.12 ± 0.19[b]	3.89 ± 0.47[ab]	3.61 ± 0.53[ab]
period 6	4.66 ± 0.34	3.62 ± 0.91	3.81 ± 0.44	5.00 ± 0.50*

[abcd]Means with alphabetic superscripts indicate differences between groups ($P < 0.05$).

*Means with asterisks indicate differences to previous period ($P < 0.05$).

1.4. Course of blood serum parameters during 22 weeks of lactation

Blood serum glucose

Blood serum glucose (table 7, figure 15), showed only difference between groups at day of parturition. Glucose levels were higher in mp-cows (5.48 ± 1.28 mmol/L) compared to MP- (4.44 ± 0.38 mmol/L; $P < 0.01$), Mp- (4.18 ± 0.32 mmol/L; $P < 0.001$) and mP-cows (4.14 ± 0.14 mmol/L; $P < 0.001$). Two out of five mp-cows showed extraordinary high blood serum glucose levels at day of parturition (9.45 and 7.58 mmol/L).

Increase of blood serum glucose levels from d 7 ap to day of parturition was significant in mp-cows (1.29 mmol/L; $P < 0.001$). Furthermore, blood serum glucose levels decreased to d 8 pp by 0.86, 1.86 and 0.83 mmol/L in MP- (to 3.58 ± 0.17 mmol/L; $P < 0.01$), mp- (to 3.62 ± 0.26 mmol/L; $P < 0.001$) and Mp-cows (to 3.35 ± 0.23 mmol/L; $P < 0.01$), respectively.

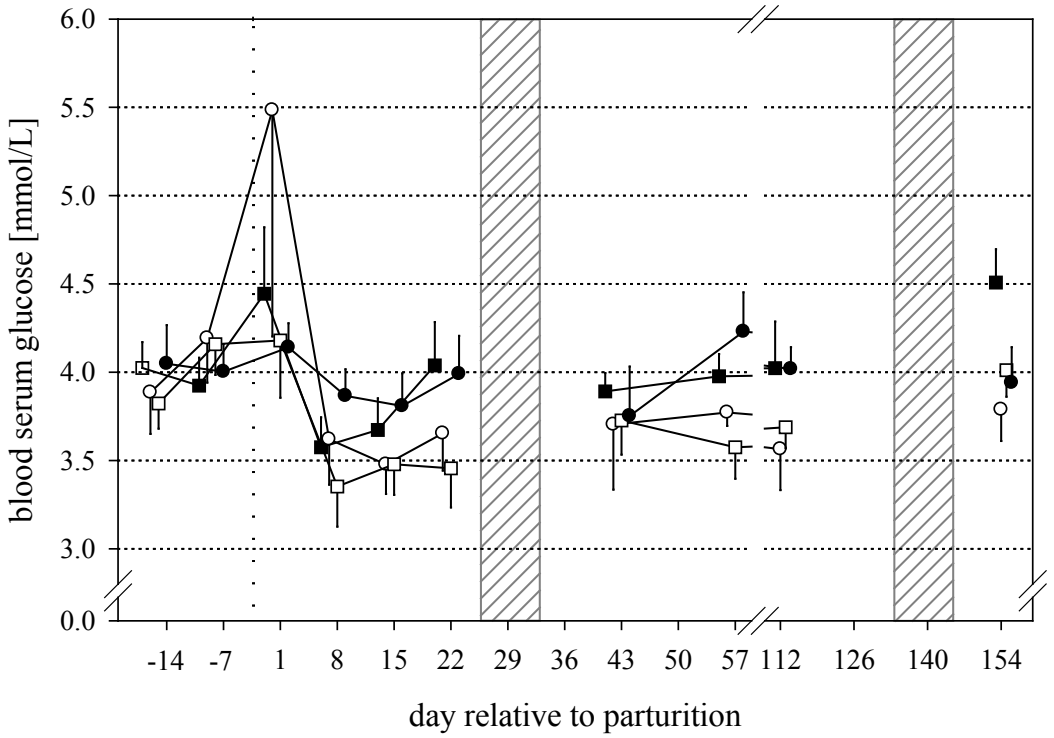

Figure 15: blood serum glucose (mmol/L) during 155 days of lactation

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.28$ and time × group $P = 0.34$. Differences between time points and between groups can be found in table 7.

Blood serum NEFA

Blood serum NEFA levels increased before parturition until d 8 pp (table 7, figure 16). At this day, 3 MP- (50%), 2 mp- (40%), 5 Mp- (71%) and 2 mP-cows (40%) showed blood serum NEFA levels above 1,000 µmol/L. Thereafter, blood serum levels of NEFA decreased throughout the remaining study period.

No differences between groups at any time point could be observed in blood serum NEFA levels, even if higher blood serum NEFA levels of MP-cows before parturition purported this ($P > 0.15$). Blood serum NEFA levels increased from d 7 ap to day of parturition by 505, 777, 601 and 730 µmol/L in MP- (404 ± 134 to 909 ± 157 µmol/L; $P < 0.01$), mp- (136 ± 137 to 913 ± 143 µmol/L; $P = 0.01$), Mp- (222 ± 116 to 823 ± 93 µmol/L; $P < 0.01$) and mP-cows (140 ± 150 to 870 ± 260 µmol/L; $P < 0.05$), respectively. In Mp-cows blood serum NEFA levels increased further to 1,228 ± 116 µmol/L at d 8 pp ($P < 0.01$). Thereafter, decrease of NEFA levels was seen in mp-cows until d 15 pp (from 1,104 ± 137 to 714 ± 165 µmol/L; $P < 0.05$) and in mP-cows until d 22 pp (from 1,067 ± 238 µmol/L to 551 ± 62 µmol/L; $P < 0.05$). In high yielding cows, blood serum NEFA levels declined between d 22 and 43 pp by 322 (to 446 ± 69 µmol/L; $P < 0.05$) and 487 µmol/L (to 380 ± 100 µmol/L; $P < 0.001$) in MP- and Mp-cows, respectively.

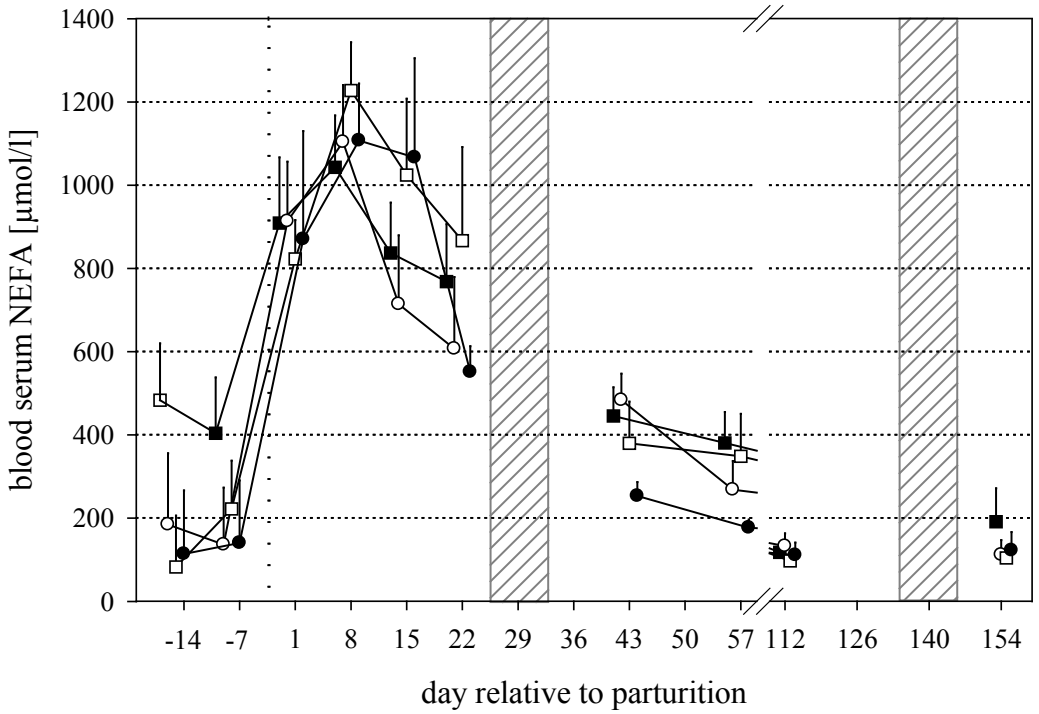

Figure 16: blood serum NEFA (µmol/L) during 155 days of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.59$ and time × group $P = 0.80$. Differences between time points and between groups can be found in table 7.

Blood serum BHBA

Course of blood serum levels of BHBA showed similar pattern to NEFA levels. Blood serum levels increased until d 8 pp and showed slow decrease until end of study (table 7, figure 17). At d 8 pp, 5 MP- (83%), 4 mp- (80%), 6 Mp- (86%) and 3 mP-cows (60%) showed BHBA levels higher 0.50 mmol/L.

At d 22 pp, three mp-cows showed extraordinary high blood serum BHBA levels (2.20, 1.36 and 1.03 mmol/L). Consequently, mp-cows (1.07 ± 0.34 mmol/L) showed higher BHBA levels at d 22 pp compared to MP- (0.62 ± 0.07 mmol/L; $P < 0.01$), Mp- (0.74 ± 0.25 mmol/L; $P < 0.01$) and mP-cows (0.50 ± 0.06 mmol/L; $P < 0.01$). Furthermore at d 43 pp, blood serum BHBA levels of mp-cows (0.73 ± 0.10 mmol/L) were higher than those of mP-cows (0.37 ± 0.03 mmol/L; $P < 0.05$). In mp-cows, BHBA levels increased from d 1 to 8 pp ($P < 0.05$) and further from d 15 to 22 pp ($P < 0.05$) each by 0.29 mmol/L, resulting in total increase from 0.69 ± 0.12 mmol/L at d 1 pp to 1.07 ± 0.34 mmol/L at d 22 pp.

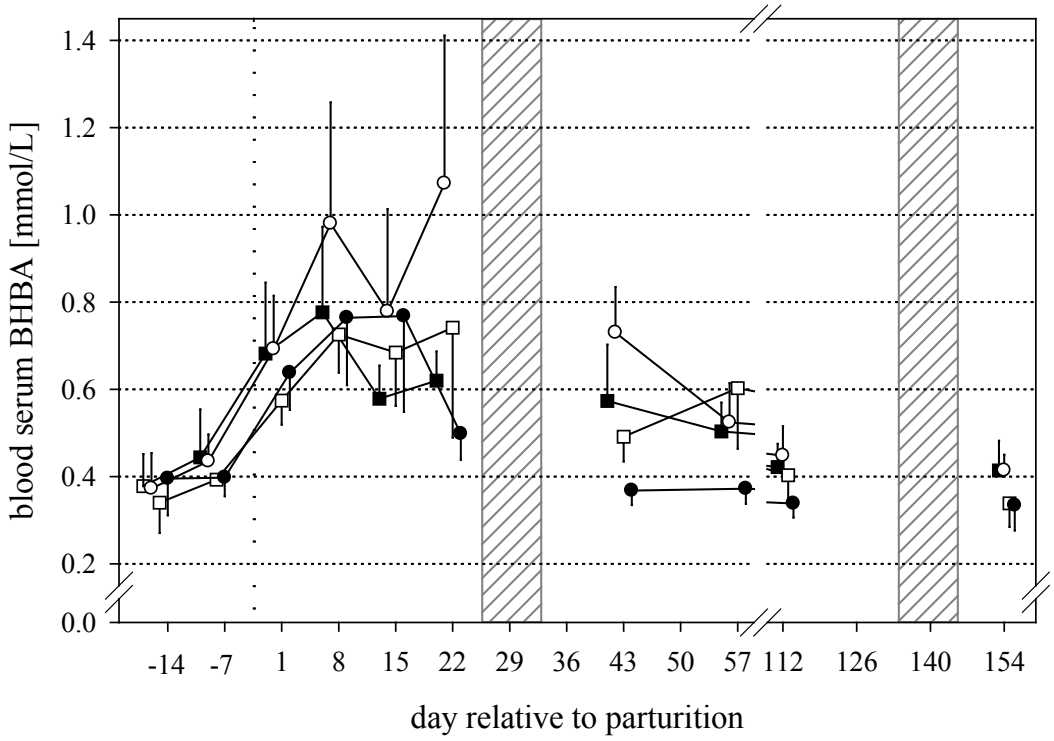

Figure 17: blood serum BHBA (mmol/L) during 155 days of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.40$ and time × group $P = 0.59$. Differences between time points and between groups can be found in table 7.

Blood serum cholesterol

At d 43 and 57 pp, Mp-cows (5.11 ± 0.35 and 5.82 ± 0.37 mmol/L) had higher blood serum cholesterol levels compared to MP- (4.59 ± 0.42 and 4.92 ± 0.53 mmol/L), mp- (4.28 ± 0.22 and 4.50 ± 0.26 mmol/L) and mP-cows (4.09 ± 0.53 and 4.25 ± 0.51 mmol/L; $P < 0.05$; table 7, figure 18). At d 113 and 155 pp, Mp-cows (6.03 ± 0.49 and 5.55 ± 0.51 mmol/L, respectively) had highest blood serum cholesterol levels compared to mp- (4.76 ± 0.17 and 4.06 ± 0.27 mmol/L; $P < 0.01$) and mP-cows (4.48 ± 0.36 and 4.22 ± 0.41 mmol/L; $P < 0.01$). Furthermore, MP-cows (5.74 ± 0.69 at d 113 pp and 5.17 ± 0.69 mmol/L at d 155 pp) had higher cholesterol levels than mp- ($P < 0.01$) and mP-cows ($P < 0.05$).

In MP-cows, blood serum cholesterol levels increased from d 8 to 15 pp by 0.86 mmol/L ($P < 0.05$), from d 22 to 43 pp by 1.56 mmol/L ($P < 0.001$) and further from d 57 to 113 pp by 0.82 mmol/L ($P < 0.001$) up to 2.75 ± 0.18, 4.59 ± 0.42 and 5.74 ± 0.69 mmol/L, respectively. In mp-cows, blood cholesterol levels increased from d 22 to 43 pp by 1.12 mmol up to 4.28 ± 0.22 mmol/L ($P < 0.001$) and declined from d 113 to 155 pp by 0.7 mmol to 4.06 ± 0.27 mmol/L ($P < 0.05$). Moreover, blood serum cholesterol levels in Mp-cows increased from d 8 to d 57 pp by 3.6 mmol up to 5.82 ± 0.37 mmol/L ($P < 0.05$) and decreased from d 113 to 155 pp from 6.03 ± 0.49 to 5.55 ± 0.51 mmol/L ($P < 0.05$). Furthermore, mP-cows showed higher blood serum cholesterol levels at d 43 pp (4.09 ± 0.53 mmol/L) than at d 22 pp (3.16 ± 0.41 mmol/L; $P < 0.05$).

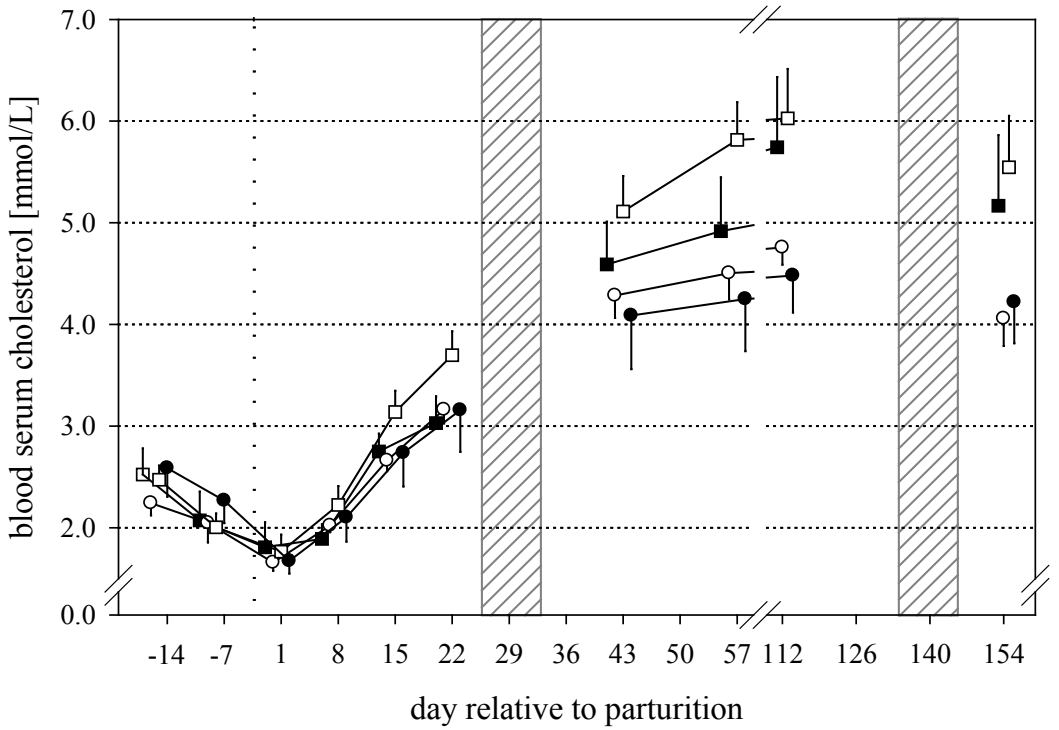

Figure 18: blood serum cholesterol (mmol/L) during 155 days of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.05$ and time × group $P < 0.001$. Differences between time points and between groups can be found in table 7.

Blood serum bilirubin

Blood serum concentrations of tBR showed no differences between groups at any time point (table 7, figure 18). From d 7 ap to day of parturition, tBR levels increased by 5.11, 6.50, 5.23 and 4.83 µmol/L in MP-, mp-, Mp- and mP-cows, respectively ($P < 0.001$). Furthermore, declining blood serum tBR levels could be observed in mp-cows from d 8 to 15 pp (by 2.08 µmol to 5.47 ± 1.33 µmol/L; $P < 0.05$) and in mP-cows from d 15 to 22 pp (by 2.94 µmol to 4.34 ± 0.38 µmol/L; $P < 0.01$).

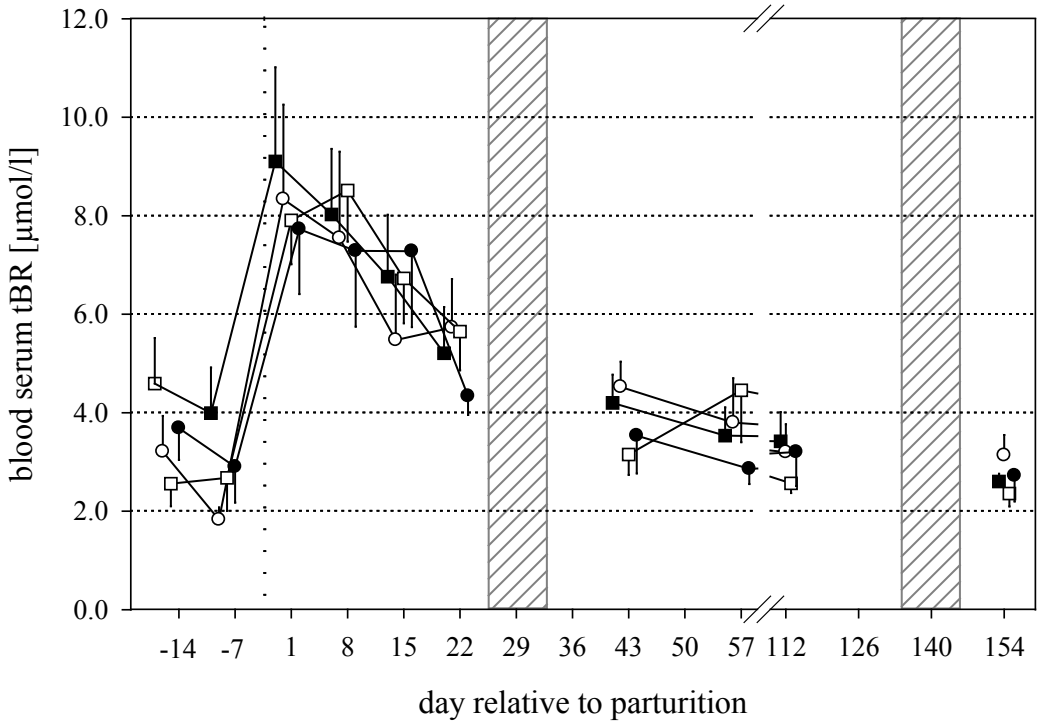

Figure 19: blood serum total bilirubin (µmol/L) during 155 days of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.79$ and time × group $P = 0.86$. Differences between time points and between groups can be found in table 7.

Table 7: Mean blood serum parameters (LSM ± SE) during 155 days of lactation.

day of lactation	MP	mp	Mp	mP
Blood serum glucose, mmol/L				
-14	4.02 ± 0.15	3.89 ± 0.23	3.82 ± 0.14	4.05 ± 0.22
-7	3.92 ± 0.16	4.19 ± 0.25	4.16 ± 0.17	4.00 ± 0.16
1	4.44 ± 0.38[a]	5.48 ± 1.28[b]*	4.18 ± 0.32[a]	4.14 ± 0.14[a]
8	3.58 ± 0.17*	3.62 ± 0.26*	3.35 ± 0.23*	3.87 ± 0.15
15	3.67 ± 0.18	3.48 ± 0.17	3.48 ± 0.17	3.81 ± 0.19
22	4.04 ± 0.24	3.65 ± 0.21	3.46 ± 0.22	3.99 ± 0.22
43	3.89 ± 0.10	3.70 ± 0.37	3.73 ± 0.19	3.75 ± 0.28
57	3.98 ± 0.13	3.77 ± 0.08	3.57 ± 0.18	4.23 ± 0.22
113	4.02 ± 0.26	3.56 ± 0.23	3.69 ± 0.12	4.02 ± 0.12
155	4.51 ± 0.19	3.79 ± 0.18	4.01 ± 0.15	3.94 ± 0.20

day of lactation	MP	mp	Mp	mP
Blood serum NEFA, µmol/L				
-14	484 ± 136	185 ± 171	83 ± 123	114 ± 152
-7	404 ± 134	136 ± 137	222 ± 116	140 ± 150
1	909 ± 157*	913 ± 143*	823 ± 93*	870 ± 260*
8	1,043 ± 124	1,104 ± 137	1,228 ± 116*	1,107 ± 137
15	837 ± 121	714 ± 165*	1,025 ± 183	1,067 ± 238
22	768 ± 138	607 ± 172	867 ± 225	551 ± 62*
43	446 ± 69*	484 ± 63	380 ± 100*	254 ± 33
57	381 ± 74	269 ± 69	349 ± 102	177 ± 19
113	118 ± 15	133 ± 31	97 ± 18	111 ± 30
155	191 ± 80	112 ± 35	105 ± 11	123 ± 44
Blood serum BHBA, mmol/L				
-14	0.38 ± 0.07	0.37 ± 0.08	0.34 ± 0.07	0.40 ± 0.08
-7	0.44 ± 0.11	0.44 ± 0.06	0.39 ± 0.01	0.40 ± 0.04
1	0.68 ± 0.16	0.69 ± 0.12	0.57 ± 0.06	0.64 ± 0.09
8	0.78 ± 0.20	0.98 ± 0.28*	0.73 ± 0.09	0.76 ± 0.15
15	0.58 ± 0.08	0.78 ± 0.24	0.68 ± 0.12	0.77 ± 0.22
22	0.62 ± 0.07^{a}	1.07 ± 0.34^{b}*	0.74 ± 0.25^{a}	0.50 ± 0.06^{a}
43	0.57 ± 0.13^{ab}	0.73 ± 0.10^{ab}*	0.49 ± 0.06^{a}	0.37 ± 0.03^{b}
57	0.50 ± 0.07	0.52 ± 0.09	0.60 ± 0.14	0.37 ± 0.03
113	0.42 ± 0.05	0.45 ± 0.07	0.40 ± 0.06	0.34 ± 0.03
155	0.41 ± 0.07	0.41 ± 0.04	0.34 ± 0.05	0.33 ± 0.06
Blood serum cholesterol, mmol/L				
-14	2.52 ± 0.26	2.24 ± 0.12	2.47 ± 0.14	2.59 ± 0.28
-7	2.07 ± 0.28	2.05 ± 0.19	2.01 ± 0.14	2.27 ± 0.22
1	1.81 ± 0.25	1.66 ± 0.08	1.77 ± 0.17	1.67 ± 0.12
8	1.89 ± 0.14	2.02 ± 0.06	2.22 ± 0.19	2.10 ± 0.24
15	2.75 ± 0.18*	2.66 ± 0.10	3.14 ± 0.21*	2.74 ± 0.33
22	3.03 ± 0.26	3.16 ± 0.13	3.70 ± 0.23*	3.16 ± 0.41
43	4.59 ± 0.42^{a}*	4.28 ± 0.22^{a}*	5.11 ± 0.35^{b}*	4.09 ± 0.53^{a}*
57	4.92 ± 0.53^{a}	4.50 ± 0.26^{a}	5.82 ± 0.37^{b}*	4.25 ± 0.51^{a}
113	5.74 ± 0.69^{a}*	4.76 ± 0.17^{b}	6.03 ± 0.49^{a}	4.48 ± 0.36^{b}
155	5.17 ± 0.69^{a}	4.06 ± 0.27^{b}*	5.55 ± 0.51^{a}*	4.22 ± 0.41^{b}
Blood serum bilirubin, µmol/L				
-14	4.59 ± 0.92	3.21 ± 0.72	2.56 ± 0.46	3.69 ± 0.65
-7	3.99 ± 0.93	1.83 ± 0.25	2.68 ± 0.67	2.90 ± 0.72
1	9.10 ± 1.91*	8.33 ± 1.92*	7.91 ± 0.89*	7.73 ± 1.32*
8	8.03 ± 1.33	7.55 ± 1.75	8.51 ± 1.04	7.29 ± 1.54
15	6.76 ± 1.26	5.47 ± 1.33*	6.73 ± 0.91	7.28 ± 1.54
22	5.21 ± 0.94	5.73 ± 0.99	5.65 ± 0.79	4.34 ± 0.38*
43	4.20 ± 0.57	4.52 ± 0.51	3.15 ± 0.41	3.53 ± 0.77
57	3.53 ± 0.57	3.80 ± 0.90	4.46 ± 1.05	2.86 ± 0.31
113	3.42 ± 0.59	3.20 ± 0.56	2.57 ± 0.20	3.20 ± 0.69
155	2.60 ± 0.15	3.13 ± 0.41	2.36 ± 0.26	2.72 ± 0.52

^{abcd}Means with alphabetic superscripts indicate differences between groups ($P < 0.05$).

*Means with asterisks indicate differences to previous sampling time point ($P < 0.05$).

1.5. Body weight, body condition score and back-fat-thickness

Body weight

All cows showed higher body weights prior to parturition and lost body weight during the first six weeks of lactation. Afterwards cows remained on low body weight levels or showed slowly increasing body weights.

In all groups, body weight decreased from wk 1 ap to wk 2 pp and further to wk 4 pp (823 ± 23 to 689 ± 21 kg for MP-cows, 774 ± 54 to 626 ± 46 kg mp-cows, 773 ± 27 to 642 ± 20 kg for Mp- and 792 ± 28 to 677 ± 32 kg for mP-cows; $P < 0.001$, figure 20). Only in Mp-cows, weight increased from wk -3 to -1 pp (730 ± 26 to 773 ± 27 kg; $P < 0.05$). In mP-cows, body weight decreased significantly from wk 4 to 6 pp ($P < 0.05$).

From wk 2 pp on, MP-cows lost 10.3% (75 kg) of their body weight until wk 12 pp, mp-cows lost 12.9% (90 kg) until wk 10 pp, Mp-cows 10.0% (69 kg) until wk 8 pp and mP-cows lost 9.7% (70 kg) until wk 6 pp.

Only mP-cows showed slowly increasing body weights after wk 6 pp. At last weighing in wk 22 pp, they showed higher body weight (737 ± 19 kg) compared to Mp-cows (606 ± 29 kg; $P < 0.05$).

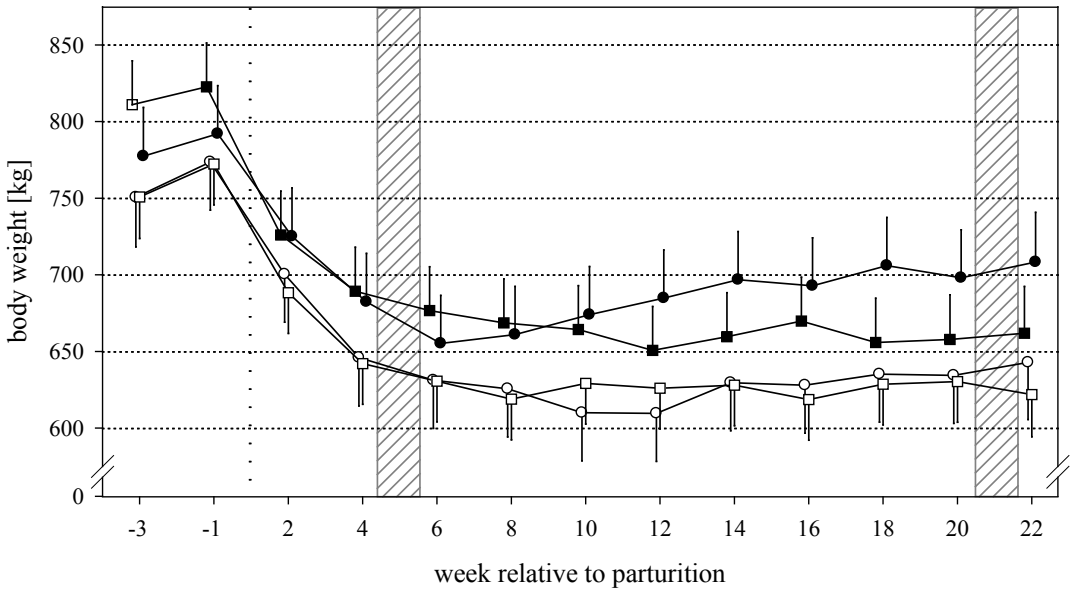

Figure 20: body weight during two weeks before and 22 weeks after parturition

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions and dashed line parturition. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.42$ and time × group $P = 0.72$.

Body condition score

From wk 3 ap on, all cows showed decreasing BCS until wk 10 pp. Only mP-cows had slowly increasing BCS from wk 12 pp until end of study (figure 21).

In MP-cows, BCS decreased from wk 3 to 1 ap by 0.36 points to 4.04 ± 0.24 ($P < 0.05$). Further decrease from wk 1 ap to wk 2 pp was seen in MP-, Mp- and mP-cows. BCS decreased from

4.04 ± 0.24 to 3.21 ± 0.10, from 3.57 ± 0.13 to 3.04 ± 0.10 and from 3.85 ± 0.26 to 3.30 ± 0.20 in MP-, Mp- and mP-cows, respectively ($P < 0.001$). In mp- and mP-cows, BCS decreased from wk 2 to 4 pp by 0.25 points to 3.25 ± 0.32 and 3.05 ± 0.12 ($P < 0.05$). Furthermore, BCS decreased in MP-, mp- and Mp-cows from wk 4 to 6 pp by 0.29, 0.40 and 0.21 points from 3.13 ± 0.14 to 2.83 ± 0.15 ($P < 0.01$), 3.25 ± 0.32 to 2.85 ± 0.28 ($P < 0.001$) and from 2.89 ± 0.12 to 2.68 ± 0.12 ($P < 0.05$) for MP-, mp- and Mp-cows, respectively. Moreover, BCS of mP-cows decreased from wk 6 to 8 pp (2.90 ± 0.23 to 2.60 ± 0.22; $P < 0.01$) and from wk 10 to 12 pp (2.70 ± 0.20 to 2.45 ± 0.23; $P < 0.05$).

In MP-cows, BCS decreased from wk 3 ap until wk 18 pp by 38.6% (1.65). Moreover, BCS decreased from wk 3 ap to wk 12 pp by 37.7% (1.48) in mp-cows, by 31.2% (1.07) in Mp-cows and by 36.4% (1.40) in mP-cows.

At first estimation of BCS in wk 3 ap, Mp-cows (3.50 ± 0.08) showed lower BCS compared to MP-cows (4.40 ± 0.19; $P < 0.01$). Differences at following measurements proved not to be significant.

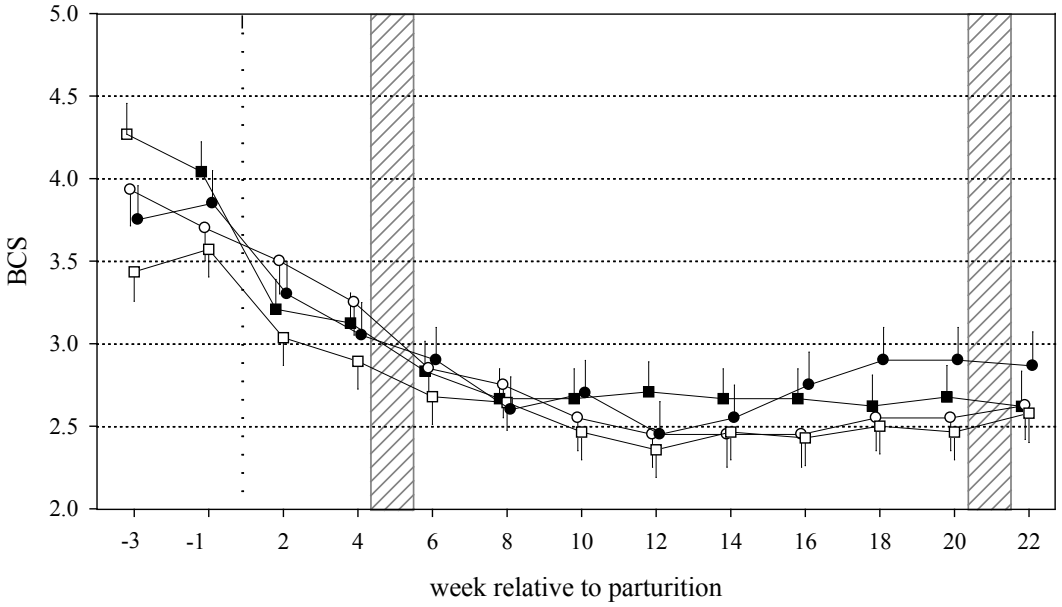

Figure 21: body condition score during two weeks before and 22 weeks after parturition

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions and dashed line parturition. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.49$ and time × group $P = 0.12$.

Back-fat thickness

Similar to BCS, all cows showed declining BFT from wk 3 ap until wk 12 pp and afterwards remained low until end of study.

No differences in BFT between cows could be seen during all estimations, except of one difference at wk 3 ap between MP- (25.0 ± 3.8 mm) and mP-cows (20.3 ± 5.1; $P < 0.05$; figure 22). In mp-cows, there was a decrease in BFT from wk 2 to 4 pp by 5.2 mm (21.0 ± 5.0 mm to 15.8 ± 3.3 mm; $P < 0.001$) and from wk 6 to 8 pp by 3.4 mm (15.2 ± 3.7 mm to 11.8 ± 2.1 mm; $P < 0.05$). Furthermore, BFT declined in Mp-cows from wk 1 ap to wk 4 pp by 7 mm (24.8 ± 2.6 mm to 17.8 ± 1.6 mm; $P < 0.01$) and from wk 6 to 8 pp by 3.8 mm (12.8 ± 1.9 mm to 9.0 ± 1.2 mm; $P < 0.05$). From wk 3 to 1 before parturition, BFT of mP-cows increased by 1.9 mm

(20.3 ± 5.1 mm to 22.2 ± 5.6 mm; P < 0.01). Afterwards, BFT of mP-cows decreased from wk 2 until wk 4 pp (to 15.6 ± 2.8 mm; P < 0.01).

Before parturition until wk 12 (MP-), 20 (mp-), 18 (Mp-) and 6 pp (mP-cows), BFT declined by 69.4% (17.7 mm) in MP-cows, by 54.9% (11.2 mm) in mp-cows, by 70.0% (14.9 mm) in Mp-cows and by 52.3% (11.6 mm) in mP-cows.

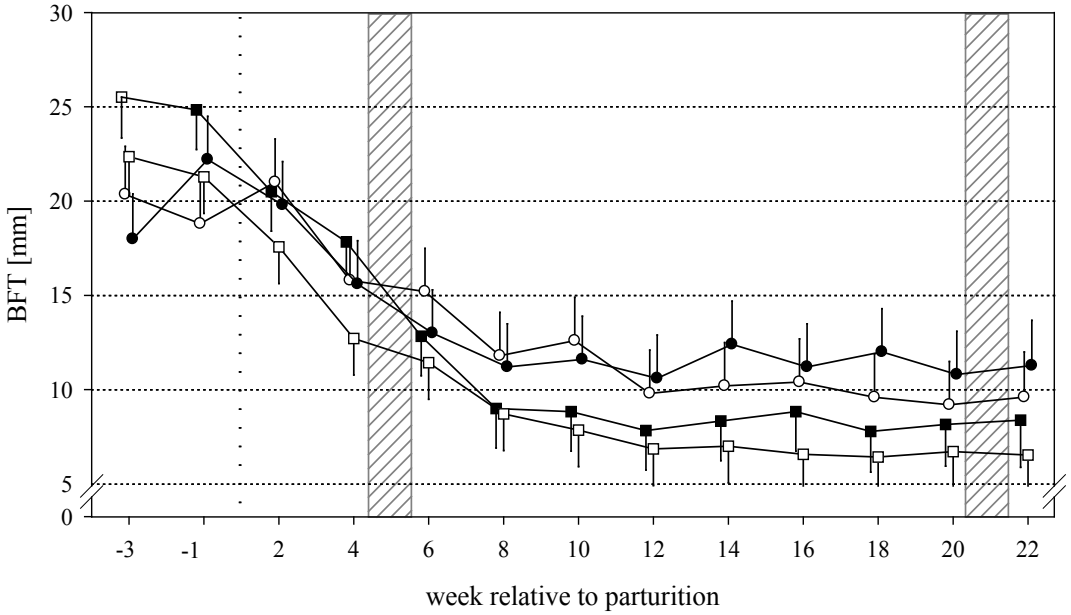

Figure 22: Back-fat-thickness during two weeks before and 22 weeks after parturition

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Shaded areas show feed restrictions and dashed line parturition. Values are presented as LSM ± SEM. Fixed effects in model: time P < 0.001, group P = 0.50 and time × group P = 0.31.

1.6. Cyclicity and health status

Ovarial cysts occurred in one mp- and two Mp-cows. These cows had to be treated and were not considered for statistical analysis of cyclicity. Day of first ovulation was comparable among groups (24 ± 4, 24 ± 6, 31 ± 4 and 22 ± 3 d pp for MP-, mp-, Mp- and mP-cows, respectively). First ovulation prior to d 30 pp was found in 67% of MP-, 75% of mp-, 40% of Mp- and 100% of mP-cows (table 8).

Table 8: Cyclicity

	MP	mp	Mp	mP
Ovarial cysts, %	0	20	29	0
First ovulation, d	24 ± 4	24 ± 6	31 ± 4	22 ± 3
First ovulation before d 30 pp, %	67	75	40	100

In this study, one mP-cow suffered on mastitis (fever, turgid mammary gland and strongly positive California Mastitis Test; Barnum and Newbould, 1961), 7 cows on retained placenta (retention > 8 hours, veterinary intervention necessary; 3 MP-, 1 mp-, 2 Mp- and 1 mP-cow) and one Mp-cow had a clinical ketosis diagnosed by clinical signs (inappetence, declining milk yield) and urinary ketone bodies. Lameness occurred in 11 cows, thereof 3 MP-cows, 1 mp-cow, 4 Mp-, and 3 mP-cows (table 9).

Table 9: Occurrence of health disorders and first day of diagnosis[1].

	MP	mp	Mp	mP
Mastitis				1 (32)
Retained placenta	3 (1)	1 (1)	2 (1)	1 (1)
Ketosis	1 (5)			
Lameness	3 (-2, 19, 50)	1 (5)	4 (-7, -2, 20, 48)	3 (7, 11, 18)

[1]Data in parentheses indicate the first day of diagnosis relative to calving.

1.7. 100 day milk performance in previous and current lactation

Mean 100-d-performances of all animals during the previous (farm in Saxony) and the current (Veitshof, Bavaria) lactation are shown in table 10.

100-d-performance of milk yield increased in Mp-cows by 715 kg ($P < 0.05$). Consequently, milk fat and protein yield increased by 43 and 26 kg ($P < 0.001$ and $P < 0.01$) in these cows. Furthermore, milk fat yield increased in MP-cows by 38 kg ($P < 0.01$).

Table 10: 100-d-performance of cows in previous lactation (Saxony, SN) and current lactation (Bavaria, BY).

Parameter	Lact.	MP	mp	Mp	mP
Milk yield, kg	SN	3,519 ± 303	3,637 ± 270	3,711 ± 334	3,478 ± 208
	BY	4,054 ± 171^{a}	3,448 ± 176^{b}	4,426 ± 137^{a}*	3,205 ± 135^{b}
Milk protein content, %	SN	3.4 ± 0.1^{a}	3.0 ± 0.1^{b}	2.9 ± 0.1^{b}	3.4 ± 0.2^{a}
	BY	3.2 ± 0.1^{a}	3.0 ± 0.1^{ab}	3.0 ± 0.0^{b}	3.5 ± 0.1^{c}
Milk protein yield, kg	SN	118 ± 9	109 ± 11	106 ± 9	119 ± 10
	BY	131 ± 7^{a}	103 ± 7^{b}	132 ± 3^{a}*	113 ± 6^{b}
Milk fat content, %	SN	4.4 ± 0.2^{a}	4.0 ± 0.1^{ab}	3.8 ± 0.2^{b}	4.4 ± 0.2^{a}
	BY	4.8 ± 0.2^{a}	4.0 ± 0.2^{b}	4.1 ± 0.3^{ab}	4.4 ± 0.3^{ab}
Milk fat yield, kg	SN	156 ± 15	145 ± 11	138 ± 10	152 ± 6
	BY	194 ± 8^{a}*	139 ± 14^{b}	181 ± 8^{a}*	142 ± 9^{b}

[abcd]Means with alphabetic superscripts indicate differences between groups ($P < 0.05$).
*Means with asterisks indicate differences to previous lactation ($P < 0.05$).
Fixed effects in model milk yield: group $P = 0.06$, lactation $P = 0.20$, lactation × group $P = 0.06$. Fixed effects in model milk fat yield: group $P = 0.07$, lactation $P < 0.05$, lactation × group $P < 0.01$. Fixed effects in model milk protein yield: group $P = 0.27$, lactation $P = 0.19$, lactation × group $P = 0.08$. Fixed effects in model milk fat content: group $P < 0.05$, lactation $P = 0.10$, lactation × group $P = 0.50$. Fixed effects in model milk protein content: group $P < 0.001$, lactation $P = 0.77$, lactation × group $P = 0.15$.

2. Hepatic mRNA expression during 22 weeks of lactation

Abundance of hepatic mRNA during lactation was measured in 127 liver tissue samples of 23 animals (thereof 41 after FR) and assorted to 4 groups like described before (MP-, mp-, Mp- and mP-cows). Results of *PCK2* and *PPARG* were omitted, because five or more measurements failed in each group independent of sample day and cow. Furthermore, results of single samples were left unconsidered, if minimum five RT-qPCR runs with different primers did not work. Single results were omitted at d 1 pp (34311, mP-cow), d 29 pp (03463, MP-cow), d 57 pp (03827, Mp-cow), d 144 pp (03463, MP-cow) and at day of slaughtering, d 155 pp (03827, Mp-; 34303, mp- and 15263, mP-cow).

Lipid metabolism

For characterisation of hepatic lipid metabolism during 155 days of lactation, mRNA abundance of *ACACA*, *ACADVL*, *CPT1A*, *ECHS1* and *GPAM* was measured (table 11, figure 23). In *ACACA*, no differences were observed between groups at any time point. Transcript abundance of *ACACA* in mp-cows was higher at d 57 pp (10.48 ± 0.74) compared to d 15 pp (8.68 ± 0.69; $P < 0.05$). In Mp-cows, mRNA abundance was highest at d 57 pp (9.90 ± 0.24; $P < 0.05$) and higher at d 15 pp (8.49 ± 0.73) compared to day of slaughtering (6.54 ± 0.61; $P < 0.05$). Transcript abundance of *ACACA* in mP-cows was lowest at day of slaughtering (7.87 ± 1.85; $P < 0.05$).

Moreover, mRNA encoding for *ACADVL* showed lowest abundance in mp-cows at d 15 pp (13.09 ± 0.19; $P < 0.05$). At d 57 pp, mRNA levels were higher in Mp-cows (13.59 ± 0.17) compared to mP-cows (13.00 ± 0.21; $P < 0.05$) and at day of slaughtering, mRNA abundance was higher in MP- (13.77 ± 0.21) compared to mP-cows (13.10 ± 0.21; $P < 0.05$). In mp-cows, transcript abundance of *ACADVL* was higher at day of parturition (13.89 ± 0.19) compared to d 15 ($P < 0.01$) and 57 pp (13.27 ± 0.21; $P < 0.05$). Furthermore, in Mp-cows mRNA levels were lower at day of slaughtering (13.20 ± 0.24) compared to d 15 pp (13.85 ± 0.16; $P < 0.05$). In mP-cows, lower mRNA abundance of *ACADVL* was measured at d 57 and 155 pp compared to day of parturition (13.97 ± 0.24; $P < 0.01$) and d 15 pp (13.85 ± 0.21; $P < 0.05$).

Transcript abundance of *CPT1A* was lowest in mp-cows at d 15 pp (12.71 ± 0.25; $P < 0.05$) amongst all cows and in mP-cows lowest at day of slaughtering (12.56 ± 0.27; $P < 0.05$).

Moreover, at d 155 pp mRNA encoding for *ECHS1* showed higher abundance in MP-cows (15.08 ± 0.22) compared to mp-cows (14.44 ± 0.19; $P < 0.05$). In MP-cows, mRNA abundance of *ECHS1* was higher at d 57 pp (15.42 ± 0.14) compared to day of parturition (14.77 ± 0.21; $P < 0.05$) and day of slaughtering ($P < 0.01$). Additionally, mp-cows showed highest mRNA abundance at d 57 pp (15.51 ± 0.13; $P < 0.05$) and higher mRNA levels of *ECHS1* at d 15 pp (15.12 ± 0.21) compared to day of slaughtering ($P < 0.01$). In Mp-cows, transcript abundance of *ECHS1* was higher at d 57 pp (15.65 ± 0.11) compared to day of parturition (14.90 ± 0.22; $P < 0.01$). Moreover, mRNA levels were lower at d 155 pp (14.60 ± 0.16) compared to d 15 (15.47 ± 0.17; $P < 0.001$) and 57 pp ($P < 0.001$). In mP-cows, mRNA transcripts of *ECHS1* were lowest at d 155 pp (14.93 ± 0.19) compared to d 15 pp (15.52 ± 0.22; $P = 0.03$) and d 57 pp (15.51 ± 0.13; $P < 0.001$).

At d 155 pp, transcript abundance of *GPAM* was lower in mP-cows (10.34 ± 0.31) compared to MP- (11.66 ± 0.31; $P < 0.01$) and mp-cows (11.59 ± 0.35; $P < 0.01$). In MP-cows, mRNA level of *GPAM* was lower at day of parturition (11.25 ± 0.28) compared to d 15 pp (12.17 ± 0.31; $P < 0.01$) and d 57 pp (12.12 ± 0.31; $P < 0.05$). Furthermore, mRNA abundance in mp-cows was higher at d 57 pp (12.48 ± 0.31) compared to day of parturition (11.37 ± 0.28; $P < 0.01$) and day of slaughtering ($P < 0.05$). In Mp-cows, mRNA levels of *GPAM* were higher at d 15 pp (12.14 ± 0.24; $P < 0.01$) and d 57 pp (12.19 ± 0.25; $P < 0.001$) compared to day of slaughtering (10.93 ± 0.30). Moreover, mP-cows showed lowest transcript abundance at day of slaughtering ($P < 0.001$).

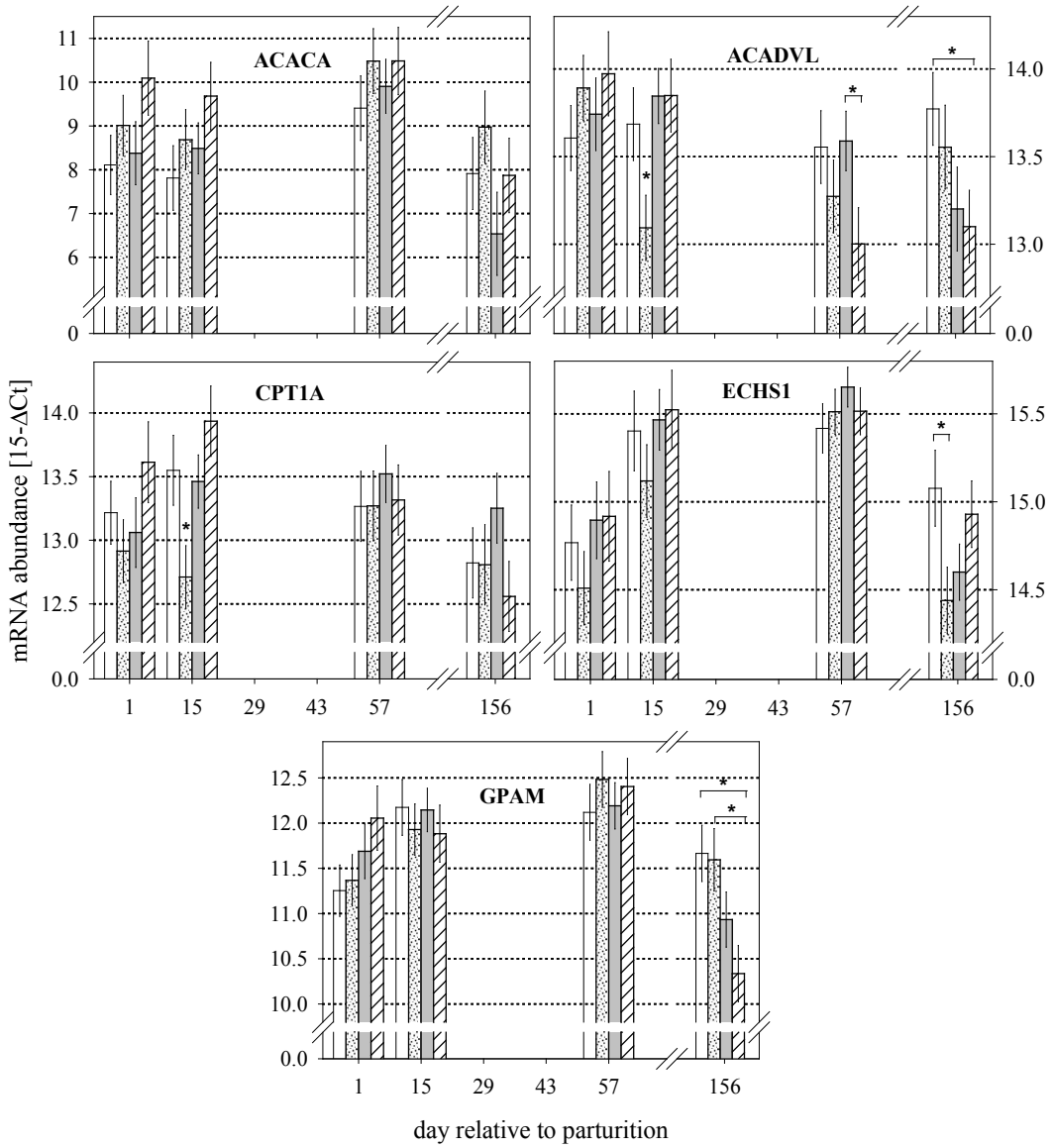

Figure 23: Changes in mRNA abundance of genes related to hepatic lipid metabolism during 155 days of lactation.

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes. Asterisks indicate differences between groups ($P < 0.05$). Differences between time points and between groups can be found in table 11. Fixed effects in model *ACACA*: time $P < 0.001$, group $P = 0.31$ and time × group $P = 0.64$. Fixed effects in model *ACADVL*: time $P < 0.05$, group $P = 0.53$ and time × group $P < 0.05$. Fixed effects in model *CPT1A*: time $P < 0.05$, group $P = 0.22$ and time × group $P = 0.23$. Fixed effects in model *ECHS1*: time $P < 0.001$, group $P = 0.29$ and time × group $P < 0.01$. Fixed effects in model *GPAM*: time $P < 0.001$, group $P = 0.94$ and time × group $P < 0.05$.

Protein metabolism

Concerning hepatic protein metabolism, mRNA abundance of *CTSL* and *TAT* was measured (table 11, figure 24).

At day of parturition and at d 15 pp, mRNA levels of *CTSL* were higher in mp- (15.79 ± 0.23 and 15.94 ± 0.22) compared to MP-cows (14.95 ± 0.23 and 15.20 ± 0.25 at day of parturition and d 15 pp, respectively; $P < 0.05$). No differences between time points were observed in transcript abundances of *CTSL* in MP-cows, whereas in mp-cows mRNA level was lowest at day of slaughtering (14.44 ± 0.28; $P < 0.001$). Furthermore in Mp- and mP-cows, transcript abundance was higher at d 57 pp (15.77 ± 0.20 and 15.78 ± 0.25) compared to day of slaughtering

(14.95 ± 0.24 and 14.93 ± 0.25 for Mp- and mP-cows; $P < 0.01$).

Transcript abundances of *TAT* showed no differences between groups during time points. In MP-, Mp- and mP-cows, mRNA levels of *TAT* were higher at day of slaughtering (16.43 ± 0.32, 16.44 ± 0.32 and 16.59 ± 0.32, for MP-, Mp- and mP-cows) compared to d 15 pp (in MP-cows 15.47 ± 0.32, $P < 0.05$; in Mp-cows 15.54 ± 0.24, $P < 0.05$ and in mP-cows 15.40 ± 0.32, $P < 0.01$). Furthermore, mRNA encoding for *TAT* showed higher abundance in Mp-cows at d 155 pp compared to day of parturition (15.36 ± 0.32; $P < 0.05$).

Carbohydrate metabolism

Regarding hepatic carbohydrate metabolism, mRNA levels of *PC* and *PCK1* were determined (table 11, figure 24).

MP-cows showed lower mRNA levels of *PC* at day of parturition (10.01 ± 0.69) compared to mP-cows (12.32 ± 0.87; $P < 0.05$) and at d 57 pp (7.61 ± 0.76) compared to Mp-cows (9.96 ± 0.63; $P < 0.05$). Furthermore transcript abundances of *PC* were higher in MP-cows at day of parturition compared to d 57 pp ($P < 0.01$). In mp-cows, highest mRNA level was observed at day of parturition (11.64 ± 0.69; $P < 0.05$). Moreover, mRNA levels of *PC* in mP-cows were higher at day of parturition compared to d 57 pp (8.31 ± 0.77; $P < 0.001$) and d 155 pp (9.67 ± 0.76; $P < 0.01$). Additionally transcript abundance in mP-cows at d 15 pp (10.36 ± 0.77) was higher compared to d 57 pp ($P < 0.05$).

At day of parturition, mRNA levels of *PCK1* were higher in mP- (16.47 ± 0.72) compared to mp-cows (14.52 ± 0.56; $P < 0.05$), whereas at day of slaughtering transcript abundance of *PCK1* was higher in mp- (17.19 ± 0.72) compared to mP-cows (15.11 ± 0.62; $P < 0.05$). MP-cows showed lower mRNA levels at day of parturition (15.16 ± 0.56) compared to d 15 pp (16.95 ± 0.62; $P < 0.05$) and d 57 pp (17.40 ± 0.62; $P < 0.01$). Furthermore transcript abundance of *PCK1* was higher in MP-cows at d 57 pp compared to day of slaughtering (15.69 ± 0.62; $P < 0.05$). In mp-cows, mRNA levels were lowest at day of parturition compared to d 57 pp (17.17 ± 0.62; $P < 0.01$) and d 155 pp ($P < 0.01$). At d 57 pp, Mp-cows showed higher mRNA levels (17.12 ± 0.51) compared to d 155 pp (15.15 ± 0.72; $P < 0.05$). Moreover, mP-cows had lowest transcript abundances of *PCK1* at d 155 pp compared to d 15 pp (17.19 ± 0.63; $P < 0.05$) and d 57 pp (16.87 ± 0.62; $P < 0.05$).

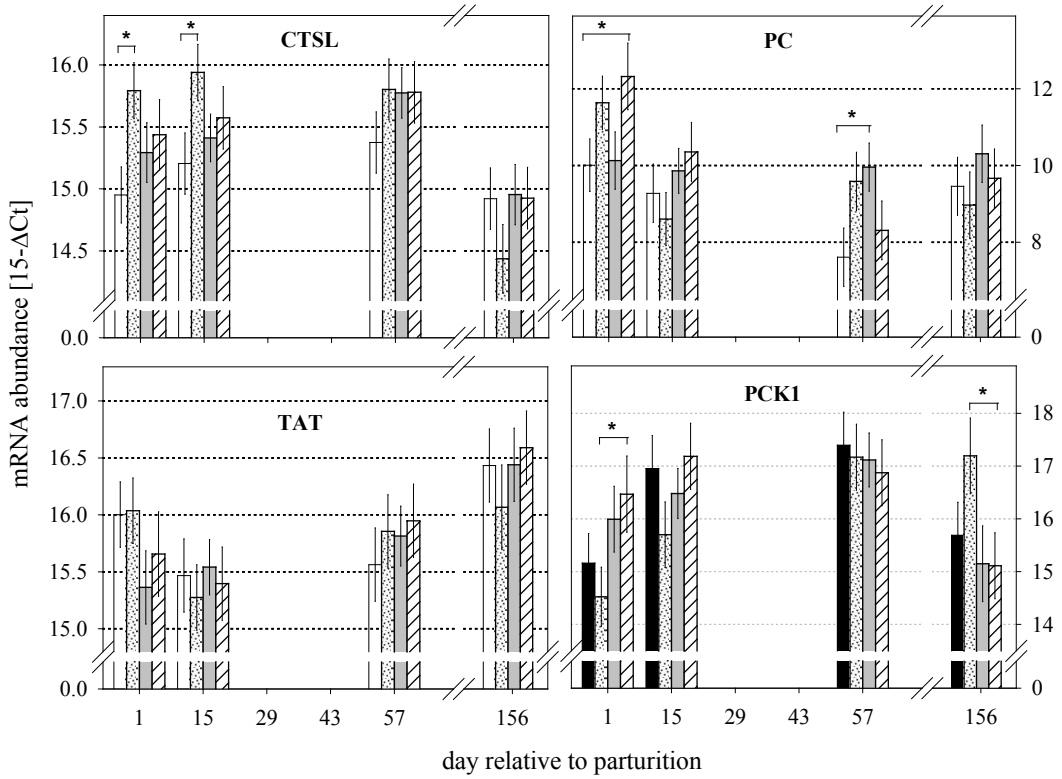

Figure 24: Changes in mRNA abundance of genes related to hepatic protein (left figures) and carbohydrate metabolism (right figures) during 155 days of lactation.

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes. Asterisks indicate differences between groups ($P < 0.05$). Differences between time points and between groups can be found in table 11. Fixed effects in model *CTSL*: time $P < 0.001$, group $P = 0.40$ and time × group $P = 0.29$. Fixed effects in model *TAT*: time $P < 0.01$, group $P = 0.96$ and time × group $P = 0.77$. Fixed effects in model *PC*: time $P < 0.001$, group $P = 0.42$ and time × group $P = 0.08$. Fixed effects in model *PCK1*: time $P < 0.01$, group $P = 0.95$ and time × group $P = 0.16$.

Glucose metabolism and ketogenesis

For better description of hepatic glucose metabolism and ketogenesis, mRNA abundance of *SLC2A2*, *INSR* and *HMGCS2* was determined (table 11, figure 25). At day of slaughtering, mRNA levels of *SLC2A2* were lowest in Mp-cows (11.98 ± 0.40) compared to mp- (13.30 ± 0.46; $P < 0.05$) and mP-cows (13.25 ± 0.40; $P < 0.05$). Furthermore mp-cows showed higher mRNA abundance of *SLC2A2* at d 57 pp (14.21 ± 0.40) compared to d 15 pp (12.90 ± 0.36; $P < 0.05$). Moreover Mp-cows had lowest transcript abundance of *SLC2A2* at day of slaughtering ($P < 0.05$).

At day of parturition, highest mRNA level of *INSR* was observed in mP-cows (12.27 ± 0.24) compared to MP- (11.33 ± 0.19; $P < 0.01$) and Mp-cows (11.58 ± 0.21; $P < 0.05$). Furthermore at d 155 pp, Mp-cows showed higher mRNA abundance compared to mP-cows (12.10 ± 0.21 and 11.50 ± 0.21 for Mp- and mP-cows; $P < 0.05$). Moreover in mP-cows, mRNA encoding for *INSR* showed lowest abundance at day of slaughtering compared to day of parturition ($P < 0.05$) and d 15 pp (12.11 ± 0.21; $P < 0.05$).

At d 57 pp, transcript abundance of *HMGCS2* was higher in MP- (16.16 ± 0.59) compared to Mp-cows (14.40 ± 0.49; $P < 0.05$). Furthermore at day of slaughtering, mRNA levels of *HMGCS2* were lower in Mp-cows (13.21 ± 0.58) compared to MP- (14.99 ± 0.59; $P < 0.05$) and mP-cows (15.33 ± 0.60; $P < 0.05$). In MP-cows, mRNA abundance of *HMGCS2* was lower at day of

parturition (14.37 ± 0.54) compared to d 15 pp (16.10 ± 0.59; P < 0.01) and d 57 pp (16.16 ± 0.59; P < 0.01). Moreover, in mp-cows mRNA levels were lowest at d 1 pp (13.08 ± 0.55; P < 0.05). Transcript abundance of *HMGCS2* in Mp-cows was highest at d 15 pp (15.08 ± 0.46) compared to d 1 pp (13.70 ± 0.58; P < 0.05) and d 155 pp (P < 0.01).

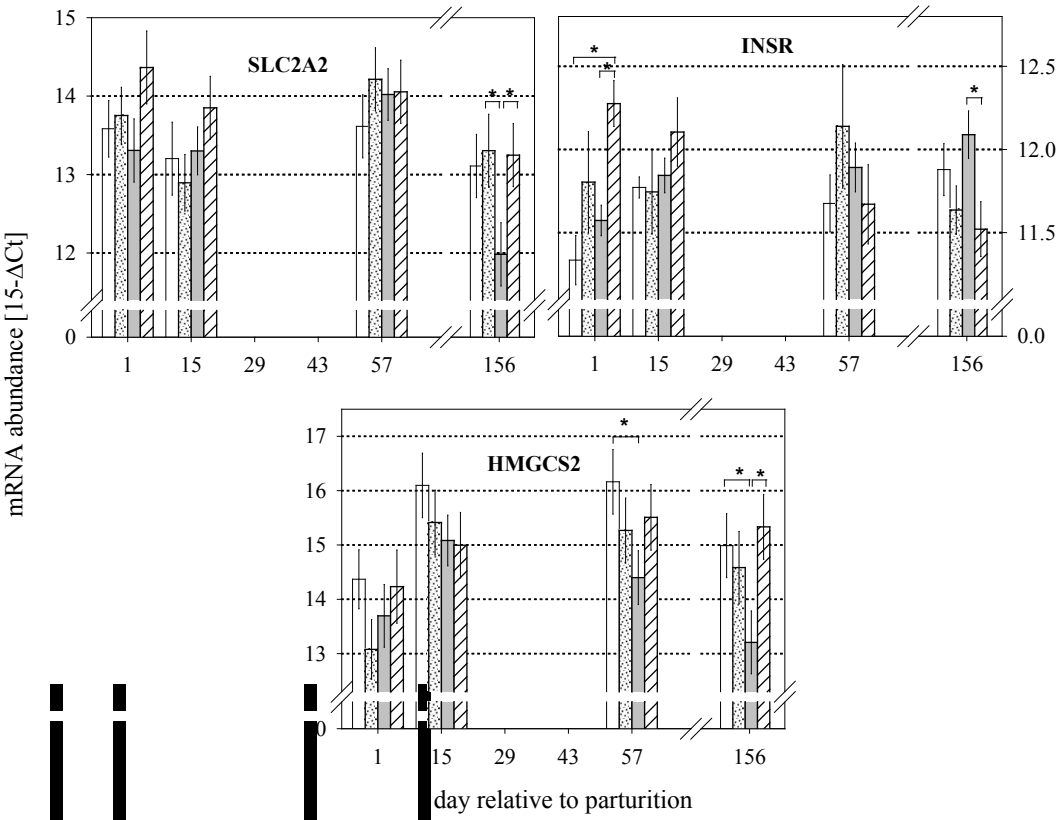

Figure 25: Changes in mRNA abundance of genes related to hepatic glucose metabolism and ketogenesis during 155 days of lactation.

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes. Asterisks indicate differences between groups (P < 0.05). Differences between time points and between groups can be found in table 11. Fixed effects in model *SLC2A2*: time P < 0.01, group P = 0.09 and time × group P = 0.48. Fixed effects in model *INSR*: time P = 0.79, group P = 0.51 and time × group P = 0.07. Fixed effects in model *HMGCS2*: time P < 0.001, group P = 0.15 and time × group P = 0.36.

Translation and transcription factors

For hepatic translation factors and transcription regulating factors mRNA encoding for *EIF4B*, *HNF4A*, *PPARA* and *SREBF1* was measured (table 11, figure 26).

Hepatic transcript abundance of translation factor *EIF4B* showed no differences between groups at any time point. In Mp- and mP-cows, mRNA levels at day of parturition (13.80 ± 0.32 and 13.68 ± 0.37 for Mp- and mP-cows) were lowest compared to d 15 pp (Mp-cows 14.57 ± 0.25, mP-cows 14.55 ± 0.33, P < 0.05) and d 155 pp (Mp-cows 14.95 ± 0.32, mP-cows 15.13 ± 0.33, P < 0.01). Furthermore mRNA abundance in mP-cows at d 155 pp was higher compared to d 57 pp (14.16 ± 0.33; P < 0.01).

Moreover mRNA encoding for *HNF4A* showed higher abundance at d 15 pp in MP-cows (13.50 ± 0.34) compared to mp-cows (12.52 ± 0.32; P < 0.05). In MP-cows mRNA levels were higher at d 155 pp (13.85 ± 0.34) compared to d 57 pp (12.99 ± 0.34; P < 0.01). Furthermore in mp-cows mRNA abundance at d 15 pp was lower compared to d 57 pp (13.28 ± 0.34; P < 0.01)

and day of slaughtering (13.67 ± 0.37; P < 0.01). Mp-cows had highest mRNA levels at day of slaughtering (14.39 ± 0.33; P < 0.01). In mP-cows hepatic mRNA abundance of *HNF4A* was lower at d 57 pp (12.71 ± 0.34) compared to day of parturition (13.64 ± 0.39; P < 0.05) and day of slaughtering (13.91 ± 0.34; P < 0.001).

Additionally hepatic mRNA encoding for *PPARA* showed no differences between groups at any time point. In mp-cows mRNA levels at d 1 pp (13.17 ± 0.17) and d 15 pp (13.30 ± 0.17) were lower compared to d 57 pp (13.90 ± 0.19; P < 0.05) and d 155 pp (13.91 ± 0.22; P < 0.05). Transcript abundance of *PPARA* in Mp-cows was higher at d 57 pp (14.05 ± 0.16) compared to day of parturition (13.44 ± 0.19; P < 0.05).

Hepatic mRNA transcripts of *SREBF1* showed no differences between groups at any time point, whereas in MP-cows mRNA levels at day of parturition were lowest (6.63 ± 0.48; P < 0.01). Additionally mRNA transcripts in MP-cows were lower at d 57 pp (8.09 ± 0.52) compared to d 155 pp (9.47 ± 0.51; P < 0.05). In mp-cows hepatic mRNA abundance of *SREBF1* was lower at d 1 and 15 pp (7.13 ± 0.49 and 7.26 ± 0.53) compared to d 57 (8.95 ± 0.52; P < 0.01) and 155 pp (8.90 ± 0.58; P < 0.01). Furthermore mRNA level of *SREBF1* was lowest in Mp-cows at day of parturition (6.52 ± 0.50; P < 0.01) and concurrently mRNA abundance at d 15 pp (8.17 ± 0.41) was lower compared to d 155 pp (9.24 ± 0.50; P < 0.05).

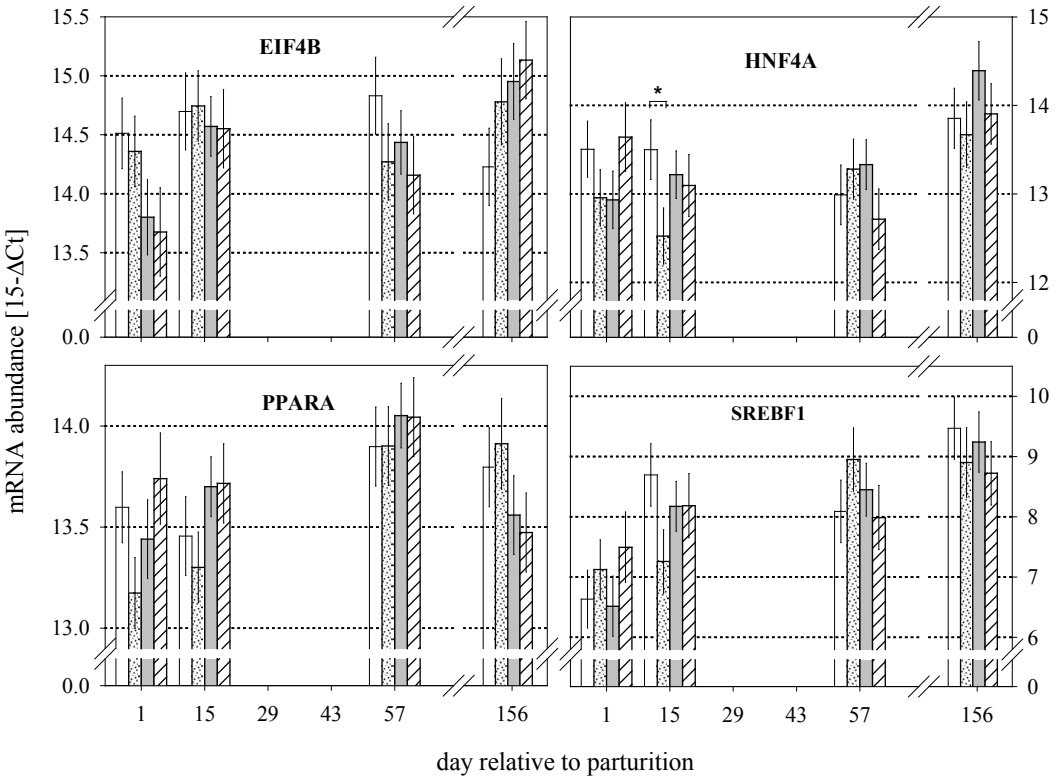

Figure 26: Changes in mRNA abundance of genes related to hepatic translation and transcription regulation during 155 days of lactation.

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes. Asterisks indicate differences between groups (P < 0.05). Differences between time points and between groups can be found in table 11. Fixed effects in model *EIF4B*: time P < 0.01, group P = 0.92 and time × group P = 0.13. Fixed effects in model *HNF4A*: time P < 0.001, group P = 0.72 and time × group P = 0.07. Fixed effects in model *PPARA*: time P < 0.01, group P = 0.59 and time × group P = 0.38. Fixed effects in model *SREBF1*: time P < 0.001, group P = 0.99 and time × group P = 0.09.

Common hepatic metabolism

No differences were found in hepatic mRNA abundance of *CS* (table 11, figure 27).

Hepatic mRNA abundance of *TNFA* showed higher abundance at d 57 pp in mp- compared to MP-cows (8.31 ± 0.34 and 7.12 ± 0.38 for mp- and MP-cows; $P < 0.05$). At day of slaughtering mP-cows showed lower mRNA levels compared to d 1 pp (7.45 ± 0.24 and 8.55 ± 0.56; $P < 0.01$).

Moreover mRNA encoding for *IGF1* showed no differences between groups at any time point. In mp-cows, mRNA levels at d 57 pp (13.00 ± 0.41) were higher compared to d 1 (11.55 ± 0.38; $P < 0.01$) and d 15 pp (11.88 ± 0.38; $P < 0.05$). Furthermore, mP-cows had lower hepatic mRNA abundance at d 1 (11.87 ± 0.48) compared to d 57 pp (13.32 ± 0.42; $P < 0.05$).

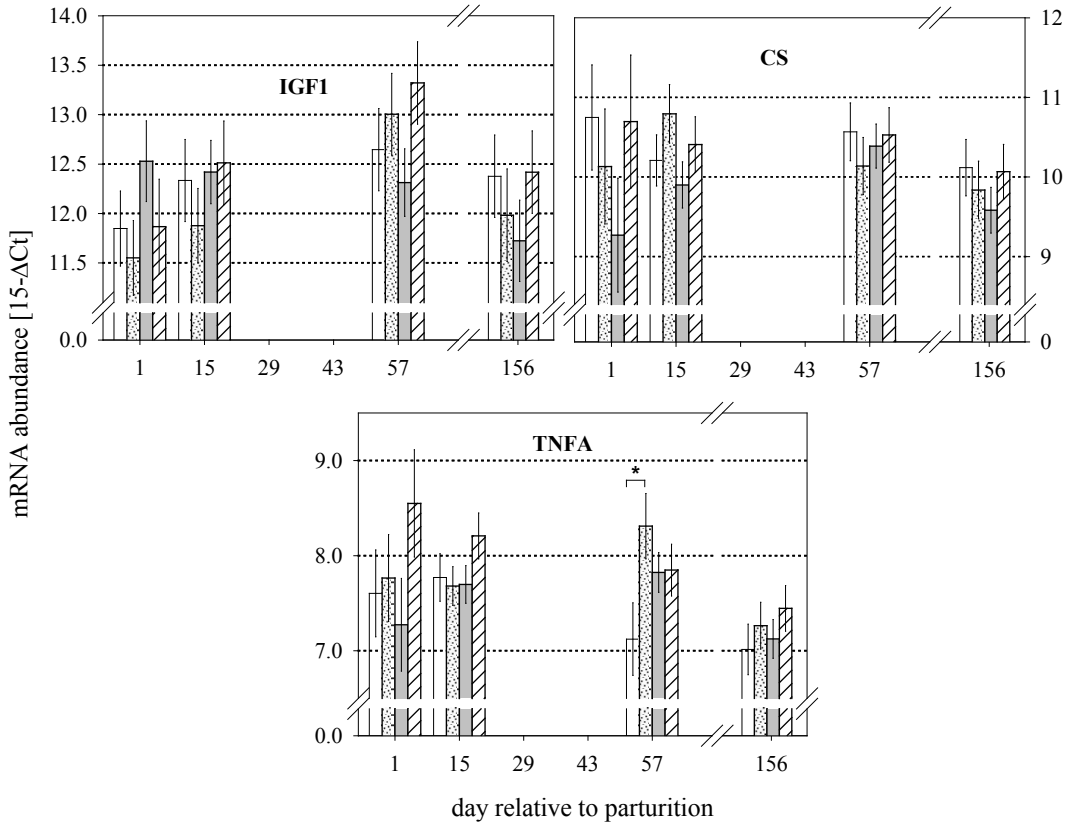

Figure 27: Changes in mRNA abundance of genes related to hepatic metabolism during 155 days of lactation.

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes. Asterisks indicate differences between groups ($P < 0.05$). Differences between time points and between groups can be found in table 11. Fixed effects in model *CS*: time $P = 0.32$, group $P < 0.05$ and time × group $P = 0.76$. Fixed effects in model *IGF1*: time $P < 0.01$, group $P = 0.75$ and time × group $P = 0.44$. Fixed effects in model *TNFA*: time $P < 0.001$, group $P = 0.14$ and time × group $P < 0.05$.

Table 11: Hepatic mRNA expression (15-ΔCt) at day of parturition and day 15, 57 and 155 of lactation.

Gene[1]	MP	mp	Mp	mP
Lipid metabolism				
ACACA				
d 1 pp	8.11 ± 0.68	9.01 ± 0.69*°	8.38 ± 0.23*#	10.09 ± 0.85°
d 15 pp	7.81 ± 0.74	8.68 ± 0.69*	8.49 ± 0.73*	9.68 ± 0.77°
d 57 pp	9.41 ± 0.74	10.48 ± 0.74°	9.90 ± 0.24°	10.48 ± 0.77°
d 155 pp	7.91 ± 0.82	8.97 ± 0.82*°	6.54 ± 0.61#	7.87 ± 1.85*
ACADVL				
d 1 pp	13.61 ± 0.19	13.89 ± 0.19*	13.74 ± 0.21*°	13.97 ± 0.24*
d 15 pp	13.69 ± 0.21a	13.09 ± 0.19b°	13.85 ± 0.16a*	13.85 ± 0.21a*
d 57 pp	13.55 ± 0.21ab	13.27 ± 0.21ab°	13.59 ± 0.17a*°	13.00 ± 0.21b°
d 155 pp	13.77 ± 0.21a	13.55 ± 0.24ab*°	13.20 ± 0.24ab°	13.10 ± 0.21b°
CPT1A				
d 1 pp	13.22 ± 0.25	12.91 ± 0.25	13.06 ± 0.27	13.61 ± 0.32°
d 15 pp	13.55 ± 0.27a	12.71 ± 0.25b	13.46 ± 0.21a	13.94 ± 0.28a°
d 57 pp	13.27 ± 0.27	13.27 ± 0.27	13.52 ± 0.22	13.32 ± 0.27°
d 155 pp	12.82 ± 0.27	12.81 ± 0.32	13.25 ± 0.27	12.56 ± 0.27*
ECHS1				
d 1 pp	14.77 ± 0.21*	14.51 ± 0.21*#	14.90 ± 0.22*#	14.92 ± 0.25*°
d 15 pp	15.40 ± 0.23°*	15.12 ± 0.21*	15.47 ± 0.17*°	15.52 ± 0.22°
d 57 pp	15.42 ± 0.14°	15.51 ± 0.13°	15.65 ± 0.11°	15.51 ± 0.13°
d 155 pp	15.08 ± 0.22a*	14.44 ± 0.19b#	14.60 ± 0.16ab#	14.93 ± 0.19ab*
GPAM				
d 1 pp	11.25 ± 0.28*	11.37 ± 0.28*	11.69 ± 0.30*°	12.05 ± 0.36*
d 15 pp	12.17 ± 0.31°	11.93 ± 0.28°*	12.14 ± 0.24*	11.88 ± 0.32*
d 57 pp	12.12 ± 0.31°	12.48 ± 0.31°	12.19 ± 0.25*	12.40 ± 0.31*
d 155 pp	11.66 ± 0.31a*°	11.59 ± 0.35a*	10.93 ± 0.30ab°	10.34 ± 0.31b°
Protein metabolism				
CTSL				
d 1 pp	14.95 ± 0.23a	15.79 ± 0.23b*	15.29 ± 0.24ab*°	15.44 ± 0.28ab*°
d 15 pp	15.20 ± 0.25a	15.94 ± 0.22b*	15.41 ± 0.19ab*°	15.57 ± 0.25ab*°
d 57 pp	15.37 ± 0.25	15.80 ± 0.25*	15.77 ± 0.20*	15.78 ± 0.25*
d 155 pp	14.92 ± 0.25	14.44 ± 0.28°	14.95 ± 0.24°	14.93 ± 0.25°
TAT				
d 1 pp	16.00 ± 0.29*°	16.04 ± 0.29	15.36 ± 0.32*	15.66 ± 0.37*°
d 15 pp	15.47 ± 0.32*	15.28 ± 0.29	15.54 ± 0.24*	15.40 ± 0.32*
d 57 pp	15.56 ± 0.32*°	15.85 ± 0.32	15.81 ± 0.26*°	15.95 ± 0.32*°
d 155 pp	16.43 ± 0.32°	16.07 ± 0.37	16.44 ± 0.32°	16.59 ± 0.32°

Gene[1]	MP	mp	Mp	mP
Carbohydrate metabolism				
PC				
d 1 pp	10.01 ± 0.69[a]*	11.64 ± 0.69[ab]*	10.13 ± 0.75[ab]	12.32 ± 0.87[b]*
d 15 pp	9.27 ± 0.76*°	8.60 ± 0.69°	9.86 ± 0.58	10.36 ± 0.77*[#]
d 57 pp	7.61 ± 0.76[a]°	9.59 ± 0.76[ab]°	9.96 ± 0.63[b]	8.31 ± 0.77[ab]°
d 155 pp	9.46 ± 0.76*°	8.97 ± 0.86°	10.31 ± 0.75	9.67 ± 0.76°[#]
PCK1				
d 1 pp	15.16 ± 0.56[ab]*	14.52 ± 0.56[a]*	15.99 ± 0.62[ab]*°	16.47 ± 0.72[b]*°
d 15 pp	16.95 ± 0.62°[#]	15.70 ± 0.62*°	16.48 ± 0.47*°	17.19 ± 0.63*
d 57 pp	17.40 ± 0.62[#]	17.17 ± 0.62°	17.12 ± 0.51*	16.87 ± 0.62*
d 155 pp	15.69 ± 0.62[ab]*°	17.19 ± 0.72[a]°	15.15 ± 0.72[ab]°	15.11 ± 0.62[b]°
Glucose transport				
SLC2A2				
d 1 pp	13.58 ± 0.36	13.75 ± 0.36*°	13.31 ± 0.40*	14.36 ± 0.46
d 15 pp	13.20 ± 0.46	12.90 ± 0.36*	13.30 ± 0.30*	13.85 ± 0.40
d 57 pp	13.62 ± 0.40	14.21 ± 0.40°	14.02 ± 0.33*	14.05 ± 0.40
d 155 pp	13.11 ± 0.40[ab]	13.30 ± 0.46[a]*°	11.98 ± 0.40[b]°	13.25 ± 0.40[a]
Hormone receptor				
INSR				
d 1 pp	11.33 ± 0.19[a]	11.81 ± 0.19[ab]	11.58 ± 0.21[a]	12.27 ± 0.24[b]*
d 15 pp	11.78 ± 0.21	11.75 ± 0.18	11.84 ± 0.16	12.11 ± 0.21*
d 57 pp	11.67 ± 0.21	12.11 ± 0.21	11.90 ± 0.17	11.68 ± 0.21*°
d 155 pp	11.88 ± 0.21[ab]	11.62 ± 0.24[ab]	12.10 ± 0.21[a]	11.50 ± 0.21[b]°
Ketogenesis				
HMGCS2				
d 1 pp	14.37 ± 0.54*	13.08 ± 0.55*	13.70 ± 0.58*	14.23 ± 0.67
d 15 pp	16.10 ± 0.59°	15.41 ± 0.60°	15.08 ± 0.46°	15.00 ± 0.60
d 57 pp	16.16 ± 0.59[a]°	15.27 ± 0.60[ab]°	14.40 ± 0.49[b]*°	15.51 ± 0.60[ab]
d 155 pp	14.99 ± 0.59[a]*°	14.58 ± 0.67[ab]°	13.21 ± 0.58[b]*	15.33 ± 0.60[a]
Translation				
EIF4B				
d 1 pp	14.51 ± 0.30	14.36 ± 0.30	13.80 ± 0.32*	13.68 ± 0.37*
d 15 pp	14.70 ± 0.33	14.75 ± 0.30	14.57 ± 0.25°	14.55 ± 0.33°[#]
d 57 pp	14.83 ± 0.33	14.27 ± 0.32	14.44 ± 0.27*°	14.16 ± 0.33*°
d 155 pp	14.23 ± 0.33	14.78 ± 0.36	14.95 ± 0.32°	15.13 ± 0.33[#]
Transcription regulation				
HNF4A				
d 1 pp	13.50 ± 0.32*°	12.96 ± 0.32*°	12.93 ± 0.32*	13.64 ± 0.39*
d 15 pp	13.50 ± 0.34[a]*°	12.52 ± 0.32[b]*	13.22 ± 0.27[ab]*	13.09 ± 0.35[ab]*°
d 57 pp	12.99 ± 0.34*	13.28 ± 0.34°	13.30 ± 0.28*	12.71 ± 0.34°
d 155 pp	13.85 ± 0.34°	13.67 ± 0.37°	14.39 ± 0.33°	13.91 ± 0.34*

Gene[1]	MP	mp	Mp	mP
PPARA				
d 1 pp	13.60 ± 0.17	13.17 ± 0.17*	13.44 ± 0.19*	13.74 ± 0.23
d 15 pp	13.46 ± 0.20	13.30 ± 0.17*	13.70 ± 0.15*°	13.72 ± 0.20
d 57 pp	13.90 ± 0.20	13.90 ± 0.19°	14.05 ± 0.16°	14.04 ± 0.20
d 155 pp	13.80 ± 0.20	13.91 ± 0.22°	13.56 ± 0.19*°	13.47 ± 0.20
SREBF1				
d 1 pp	6.63 ± 0.48*	7.13 ± 0.49*	6.52 ± 0.50*	7.50 ± 0.58
d 15 pp	8.70 ± 0.52°[#]	7.26 ± 0.53*	8.17 ± 0.41°	8.19 ± 0.53
d 57 pp	8.09 ± 0.52°	8.95 ± 0.52°	8.45 ± 0.44°[#]	7.99 ± 0.53
d 155 pp	9.47 ± 0.51[#]	8.90 ± 0.58°	9.24 ± 0.50[#]	8.72 ± 0.52
Final metabolism				
CS				
d 1 pp	10.75 ± 0.66	10.13 ± 0.72	9.27 ± 0.71	10.70 ± 0.83
d 15 pp	10.21 ± 0.32	10.79 ± 0.37	9.90 ± 0.29	10.41 ± 0.35
d 57 pp	10.57 ± 0.36	10.14 ± 0.36	10.39 ± 0.28	10.53 ± 0.34
d 155 pp	10.12 ± 0.35	9.84 ± 0.36	9.58 ± 0.29	10.07 ± 0.34
Inflammation				
TNFA				
d 1 pp	7.60 ± 0.46	7.76 ± 0.46	7.27 ± 0.49	8.55 ± 0.56*
d 15 pp	7.77 ± 0.25	7.68 ± 0.20	7.70 ± 0.20	8.21 ± 0.24*°
d 57 pp	7.12 ± 0.38[a]	8.31 ± 0.34[b]	7.82 ± 0.21[ab]	7.85 ± 0.27[ab]*°
d 155 pp	7.01 ± 0.27	7.26 ± 0.25	7.13 ± 0.20	7.45 ± 0.24°
Anabolism				
IGF1				
d 1 pp	11.85 ± 0.38	11.55 ± 0.38[a]*	12.53 ± 0.41	11.87 ± 0.48*
d 15 pp	12.33 ± 0.42	11.88 ± 0.38*	12.42 ± 0.32	12.51 ± 0.42*°
d 57 pp	12.65 ± 0.42	13.00 ± 0.41°	12.31 ± 0.34	13.32 ± 0.42°
d 155 pp	12.38 ± 0.42	11.98 ± 0.47*°	11.72 ± 0.41	12.42 ± 0.42*°

[abcd]Means with alphabetic superscripts indicate differences between groups ($P < 0.05$).
*°[#+]Means with symbolic superscripts indicate differences between time points ($P < 0.05$).
[1]*ACACA* = acyl-CoA carboxylase α; *ACADVL* = acyl-CoA dehydrogenase, very long chain; *CPT1A* = carnitine palmitoyltransferase; *ECHS1* = enoyl CoA hydratase 1; *GPAM* = glycerol-3-phosphate acyltransferase, mitochondrial; *CTSL* = cathepsin L; *TAT* = tyrosine aminotransferase; *PC* = pyruvate carboxylase; *PCK1* = phosphoenolpyruvate carboxykinase, cytosolic; *SLC2A2* = facilitated glucose transporter, member 2; *INSR* = insulin receptor; *HMGCS2* = 3-hydroxy-3-methylglutary-coenzyme A synthase 2; *EIF4B* = eukaryotic translation initiation factor 4B; *HNF4A* = hepatocyte nuclear factor-4A (Loor et al., 2005); *PPARA* = peroxisome proliferator activated receptor-α (Sigl et al., 2010); *SREBF1* = sterol regulatory element binding transcription factor 1 (Van Dorland et al., 2009); *CS* = citrate synthase; *TNFA* = tumour necrosis factor α; *IGF1* = insulin-like growth factor 1.

3. Feed restrictions

All 23 animals were subjected to a FR in early lactation (d 26 to 28 pp) and another FR in mid-lactation (d 141 to 143 pp).

3.1. DMI intake and energy balance

Dry matter intake

During both FRs, DMI was lower at last day during FR (d 28 and 143 pp) compared to last day before FR (d 25 and 140 pp) and last day of realimentation (d 31 and 146 pp; $P < 0.05$ for mp- and mP-cows, $P < 0.01$ for Mp-cows, $P < 0.001$ for MP-cows; table 12, figure 28). Totally, DMI during FR in early lactation was reduced by 34.1 ± 5.5% in MP-, by 33.5 ± 6.7% in mp-, by 28.1 ± 4.0% in Mp- and by 27.9 ± 3.6% in mP-cows, resulting in 69.1% mean DMI. During FR in mid-lactation cows showed 67.9% mean DMI after reduction by 31.1 ± 2.7% in MP-, by 31.0 ± 2.7% in mp-, by 34.4 ± 2.8% in Mp- and by 31.8 ± 3.5% in mP-cows.

After three days of restricted feeding (d 29 and 144 pp), DMI increased steeply in all cows ($P < 0.001$). At this time point in early lactation, high yielding cows showed higher DMI (22.2 ± 1.1 and 23.4 ± 1.3 kg at d 29 pp in MP- and Mp-cows) than before FR (18.7 ± 0.3 kg and 18.7 ± 0.9 kg at d 25 pp in MP- and Mp-cows; $P < 0.05$ for MP- and $P < 0.01$ for Mp-cows). In mid-lactation, low protein cows showed higher DMI after FR (21.6 ± 1.5 and 24.7 ± 1.0 kg at d 144 pp in mp- and Mp-cows) than before FR (at d 140 pp in mp-cows 17.8 ± 1.3 kg, $P < 0.05$; in Mp-cows 20.8 ± 1.0 kg, $P < 0.01$). Nevertheless, DMI at last day after FR (d 31 and 146 pp) was comparable to DMI before FR (d 25 and 140 pp) in all cows.

Furthermore mP-cows showed lower DMI at the day before FR in early lactation (17.8 ± 1.6 kg at d 25 pp) compared to the day before FR in mid-lactation (21.4 ± 0.6 kg at d 140 pp; $P < 0.05$).

Differences between cows during FR in early lactation could be observed as follows: before FR, high yielding cows showed higher mean DMI (18.9 ± 0.3 and 18.3 ± 0.9 kg/d in MP- and Mp-cows) compared to mp-cows (15.4 ± 0.6 kg/d; $P < 0.05$). During restricted feeding, DMI was comparable amongst all groups. After FR at d 29 pp, MP- (22.16 ± 1.13 kg) and Mp-cows (23.36 ± 1.35 kg) showed higher DMI compared to mp- (18.63 ± 1.22 kg; $P < 0.05$) and mP-cows (18.39 ± 1.89 kg; $P < 0.05$) and at d 31 pp MP-cows (20.2 ± 2.1 kg) showed higher DMI compared to mp-cows (15.3 ± 0.7 kg; $P < 0.05$).

Before FR in mid-lactation, mp-cows showed lower mean DMI compared to MP- and mP-cows (18.4 ± 1.0, 22.0 ± 1.7 and 21.8 ± 0.9 kg/d in mp-, MP- and mP-cows; $P < 0.05$). During restricted feeding, DMI of all cows was comparable. At d 144 pp, DMI was comparable amongst all cows. Afterwards, DMI declined in mp-cows, resulting in lower DMI of mp-cows compared to Mp- and mP-cows at d 145 pp (17.2 ± 1.5, 20.7 ± 1.6 and 21.1 ± 0.8 kg in mp-, Mp- and mP-cows; $P < 0.05$) and compared to MP-cows at d 146 pp (16.1 ± 1.1 and 20.9 ± 1.0 kg in mp- and MP-cows; $P < 0.05$).

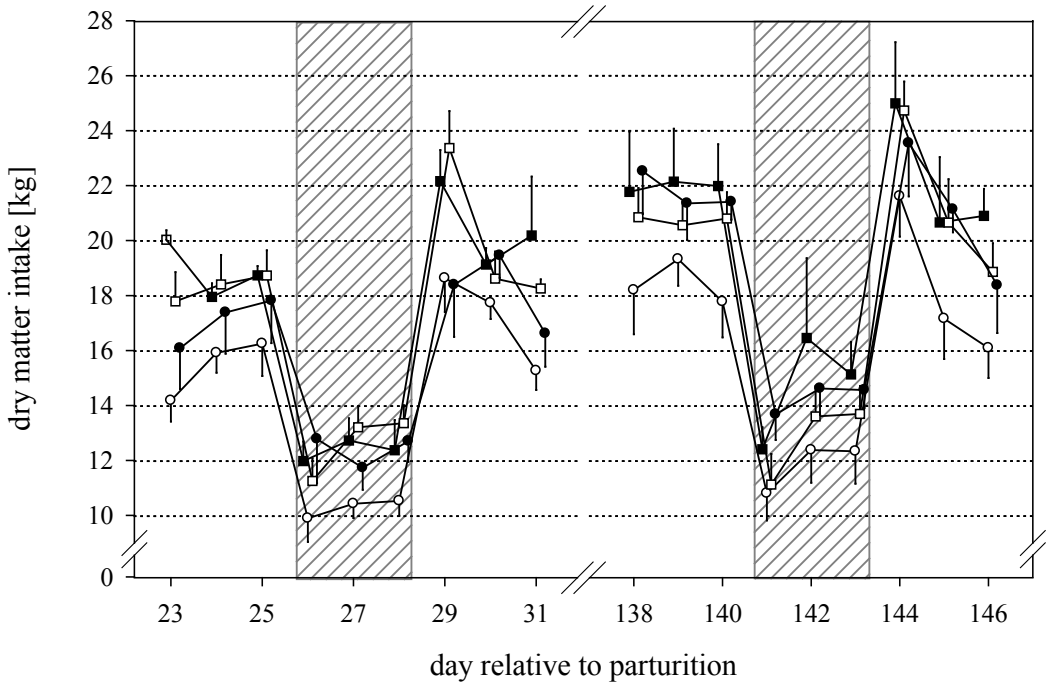

Figure 28: Dry matter intake (kg) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presenteds as LSM ± SEM. Fixed effects in model dry matter intake: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.64$.

Energy balance

All cows showed negative EB before FR in early lactation (table 12, figure 29). Mean EB before FR in early lactation was more negative in MP- (-72.4 ± 6.3 MJ NE_L/d) compared to mP-cows (-46.4 ± 10.8 MJ NE_L/d; $P < 0.05$). During first day of restricted feeding (d 26 pp), EB declined in all cows (by 58.8 ± 2.6, 46.7 ± 14.6, 53.0 ± 6.0 and by 37.5 ± 14.3 MJ NE_L/d in MP-, mp-, Mp- and mP-cows, respectively; $P < 0.001$), resulting in lower EB of MP-cows (-123.7 ± 6.1 MJ NE_L) compared to mp- (-93.9 ± 8.5 MJ NE_L; $P < 0.05$) and mP-cows (-79.0 ± 10.9 MJ NE_L; $P < 0.01$) and lower EB in Mp- (-116.3 ± 5.3 MJ NE_L) compared to mP-cows ($P < 0.01$). During restricted feeding in early lactation, EB increased in Mp-cows from d 26 to 28 pp by 27.1 MJ NE_L ($P < 0.05$). Furthermore mP-cows (-77.9 ± 4.2 MJ NE_L/d) showed higher mean EB during restricted feeding compared to MP- (-111.6 ± 7.6 MJ NE_L/d; $P < 0.01$) and Mp-cows (-102.3 ± 4.9 MJ NE_L/d; $P < 0.05$). During first day after early FR (d 29 pp), EB increased in MP-, mp-, Mp- and mP-cows by 52.1 ($P < 0.001$), 60.6 ($P < 0.001$), 71.3 ($P < 0.001$) and 33.0 MJ NE_L ($P < 0.01$), respectively. At this time point, MP-cows (-49.3 ± 7.7 MJ NE_L) showed lower EB compared to Mp-cows (-17.9 ± 10.3 MJ NE_L; $P < 0.05$). After FR in early lactation, MP-cows (-55.5 ± 10.2 MJ NE_L/d) showed lower mean EB compared to mP-cows (-30.7 ± 8.6 MJ NE_L/d; $P < 0.05$). Solely in Mp-cows, EB decreased from d 29 pp (-17.9 ± 10.3 MJ NE_L) to d 30 pp (-45.6 ± 8.5 MJ NE_L; $P < 0.01$). In mp- and Mp-cows, mean EB showed higher levels after FR (-29.9 ± 4.1 and -32.9 ± 6.8 MJ NE_L/d) compared to mean EB before FR (-55.7 ± 6.2 MJ NE_L/d in mp-cows, $P < 0.05$; -62.6 ± 6.9 MJ NE_L/d in Mp-cows, $P < 0.01$).

Mean levels of EB before, during and after FR in mid-lactation were higher compared to early lactation ($P < 0.001$ for MP and Mp-cows; $P < 0.01$ for mp- and mP-cows).

Before restricted feeding in mid-lactation, mean levels of EB were comparable amongst cows.

During first day of restricted feeding in mid-lactation, EB declined by 67.5, 42.7, 62.1 and 48.7 MJ NE_L in MP-, mp-, Mp- and mP-cows ($P < 0.001$), resulting in more negative EB at d 141 pp in Mp- (-73.8 ± 8.4 MJ NE_L) compared to mP-cows (-44.2 ± 7.4 MJ NE_L; $P < 0.05$). Increase of EB from d 141 to 142 pp was observed in MP- (from -67.2 ± 12.4 to -26.1 ± 19.6 MJ NE_L; $P < 0.001$) and in Mp-cows (from -73.8 ± 8.4 to -52.1 ± 6.0 MJ NE_L; $P < 0.05$), whereas mean EB during restricted feeding was comparable amongst cows. At d 144 pp, EB increased by 65.6, 66.8, 74.3 and 63.4 MJ NE_L in MP-, mp-, Mp- and mP-cows ($P < 0.001$). No differences between cows were observed in mean EB after FR in mid-lactation.

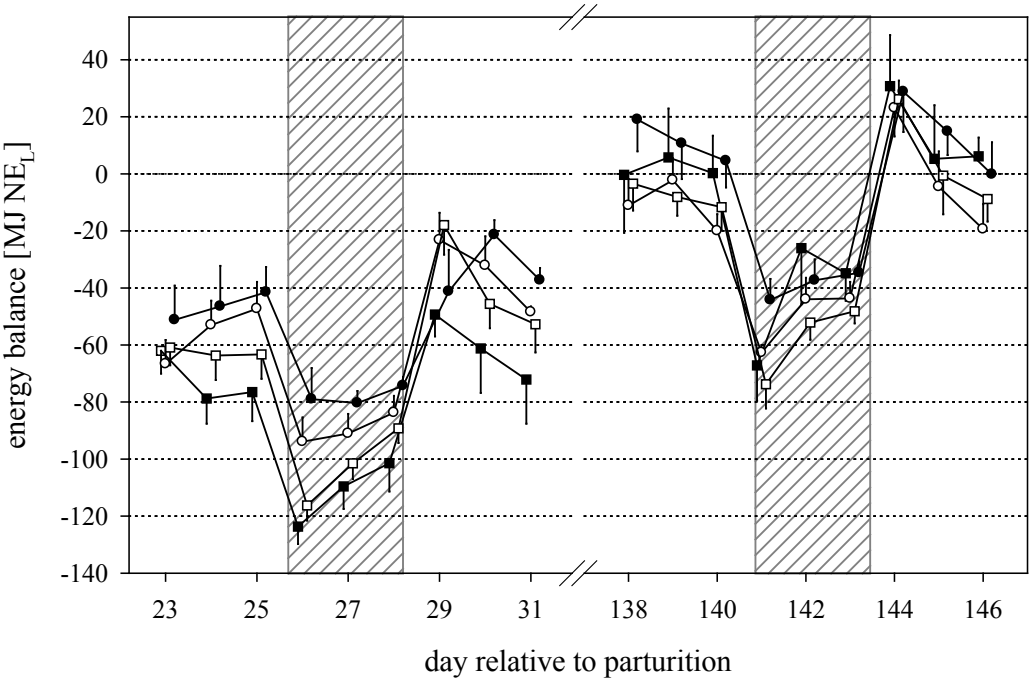

Figure 29: Energy balance (MJ NE_L) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model energy balance: time $P < 0.001$, group $P < 0.05$ and time × group $P = 0.57$.

Table 12: Mean feed parameters (LSM ± SE) at last day before, during and after three days of feed restriction in early and mid-lactation.

	MP	mp	Mp	mP
dry matter intake, kg				
d 25 pp	18.7 ± 0.3*	16.3 ± 1.2*	18.7 ± 0.9*	17.8 ± 1.6*[1]
d 28 pp	12.4 ± 1.1°	10.5 ± 0.5°	13.4 ± 0.7°	12.7 ± 0.8°
d 31 pp	20.2 ± 2.1[a]*	15.3 ± 0.7[b]*	18.3 ± 0.3[ab]*	16.6 ± 1.2[ab]*
d 140 pp	22.0 ± 1.5[a]*	17.8 ± 1.3[b]*	20.8 ± 1.0[ab]*	21.4 ± 0.6[a]*
d 143 pp	15.1 ± 1.2°	12.3 ± 1.2°	13.7 ± 1.0°	14.6 ± 0.7°
d 146 pp	20.9 ± 1.0[a]*	16.1 ± 1.1[b]*	18.9 ± 1.0[ab]*	18.4 ± 1.7[ab]*

	MP	mp	Mp	mP
energy balance, MJ NE_L				
d 25 pp	-76.5 ± 10.2^{a}*°^{1}	-47.3 ± 9.4^{b}*	-63.3 ± 8.5^{ab}*^{1}	-41.5 ± 8.8^{b}*^{1}
d 28 pp	-101.4 ± 9.9*^{1}	-83.7 ± 5.9°^{1}	-89.2 ± 5.1°^{1}	-74.3 ± 3.1°^{1}
d 31 pp	-72.1 ± 15.5°^{1}	-48.4 ± 1.9*	-52.7 ± 9.9*	-37.3 ± 4.3*^{1}
d 140 pp	0.3 ± 13.1*	-20.0 ± 5.9	-11.7 ± 8.2*	4.6 ± 9.3*
d 143 pp	-34.8 ± 9.6°	-43.7 ± 5.8	-48.2 ± 4.3°	-34.7 ± 3.9°
d 146 pp	6.2 ± 6.5*	-19.4 ± 8.7	-8.8 ± 7.9*	-0.2 ± 11.2*

^{abcd}Alphabetic superscripts indicate differences between groups ($P < 0.05$).
*°$^{\#}$Symbolic superscripts indicate differences between days within a feed restriction ($P < 0.05$).
^{1}Superscripted 1 indicates difference ($P < 0.05$) of day in early lactation feed restriction (d 25, 28 and 31 pp) compared to day in mid-lactation feed restriction (d 140, 143 and 146 pp, respectively).

3.2. Milk composition

Milk yield

Before, during and after FR in early lactation, milk yields were higher in all cows compared to FR in mid-lactation (table 13, figure 30).

Before FR in early lactation, milk yield was higher in high yielding cows compared to low yielding cows ($P < 0.05$). In high yielding cows and in mp-cows, milk yield declined during FR by 5.8, 7.8 and 5.5 kg in MP-, Mp- and mp-cows ($P < 0.01$). At d 28 pp, Mp-cows (38.9 ± 1.3 kg) showed higher milk yield compared to low yielding cows (31.3 ± 1.6 and 30.5 ± 1.4 kg in mp- and mP-cows; $P < 0.01$) and MP-cows (36.0 ± 1.0 kg) showed higher milk yield compared to mP-cows ($P < 0.05$). Solely in MP-cows, milk yield increased from d 28 to 29 pp (by 2.5 kg; $P < 0.05$), resulting in only significant increase after FR in early lactation ($P < 0.01$). At d 31 pp, high yielding cows showed higher milk yields compared to low yielding cows ($P < 0.05$).

Before FR in mid-lactation, Mp-cows (37.0 ± 2.4 kg) showed higher milk yield compared to low yielding cows (31.2 ± 2.4 and 26.6 ± 2.4 kg in mp- and mP-cows; $P < 0.05$) and MP-cows (32.1 ± 2.2 kg) showed higher milk yield compared to mP-cows ($P < 0.05$). Milk yield declined from d 140 to 143 pp by 4.3, 5.7 and 5.4 kg in MP- ($P < 0.05$), mp- and Mp-cows ($P < 0.01$). At d 143 pp, Mp-cows (31.6 ± 2.2 kg) showed higher milk yields compared to mp- (25.5 ± 2.5 kg; $P < 0.05$) and mP-cows (23.5 ± 1.7 kg; $P < 0.01$). After FR in mid-lactation, milk yields of all cows stayed on similar levels.

FCM

Before, during and after FR in mid-lactation all cows showed lower FCM compared to early FR ($P < 0.05$). Solely mp-cows tended to have similar FCM before FRs ($P = 0.07$; table 13, figure 30).

Before FR in early lactation, high yielding cows showed higher FCM compared to low yielding cows (53.7 ± 4.1, 49.9 ± 1.9, 39.3 ± 1.7 and 38.7 ± 1.1 kg in MP-, Mp-, mp- and mP-cows; $P < 0.01$). In high yielding cows, FCM declined during FR in early lactation by 7.3 kg in MP-cows to 46.4 ± 2.5 kg ($P < 0.01$) and by 5.5 kg in Mp-cows to 44.4 ± 1.9 kg ($P < 0.05$). At d 28 pp, high yielding cows had still higher FCM compared to low yielding cows (46.4 ± 2.5, 44.4 ± 1.9, 36.7 ± 1.6 and 37.0 ± 2.2 kg in MP-, Mp-, mp- and mP-cows; $P < 0.05$). Solely in MP-cows, FCM increased after FR in early lactation from d 28 to 29 pp (by 7.5 kg; $P < 0.001$), leading to only significant increase by 5.9 kg to 52.3 ± 2.9 kg ($P < 0.05$).

Before, during and after FR in mid-lactation, FCM was similar amongst all cows and throughout days.

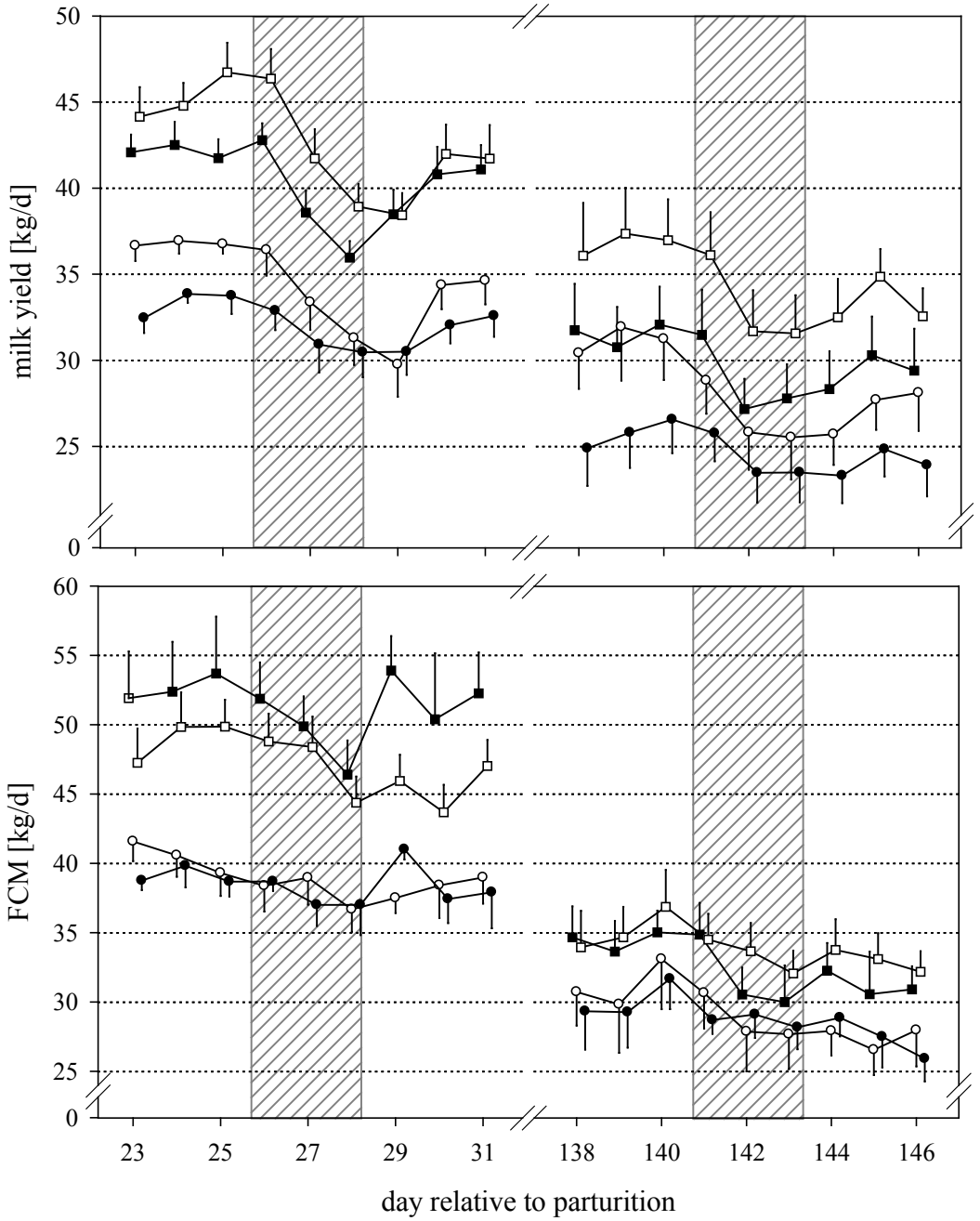

Figure 30: Milk yield (kg) and FCM (kg) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model milk yield: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.92$. Fixed effects in model FCM: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.82$.

ECM

All cows showed lower ECM before, during and after FR in mid-lactation compared to early FR ($P < 0.05$). Solely mp-cows had similar ECM before FRs ($P = 0.11$; table 13).

Before FR in early lactation, ECM was higher in high yielding cows compared to low yielding cows ($P < 0.001$) and decreased by 8.2 and 6.4 kg until d 28 pp in MP- (to 47.7 ± 2.5 kg; $P < 0.01$)

and Mp-cows (to 46.0 ± 1.8 kg; $P < 0.01$). At d 28 pp, high yielding cows showed still higher ECM compared to low yielding cows ($P < 0.05$). After FR, ECM increased in MP-cows until d 29 pp by 6.8 kg ($P < 0.001$), resulting in only significant increase until d 31 pp (to 53.8 ± 2.8 kg; $P < 0.05$). Furthermore, ECM of high yielding cows was still higher after FR compared to low yielding cows ($P < 0.01$).

Amongst cows, ECM was similar at all days of FR during mid-lactation. Nevertheless, ECM decreased in MP-, mp- and Mp-cows from d 140 to 143 pp by 5.9, 6.1 and 5.7 kg ($P < 0.05$).

Milk protein concentration

In high protein cows, milk protein concentration was higher at days of FR in mid-lactation compared to FR in early lactation ($P < 0.01$ and $P < 0.001$ for MP- and mP-cows; table 13, figure 31). In low protein cows, protein concentration was higher at d 140 and 146 pp compared to respective days of FR in early lactation ($P < 0.01$ and $P < 0.05$ for mp- and Mp-cows).

Before FR in early lactation, milk protein concentration was higher in high protein cows (3.28 ± 0.07 and 3.42 ± 0.04% in MP- and mP-cows) compared to low protein cows (2.83 ± 0.06 and 2.92 ± 0.06 in mp- and Mp-cows; $P < 0.01$). Afterwards, protein concentration decreased in high protein cows (to 3.10 ± 0.08 and 3.22 ± 0.08% in MP- and mP-cows; $P < 0.05$) and was at d 28 pp higher in mP- compared to Mp-cows (2.90 ± 0.06%; $P < 0.05$). After FR in early lactation, protein concentration of high protein cows stayed on previous low levels (3.06 ± 0.07 and 3.11 ± 0.07% in MP- and mP-cows; $P < 0.05$) and was higher in mP-cows compared to mp-cows (2.79 ± 0.10%; $P < 0.05$).

Before FR in mid-lactation (at d 140 pp), milk protein content was higher in high protein cows (3.66 ± 0.18 and 3.91 ± 0.14% in MP- and mP-cows) compared to low protein-cows (3.23 ± 0.07 and 3.18 ± 0.06% in mp- and Mp-cows; $P < 0.01$). Afterwards, milk protein concentration declined only in MP-cows from d 140 pp to d 143 pp (to 3.43 ± 0.20%; $P < 0.01$). Furthermore, mP-cows showed declining protein concentrations from d 141 to 143 pp (3.95 ± 0.16 to 3.80 ± 0.14%; $P < 0.05$). At d 143 pp mP-cows showed overall highest milk protein content ($P < 0.05$) and MP-cows (3.43 ± 0.20%) showed higher protein content compared to low protein-cows (3.15 ± 0.08 and 3.06 ± 0.06% in mp- and Mp-cows; $P < 0.05$). After FR in mid-lactation, milk protein content stayed on similar levels with high protein cows showing higher concentrations compared to low protein cows (3.58 ± 0.19, 3.87 ± 0.15, 3.26 ± 0.05 and 3.17 ± 0.05% in MP-, mP-, mp- and Mp-cows; $P < 0.05$).

Milk protein yield

Compared to respective day of FR in early lactation, high yielding cows showed lower milk protein yields during FR in mid-lactation ($P < 0.05$; table 13, figure 31).

At d 25 pp, high yielding cows showed higher milk protein yields compared to low yielding cows ($P < 0.05$). Afterwards, declining protein yields were observed in high yielding cows (by 257 and 238 g in MP- and Mp-cows; $P < 0.001$) and in mP-cows (by 170 g; $P < 0.05$). At d 28 pp, mp-cows had lower milk protein yields compared to high yielding cows (922 ± 56, 1,116 ± 55 and 1,127 ± 38 g in mp-, MP- and Mp-cows; $P < 0.05$) and showed numerically further declining protein yields at d 29 pp (850 ±71 g; $P = 0.10$). Only in MP-cows, milk protein yields increased after FR by 140 g until d 146 pp ($P < 0.05$). After FR in early lactation, high yielding cows showed higher milk protein yields compared to low yielding cows ($P < 0.05$).

Before FR in mid-lactation, milk protein yields were similar in all cows and declined in all cows during FR (by 221, 202, 213 and 143 g in MP-, mp-, Mp- and mP-cows; $P < 0.05$) to similar levels at d 143 (939 ± 27, 810 ± 98, 962 ± 62 and 884 ± 34 g in MP-, mp-, Mp- and mP-cows). After FR,

protein yields stayed in all cows on similar levels, resulting in similar protein yields at d 146 pp (1,034 ± 44 g, 917 ± 79 g, 1,033 ± 57 g and 916 ± 35 g in MP-, mp-, Mp- and mP-cows).

Milk fat content

In all cows, milk fat concentration showed varying amounts with numerically higher levels in high protein cows compared to low protein cows and higher levels during the days of FR period in early lactation compared to those in mid-lactation (table 13, figure 32).

Before, during and after FR in early lactation, only MP-cows showed higher milk fat content compared to FR in mid-lactation ($P < 0.01$). Additionally, Mp-cows had higher fat concentration in milk after FR in early lactation compared to FR in early lactation (4.93 ± 0.43 and 13.94 ± 0.15%; $P < 0.05$).

At d 25 pp, milk fat content was overall highest in MP-cows (5.93 ± 0.72%; $P < 0.05$), declined by 0.52%-points ($P = 0.10$) and was still higher at d 26 pp compared to low protein cows (5.41 ± 0.33%; $P < 0.05$). Although numerical increases of milk fat concentrations were observed in all cows during FR in early lactation, these increases proved not to be significant. At d 28 pp, MP-cows showed higher fat content compared to Mp-cows (5.92 ± 0.32 and 4.95 ± 0.3% in MP- and Mp-cows; $P < 0.05$).

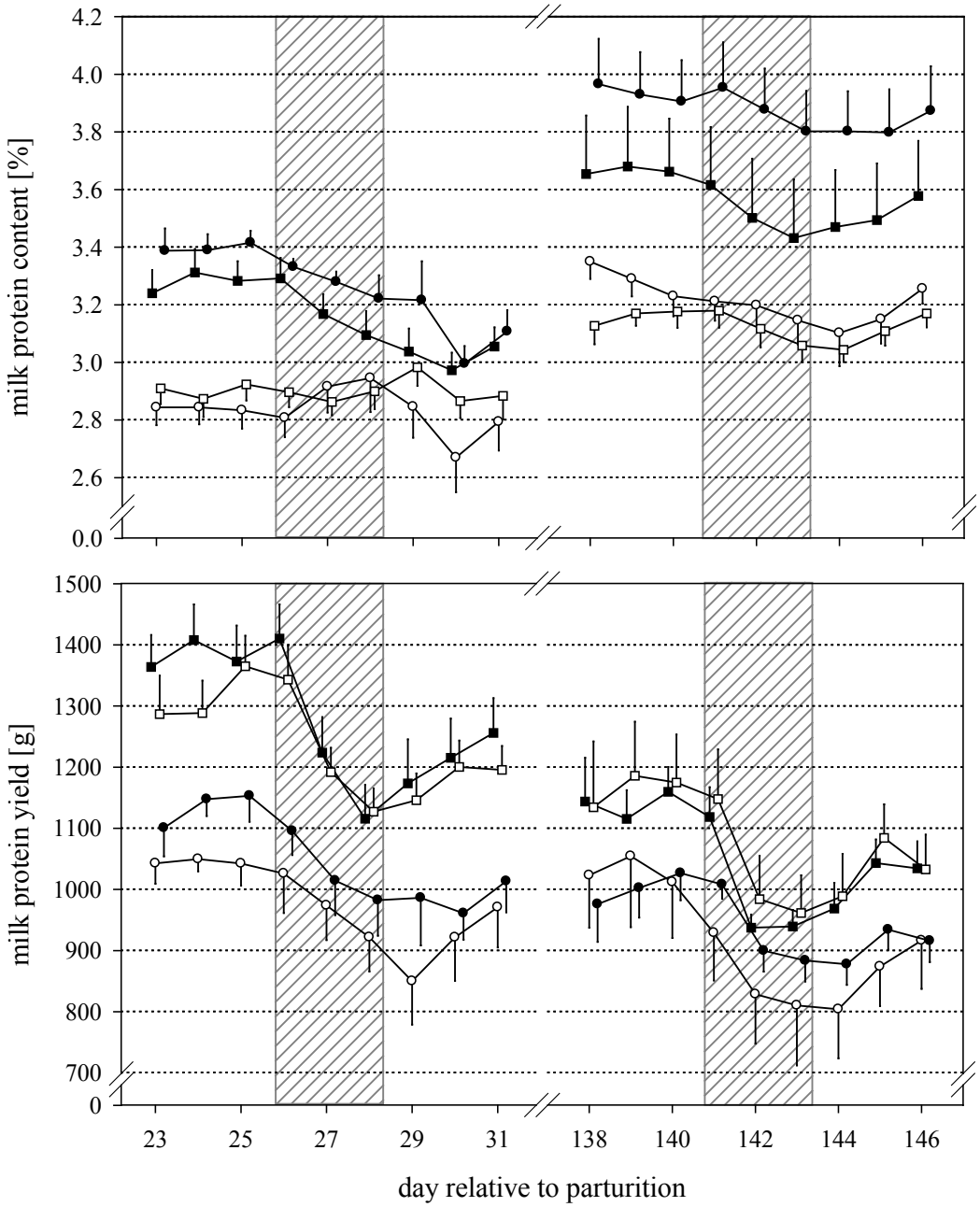

Figure 31: Milk protein content (%) and protein yield (g) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model protein content: time $P < 0.001$, group $P < 0.001$ and time × group $P < 0.001$. Fixed effects in model protein yield: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.74$.

After FR, fat content increased further until d 29 pp in high protein cows (6.71 ± 0.46 in MP-cows, $P < 0.05$ and 6.39 ± 0.52 in mP-cows, $P < 0.01$) and numerically in low protein cows ($P = 0.07$ and $P = 0.24$ for mp- and Mp-cows). Additionally, milk fat concentration decreased until d 30 pp in all cows ($P < 0.001$ for MP-, Mp- and mP-cows, $P < 0.1$ for mp-cows). At d 31 pp, MP- cows (5.80 ± 0.33%) showed higher milk fat content compared to Mp-cows (4.93 ± 0.43%; $P < 0.05$) and tended to be higher compared to mp-cows (4.88 ± 0.46%; $P < 0.1$).

Before FR in mid-lactation, mP-cows (5.39 ± 0.62%) showed higher fat content compared to mp-

(4.34 ± 0.40%; P < 0.05) and Mp-cows (3.97 ± 0.11%; P < 0.01). Between d 141 and 142 pp, fat concentration increased in Mp- and mP-cows (P < 0.05), but overall increase from d 140 or 141 pp until d 143 pp proved not to be significant. At d 143 pp, milk fat content was higher in mP- (5.38 ± 0.26%) compared to Mp-cows (4.16 ± 0.14%; P < 0.01). after FR in mid-lactation, fat content declined from d 144 to 145 pp in all cows (P < 0.05), but overall decrease proved not to be significant nevertheless tended to be significant in mP-cows (P < 0.1).

Milk fat yield

Before FR in early lactation, high yielding cows had higher milk fat yields compared to FR in mid-lactation (P < 0.001; table 13, figure 32). Furthermore, milk fat yields were higher in all cows during and after FR in early compared to mid-lactation (P < 0.05).

Regarding FR in early lactation, MP-cows showed highest milk fat yields amongst groups at d 25 pp (2,466 ± 274 g; P < 0.05) and Mp-cows (2,078 ± 108 g) showed higher milk fat yields compared to low yielding cows (1,640 ± 118 and 1,678 ± 61 g in mp- and mP-cows; P < 0.05). Only decrease of milk fat yields during restricted feeding was observed in MP-cows (by 331 g, P < 0.05). At d 28 pp, milk fat yields were higher in MP-cows (2,135 ± 145 g) compared to mp- (1,611 ± 94 g; P < 0.01) and mP-cows (1,654 ± 116 g; P < 0.01). After FR, milk fat yields increased in high protein cows from d 28 to 29 pp (by 432 and 266 g in MP- and mP-cows; P < 0.05) and declined again to d 30 pp (to 2,271 ± 277 and 1,641 ± 88 g in MP- and mP-cows; P < 0.05). Furthermore, Mp-cows showed declining milk fat yields from d 29 to 30 pp by 245 g (to 1,792 ± 107 g; P < 0.05) and again inclining fat yields to d 31 pp (by 231 g; P < 0.05). This resulted in no significant difference between d 28 and 31 pp.

During FR in mid-lactation, all cows showed similar milk fat yields at all days. Additionally, no differences within groups were observed.

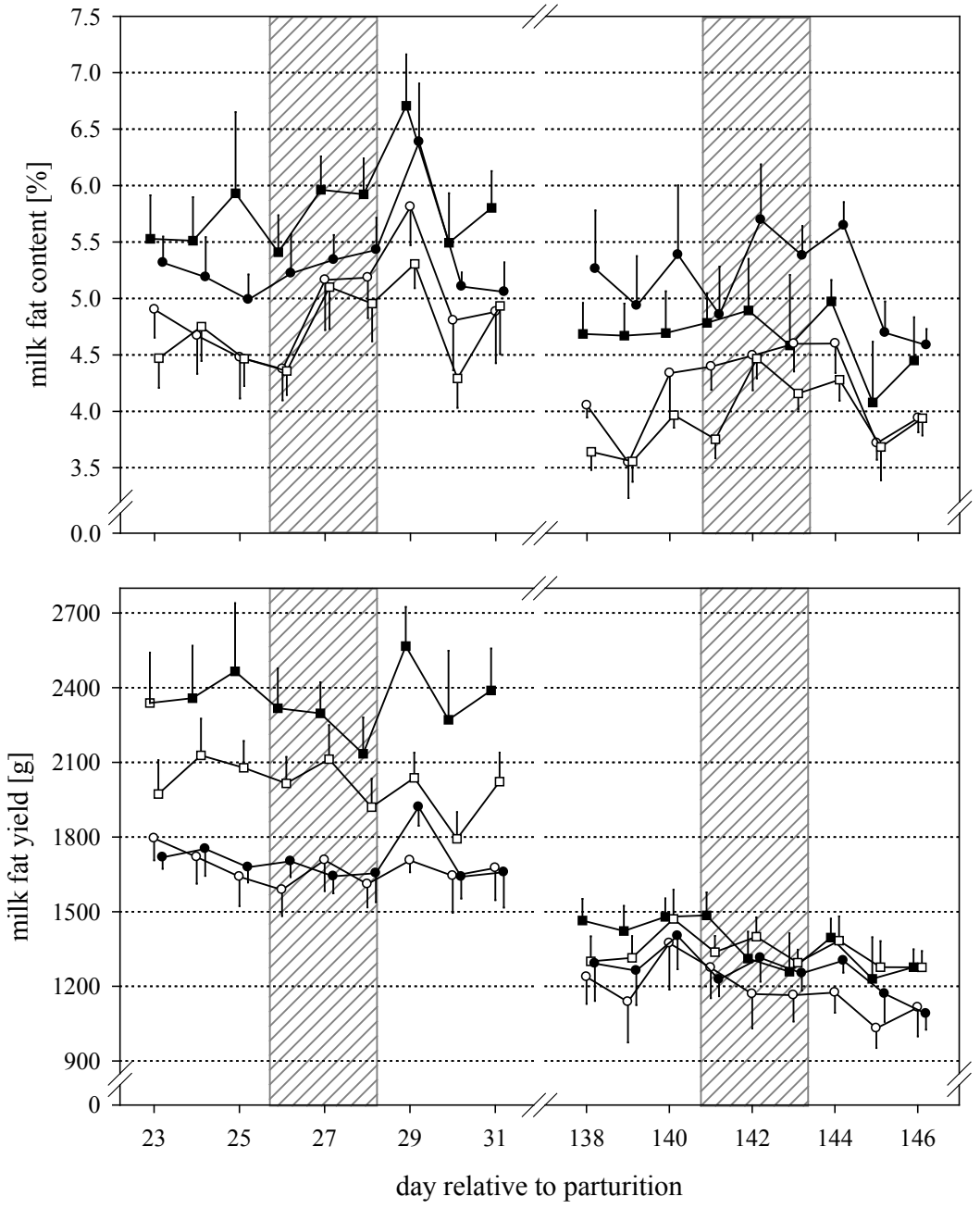

Figure 32: Milk fat content (%) and fat yield (g) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model fat content: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.88$. Fixed effects in model fat yield: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.72$.

Milk lactose concentration

Milk lactose concentration was similar during days of FR in early and mid-lactation, solely in mP-cows at d 25 pp (4.94 ± 0.031%), lactose concentration was higher compared to d 140 pp (4.72 ± 0.09%; $P < 0.05$; table 13).

Before FR in early lactation, no differences of lactose content were observed within cows. In mp-cows, milk lactose content declined from d 25 to 28 pp by 0.16%-points ($P < 0.05$), whereas in all

other cows, milk lactose content decreased numerically from d 26 to 28 and further to d 29 pp. At d 28 pp, mP-cows showed higher lactose content compared to mp-cows (4.60 ± 0.08 and 4.86 ± 0.06% in mp- and mP-cows; $P < 0.01$). After FR, milk lactose content increased in all cows from d 29 to 30 pp (to 4.88 ± 0.04, 4.80 ± 0.06, 4.83 ± 0.06 and 4.94% in MP-, mp-, Mp- and mP-cows; $P < 0.001$ for MP- and mp-cows, $P < 0.05$ for mP-cows and $P < 0.1$ for Mp-cows) and declined again to d 31 pp (4.77 ± 0.06 and 4.71 ± 0.05% in MP- and mp-cows, $P < 0.05$; 4.76 ± 0.07 in Mp-cows, $P < 0.01$ and 4.88 ± 0.06% in mP-cows, $P = 0.17$). At all days after FR in early lactation, no differences within groups were observed.

During all days of FR in mid-lactation no differences were proved to be significant within groups or days. Also numerically, milk lactose content stayed on similar levels throughout restrictive feeding.

Milk lactose yield

During both FRs, milk lactose yields showed numerically decreases during restricted feeding and higher lactose yields were observed in high yielding cows in FR during early lactation.

In MP-, Mp- and mP-cows, milk lactose yields were higher at all days during early FR compared to respective days of FR in mid-lactation ($P < 0.01$), whereas milk lactose yields in mp-cows were higher solely at d 31 pp compared to d 146 pp ($P < 0.05$) and tended to be higher at the other days ($P < 0.1$; table 13).

Regarding FR in early lactation, MP-cows (1,993 ± 82 g) showed lower milk lactose yields at d 25 pp compared to Mp- (2,250 ± 89 g; $P < 0.05$) and higher lactose yields compared to mP-cows (1,666 ± 53 g; $P < 0.05$). Furthermore, Mp-cows showed higher milk lactose yield compared to mp- (1,751 ± 42 g; $P < 0.001$) and mP-cows ($P < 0.001$). From d 25 to 28 pp, milk lactose yield decreased by 305 g in MP-cows (to 1,688 ± 53 g; $P < 0.001$), by 393 g in Mp-cows (to 1,857 ± 68 g; $P < 0.001$) and by 308 g in mp-cows (to 1,443 ± 91 g; $P < 0.01$). At d 28 pp, Mp-cows showed higher milk lactose yield compared to mp- and mP-cows (1,480 ± 62 g; $P < 0.01$). After FR milk lactose yield increased from d 29 to 31 pp in MP-, mp- and Mp-cows ($P < 0.05$), whereas increasing levels from d 28 to 31 pp could be observed solely in MP-cows (to 1,965 ± 94 g; $P < 0.01$). At d 31 pp, high yielding MP- and Mp-cows (1,993 ± 115 g) showed higher milk lactose yields compared to low yielding cows (1,632 ± 70 g, $P < 0.05$ in mp- and 1,588 ± 47 g, $P < 0.01$ in mP-cows).

Before FR in mid-lactation, milk lactose yields at d 140 pp were higher in MP-cows (1,512 ± 122 g) compared to mP-cows (1,255 ± 103 g; $P < 0.05$) and in Mp- (1,783 ± 114 g) compared to mp- (1,494 ± 133 g; $P < 0.05$) and mP-cows ($P < 0.001$). During FR, milk lactose yields declined significantly from d 141 to 142 pp, resulting in significant decreases from d 140 to 143 pp in MP-, mp-, and Mp-cows (to 1,307 ± 102, 1,214 ± 136 and 1,521 ± 109 g, respectively; $P < 0.05$). At d 143 pp, Mp-cows showed higher milk lactose yields compared to mp- ($P < 0.05$) and mP-cows (1,127 ± 87 g; $P < 0.01$). Furthermore after FR, milk lactose yields remained low and were higher in Mp- compared to mP-cows at d 146 pp (1,549 ± 84 and 1146 ± 100 g in Mp- and mP-cows; $P < 0.01$).

Milk FPR

In high yielding cows, FPR was found higher at days of early FR compared to those of FR in mid-lactation ($P < 0.001$ in MP- and $P < 0.05$ in Mp-cows; table 13).

Before FR in early lactation at d 25 pp, MP-cows (1.81 ± 0.22) showed higher FPR compared to Mp- (1.53 ± 0.10; $P < 0.05$) and mP-cows (1.46 ± 0.06; $P < 0.05$). During FR in early lactation, FPR increased numerically in all cows, but only increases from d 26 to 27 pp in high yielding

cows and from d 28 to 29 pp in MP-, mp- and mP-cows were proved to be significant ($P < 0.05$). At d 28 pp, all cows showed similar FPR. After FR, FPR increased further in all cows until d 29 pp, resulting in FPR increases of all cows from d 26 to 29 pp ($P < 0.05$) and decreased afterwards to d 30 pp ($P < 0.05$), showing similar levels to before FR. At d 31 pp, FPR was similar amongst cows.

In FR during mid-lactation, all cows showed similar levels of FPR within days. Slight numerical increases were observed in all cows throughout period before, during FR and until d 144 pp. Afterwards, FPR decreased in all cows ($P < 0.05$) to levels similar before FR in mid-lactation.

Milk SCC

Milk SCC was similar during all days of FR in early and mid-lactation. Due to two animals with constantly elevated SCC and highest SCC at d 31 pp (5,642 and 1,468 × 1,000/mL), mP-cows showed overall highest SCC at d 31 pp (1,447.6 ± 1,084.4 × 1,000/mL) compared to d 146 pp (598.8 ± 486.7 × 1,000/mL; $P < 0.05$), d 25 pp (291.2 ± 245.1 × 1,000/mL; $P < 0.001$) and d 28 pp (300.8 ± 212.6 × 1,000/mL; $P < 0.001$), as well as compared to d 31 pp in MP- (40.7 ± 17.1 × 1,000/mL; $P < 0.001$), mp- (45.6 ± 16.3 × 1,000/mL; $P < 0.001$) and Mp-cows (268.7 ± 240.6 × 1,000/mL; $P < 0.001$; table 13).

Additionally, SCC increased in one mp-cow after FR in mid-lactation from d 143 to 146 pp (from 49 to 3,104 × 1,000/mL) and therefore increased in mp-cows (from 80.2 ± 31.9 to 679.8 ± 606.3 × 1,000/mL; $P < 0.05$).

Milk urea

Amount of milk urea was lower during early FR in MP-cows at d 28 (154 ± 24 mg/L; $P < 0.05$) and 31 pp (164 ± 19 mg/L; $P < 0.05$), in mp-cows at d 28 pp (139 ± 19 mg/L; $P < 0.05$) and in Mp-cows at d 31 pp (150 ± 17 mg/L; $P < 0.01$) compared to corresponding days of FR in mid-lactation (table 13).

Milk hydrocortisone

Although concentrations of milk hydrocortisone seemed to be lower during days of FR in early lactation compared to FR in mid-lactation and during days of restricted feeding compared to periods before and after those days, differences proved not to be significant (table 13).

Milk BHBA

During days of FR in early and mid-lactation, cows showed similar levels of milk BHBA (table 13). At d 31 pp, mp-cows showed higher milk BHBA (61.3 ± 13.0 mg/L) compared to mP-cows (18.9 ± 14.1 mg/L; $P < 0.05$) and highest value during early FR ($P < 0.05$). Furthermore, milk BHBA in mp-cows was higher at d 31 compared to d 146 pp (27.4 ± 14.6 mg/L; $P < 0.001$). During FR in mid-lactation, milk BHBA in mp-cows declined from d 140 to 143 pp by 25.5 mg/L (to 13.8 ± 13.6 mg/L; $P < 0.05$).

Table 13: Mean milk parameters (LSM ± SE) at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restriction in early and mid-lactation.

	MP	mp	Mp	mP
milk yield, kg				
d 25 pp	41.7 ± 1.1^{a*1}	36.8 ± 0.6^{ac*1}	46.7 ± 1.7^{b*1}	33.7 ± 1.1^{c1}
d 28 pp	36.0 ± 1.0$^{ab\circ 1}$	31.3 ± 1.6$^{ac\circ 1}$	38.9 ± 1.3$^{b\circ 1}$	30.5 ± 1.4^{c1}
d 31 pp	41.1 ± 1.4^{a*1}	34.6 ± 1.4$^{b*\circ 1}$	41.7 ± 1.9$^{a\circ 1}$	32.6 ± 1.2^{b1}
d 140 pp	32.1 ± 2.2^{ab*}	31.2 ± 2.4^{ac*}	37.0 ± 2.4^{b*}	26.6 ± 2.0^{c}
d 143 pp	27.8 ± 2.0$^{ab\circ}$	25.5 ± 2.5$^{a\circ}$	31.6 ± 2.2$^{b\circ}$	23.5 ± 1.7^{a}
d 146 pp	29.4 ± 2.4$^{a*\circ}$	28.1 ± 2.2$^{ab*\circ}$	32.6 ± 1.6$^{a\circ}$	23.9 ± 1.8^{b}
FCM, kg				
d 25 pp	53.7 ± 4.1^{a*1}	39.3 ± 1.7^{b}	49.9 ± 1.9^{a*1}	38.7 ± 1.1^{b1}
d 28 pp	46.4 ± 2.5$^{a\circ 1}$	36.7 ± 1.6^{b1}	44.4 ± 1.9$^{a\circ 1}$	37.0 ± 2.2^{b1}
d 31 pp	52.3 ± 2.9^{a*1}	39.0 ± 1.9^{b1}	47.0 ± 1.9$^{a*\circ 1}$	37.9 ± 2.6^{b1}
d 140 pp	35.0 ± 1.6	33.1 ± 3.6	36.9 ± 2.7	31.7 ± 2.2
d 143 pp	30.0 ± 2.6	27.7 ± 2.5	32.1 ± 1.6	28.2 ± 1.6
d 146 pp	30.9 ± 1.7	28.0 ± 2.6	32.2 ± 1.5	25.9 ± 1.6
ECM, kg				
d 25 pp	55.9 ± 3.6^{a*1}	41.1 ± 1.4^{b}	52.4 ± 1.9^{a*1}	41.4 ± 1.2^{b1}
d 28 pp	47.7 ± 2.5$^{a\circ 1}$	38.0 ± 1.8^{b1}	46.0 ± 1.8$^{a\circ 1}$	38.8 ± 2.3^{b1}
d 31 pp	53.8 ± 2.8^{a*1}	40.3 ± 1.8^{b1}	48.8 ± 1.6$^{a*\circ 1}$	39.7 ± 2.6^{b1}
d 140 pp	38.4 ± 1.6*	35.6 ± 3.6*	40.0 ± 2.8*	34.6 ± 2.0
d 143 pp	32.5 ± 2.4°	29.5 ± 2.8°	34.3 ± 1.8°	30.5 ± 1.6
d 146 pp	33.9 ± 1.6*°	30.5 ± 2.8*°	35.0 ± 1.6*°	28.8 ± 1.6
milk protein content, %				
d 25 pp	3.28 ± 0.07^{a*1}	2.83 ± 0.06^{b1}	2.92 ± 0.06^{b1}	3.42 ± 0.04^{a*1}
d 28 pp	3.10 ± 0.08$^{ab\circ 1}$	2.95 ± 0.12^{ab}	2.90 ± 0.06^{a}	3.22 ± 0.08$^{b\circ 1}$
d 31 pp	3.06 ± 0.07$^{ab\circ 1}$	2.79 ± 0.10^{a1}	2.88 ± 0.08^{ab1}	3.11 ± 0.07$^{b\circ 1}$
d 140 pp	3.66 ± 0.18^{a*}	3.23 ± 0.07^{b}	3.18 ± 0.06^{b}	3.91 ± 0.14^{a}
d 143 pp	3.43 ± 0.20$^{a\circ}$	3.15 ± 0.08^{b}	3.06 ± 0.06^{b}	3.80 ± 0.14^{c}
d 146 pp	3.58 ± 0.19$^{a*\circ}$	3.26 ± 0.05^{b}	3.17 ± 0.05^{b}	3.87 ± 0.15^{a}
milk protein yield, g				
d 25 pp	1,373 ± 59^{a*1}	1,043 ± 36^{b}	1,365 ± 50^{a*1}	1,153 ± 42^{b*}
d 28 pp	1,116 ± 55$^{a\circ 1}$	922 ± 56^{b}	1,127 ± 38$^{a\circ 1}$	983 ± 58$^{ab\circ}$
d 31 pp	1,256 ± 57^{a*1}	971 ± 66^{b}	1,196 ± 39$^{a\circ 1}$	1,014 ± 51$^{b*\circ}$
d 140 pp	1,160 ± 41*	1,012 ± 91*	1,175 ± 79*	1,027 ± 44*
d 143 pp	939 ± 27°	810 ± 98°	962 ± 62°	884 ± 34°
d 146 pp	1,034 ± 44*°	917 ± 79*°	1,033 ± 57°	916 ± 35*°

	MP	mp	Mp	mP
milk fat content, %				
d 25 pp	5.93 ± 0.72^{a1}	4.48 ± 0.36^{b}	4.47 ± 0.2 ^{b}	4.99 ± 0.22 ^{b}
d 28 pp	5.92 ± 0.32^{a1}	5.18 ± 0.36^{ab}	4.95 ± 0.3^{b}	5.43 ± 0.28^{ab}
d 31 pp	5.80 ± 0.33^{a1}	4.88 ± 0.46^{ab}	4.93 ± 0.43^{b1}	5.06 ± 0.26^{ab}
d 140 pp	4.69 ± 0.37^{ab}	4.34 ± 0.40^{a}	3.97 ± 0.11^{a}	5.39 ± 0.62^{b}
d 143 pp	4.58 ± 0.62^{ab}	4.60 ± 0.24^{ab}	4.16 ± 0.14^{a}	5.38 ± 0.26^{b}
d 146 pp	4.45 ± 0.38	3.94 ± 0.13	3.94 ± 0.15	4.59 ± 0.14
milk fat yield, g				
d 25 pp	2,466 ± 274^{a*1}	1,640 ± 118^{b}	2,078 ± 108^{c1}	1,678 ± 61^{b}
d 28 pp	2,135 ± 145$^{a\circ 1}$	1,611 ± 94^{b1}	1,920 ± 114^{ab1}	1,654 ± 116^{b1}
d 31 pp	2,389 ± 168$^{a*\circ 1}$	1,675 ± 129^{b1}	2,023 ± 117^{c1}	1,659 ± 142^{b1}
d 140 pp	1,479 ± 74	1,373 ± 186	1,471 ± 117	1,403 ± 134
d 143 pp	1,259 ± 155	1,164 ± 106	1,295 ± 52	1,252 ± 70
d 146 pp	1,277 ± 72	1,115 ± 116	1,277 ± 65	1,090 ± 64
milk lactose content, %				
d 25 pp	4.77 ± 0.08	4.76 ± 0.05*	4.81 ± 0.04	4.94 ± 0.03^{1}
d 28 pp	4.69 ± 0.04^{ab}	4.60 ± 0.08$^{a\circ}$	4.77 ± 0.06^{ab}	4.86 ± 0.06^{b}
d 31 pp	4.77 ± 0.06	4.71 ± 0.05*$^{\circ}$	4.76 ± 0.07	4.88 ± 0.06
d 140 pp	4.70 ± 0.10	4.76 ± 0.07	4.82 ± 0.05	4.72 ± 0.09
d 143 pp	4.69 ± 0.11	4.73 ± 0.09	4.82 ± 0.04	4.80 ± 0.06
d 146 pp	4.76 ± 0.08	4.68 ± 0.09	4.75 ± 0.04	4.78 ± 0.08
milk lactose yield, g				
d 25 pp	1,993 ± 82^{a*1}	1,751 ± 42^{ac*}	2,250 ± 89^{b*1}	1,666 ± 53^{c1}
d 28 pp	1,688 ± 53$^{ab\circ 1}$	1,443 ± 91$^{a\circ}$	1,857 ± 68$^{b\circ 1}$	1,480 ± 62^{a1}
d 31 pp	1,965 ± 94^{a*1}	1,632 ± 70$^{b*\circ 1}$	1,993 ± 115$^{a\circ 1}$	1,588 ± 47^{b1}
d 140 pp	1,512 ± 122^{ab*}	1,494 ± 133^{ac*}	1,783 ± 114^{b*}	1,255 ± 103^{c}
d 143 pp	1,307 ± 102$^{ab\circ}$	1,214 ± 136$^{a\circ}$	1,521 ± 109$^{b\circ}$	1,127 ± 87^{a}
d 146 pp	1,403 ± 127$^{ab*\circ}$	1,320 ± 123$^{ab*\circ}$	1,549 ± 84$^{a\circ}$	1,146 ± 100^{b}
FPR				
d 25 pp	1.81 ± 0.22^{a1}	1.59 ± 0.14^{ab}	1.53 ± 0.10^{b1}	1.46 ± 0.06^{b}
d 28 pp	1.91 ± 0.08^{1}	1.76 ± 0.07	1.71 ± 0.11^{1}	1.68 ± 0.06
d 31 pp	1.90 ± 0.11^{1}	1.76 ± 0.20^{1}	1.71 ± 0.14^{1}	1.63 ± 0.06^{1}
d 140 pp	1.28 ± 0.06	1.35 ± 0.14	1.25 ± 0.03	1.38 ± 0.14
d 143 pp	1.34 ± 0.16	1.47 ± 0.10	1.36 ± 0.04	1.42 ± 0.06
d 146 pp	1.24 ± 0.08	1.21 ± 0.05	1.25 ± 0.06	1.19 ± 0.04
milk SCC, × 1,000/mL				
d 25 pp	28.5 ± 7.9	37.0 ± 6.7	24.3 ± 3.1	291.2 ± 245.1*
d 28 pp	45.7 ± 15.4	163.6 ± 122.0	26.6 ± 2.2	300.8 ± 212.6*
d 31 pp	40.7 ± 17.1^{a}	45.6 ± 16.3^{a}	268.7 ± 240.6^{a}	1,447.6 ± 1,084.4$^{b\circ 1}$
d 140 pp	54.8 ± 17.9	94.4 ± 45.2*$^{\circ}$	125.1 ± 52.7	362.2 ± 146.8
d 143 pp	54.2 ± 11.0	80.2 ± 31.9°	67.9 ± 12.4	441.4 ± 257.6
d 146 pp	68.6 ± 36.7	679.8 ± 606.3*	68.9 ± 24.0	598.8 ± 486.7

	MP	mp	Mp	mP
milk pH				
d 25 pp	6.66 ± 0.05^{ab}	6.60 ± 0.03^{a}	6.68 ± 0.01^{b}*	6.68 ± 0.03^{ab}
d 28 pp	6.66 ± 0.04^{a}	6.58 ± 0.02^{b}	6.67 ± 0.02^{a}*^{1}	6.67 ± 0.02^{a}
d 31 pp	6.66 ± 0.03^{a}	6.58 ± 0.03^{b}	6.61 ± 0.03^{ab}○	6.67 ± 0.02^{a}
d 140 pp	6.61 ± 0.03	6.59 ± 0.03	6.63 ± 0.02	6.61 ± 0.02
d 143 pp	6.60 ± 0.03^{ab}	6.59 ± 0.01^{a}	6.59 ± 0.03^{a}	6.67 ± 0.02^{b}
d 146 pp	6.62 ± 0.02	6.61 ± 0.01	6.62 ± 0.02	6.64 ± 0.03
milk urea, mg/L				
d 25 pp	185 ± 15	163 ± 24	175 ± 16	161 ± 10
d 28 pp	154 ± 24^{1}	139 ± 19^{1}	154 ± 19	175 ± 13
d 31 pp	164 ± 19^{1}	159 ± 26	150 ± 17^{1}	183 ± 22
d 140 pp	210 ± 15	190 ± 26	200 ± 22	191 ± 21
d 143 pp	220 ± 14	206 ± 21	201 ± 8	226 ± 18
d 146 pp	228 ± 16	179 ± 23	224 ± 23	217 ± 22
milk hydrocortisone, nmol/L				
d 25 pp	3.34 ± 0.79	2.61 ± 0.49	3.21 ± 0.55	4.01 ± 0.70
d 28 pp	3.06 ± 0.27	3.49 ± 0.59	2.86 ± 0.29	4.13 ± 0.72
d 31 pp	3.56 ± 0.70	3.39 ± 0.70	3.09 ± 0.59	3.66 ± 0.48
d 140 pp	4.29 ± 0.85	3.34 ± 0.40	4.97 ± 0.53	3.20 ± 0.40
d 143 pp	3.98 ± 0.88	2.99 ± 0.29	3.45 ± 0.52	3.23 ± 0.52
d 146 pp	5.35 ± 0.77	3.80 ± 0.56	4.20 ± 0.77	4.83 ± 0.79
milk BHBA, mg/L				
d 25 pp	26.5 ± 12.2	29.6 ± 13.7*	26.9 ± 12.3	13.9 ± 15.4
d 28 pp	21.9 ± 13.3	21.3 ± 12.9*	29.6 ± 12.2	29.1 ± 14.4
d 31 pp	27.7 ± 11.8^{ab}	61.3 ± 13.0^{a}○^{1}	36.8 ± 12.4^{ab}	18.9 ± 14.1^{b}
d 140 pp	41.4 ± 12.2	39.3 ± 12.6*	22.2 ± 11.6	17.1 ± 13.7
d 143 pp	33.5 ± 13.1	13.8 ± 13.6°	26.3 ± 12.1	14.6 ± 14.8
d 146 pp	26.4 ± 14.3	27.4 ± 14.6*°	28.1 ± 11.7	19.8 ± 15.1

^{abcd}Alphabetic superscripts indicate differences between groups ($P < 0.05$).

*○$^{\#}$Symbolic superscripts indicate differences between days within a feed restriction ($P < 0.05$).

^{1}Superscripted 1 indicates difference ($P < 0.05$) of day in FR during early lactation (d 25, 28 and 31 pp) compared to day of FR in mid-lactation (d 140, 143 and 146 pp, respectively).

Fixed effects in model milk lactose content: time $P < 0.001$, group $P = 0.22$ and time × group $P = 0.45$. Fixed effects in model milk lactose yield: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.87$. Fixed effects in model FPR: time $P < 0.001$, group $P = 0.14$ and time × group $P = 0.96$. Fixed effects in model ECM: time $P < 0.001$, group $P < 0.001$ and time × group $P = 0.84$. Fixed effects in model SCC: time $P = 0.11$, group $P = 0.04$ and time × group $P = 0.63$. Fixed effects in model milk pH: time $P < 0.01$, group $P = 0.08$ and time × group $P = 0.20$. Fixed effects in model milk urea: time $P < 0.001$, group $P = 0.67$ and time × group $P = 0.99$. Fixed effects in model milk hydrocortisone: time $P = 0.02$, group $P = 0.64$ and time × group $P = 0.96$. Fixed effects in model milk BHBA: time $P = 0.23$, group $P = 0.83$ and time × group $P = 0.34$.

3.3. Blood serum metabolites

Blood samples were collected in the morning before feeding, meaning that periods before restricted feeding are represented by samples at d 26 and 141 pp, days of restricted feeding by d 27 to 29 and d 142 to 144 pp and periods after restricted feeding by samples at d 32 and 147 pp.

Glucose

In MP-cows, lower concentrations of blood serum glucose were observed at all days of FR in early lactation compared to mid-lactation ($P < 0.05$), whereas in mp- and Mp-cows blood serum glucose was lower solely at d 29 pp (2.49 ± 0.52 and 2.51 ± 0.18 mmol/L) compared to d 144 pp (3.53 ± 0.21 and 3.48 ± 0.09 mmol/L; $P < 0.01$; table 14, figure 33).

Levels of blood serum glucose decreased in all cows from d 26 to 29 pp (by 0.7, 0.85, 0.99 and 0.65 mmol/L in MP-, mp-, Mp- and mP-cows; $P < 0.05$) and in all cows except of mP-cows below critical values of 3.0 mmol/L. At d 29 pp, blood serum glucose was similar in all groups with MP- and mp-cows showing levels below value of 3.0 mmol/L. After FR, glucose levels increased until d 32 pp significantly in mp-, Mp- and mP-cows (by 1.35 in mp- and by 0.76 nmol/L in Mp- and mP-cows; $P < 0.05$) and increase tended to be significant in MP-cows (by 0.48 mmol/L; $P < 0.1$). At d 32 pp, all cows showed similar blood glucose levels. During days of restricted feeding in mid-lactation, glucose levels decreased numerically, but not below critical values and increased afterwards. Furthermore at d 147 pp, blood serum glucose levels were higher in MP-cows compared to Mp-cows (4.28 ± 0.20 and 3.61 ± 0.11; $P < 0.05$).

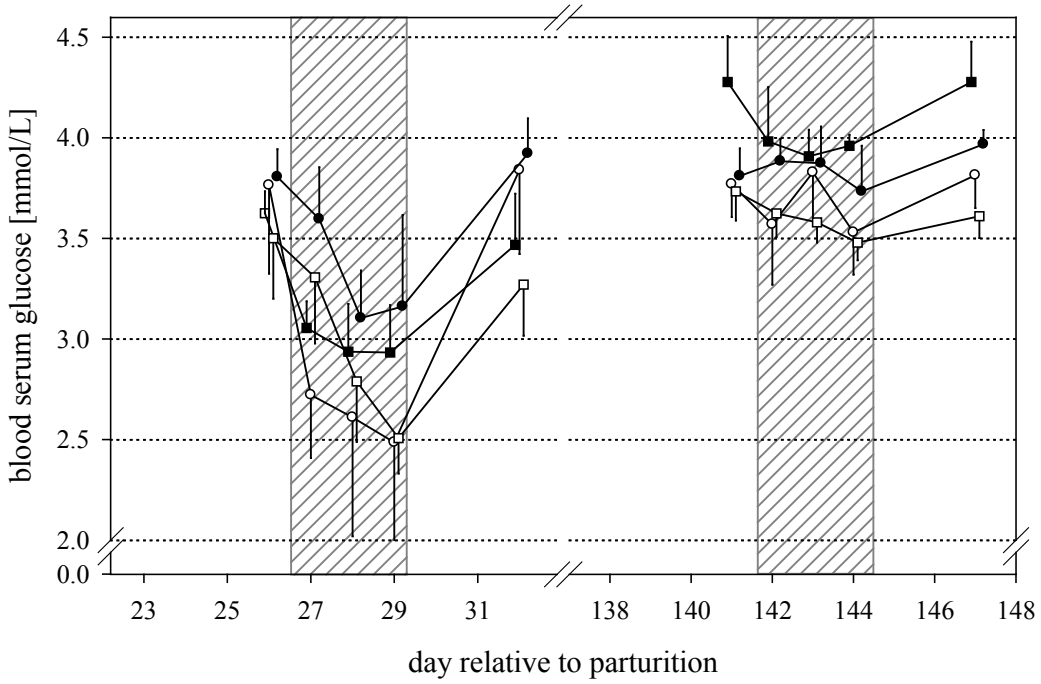

Figure 33: Blood serum glucose (mmol/L) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model glucose: time $P < 0.001$, group $P = 0.24$ and time × group $P = 0.67$.

NEFA

All cows showed higher blood serum NEFA levels during FR in early lactation compared to FR in mid-lactation ($P < 0.05$; table 14, figure 34).

Blood serum concentrations were similar amongst cows at all days during FR in early lactation. At d 26 pp, MP-cows showed levels above critical value of 600 µmol/L. In all cows, NEFA levels increased from d 26 to 28 pp above values of 600 µmol/L (to 1,238 ± 141, 1,241 ± 373, 1,089 ± 143 and 1,064 ± 133 µmol/L in MP-, mp-, Mp- and mP-cows; $P < 0.05$) and decreased until last day of restricted feeding in MP-, mp- and mP-cows by 280, 233 and 322 µmol/L ($P < 0.05$). Only Mp-cows showed increasing blood serum NEFA levels from d 26 (491 ± 37 µmol/L) to 29 pp (1,091 ± 147 µmol/L; $P < 0.001$). After days of restricted feeding, NEFA levels decreased in Mp- (to 499 ± 60 µmol/L; $P < 0.001$) and mP-cows (455 ± 57 µmol/L; $P = 0.05$). Levels of NEFA in MP-cows remained still above critical value after FR (804 ± 181 µmol/L).

Concentrations of NEFA increased in all cows during days of restricted feeding in mid-lactation and decreased afterwards, but difference proved not to be significant. No increase above critical value was measured.

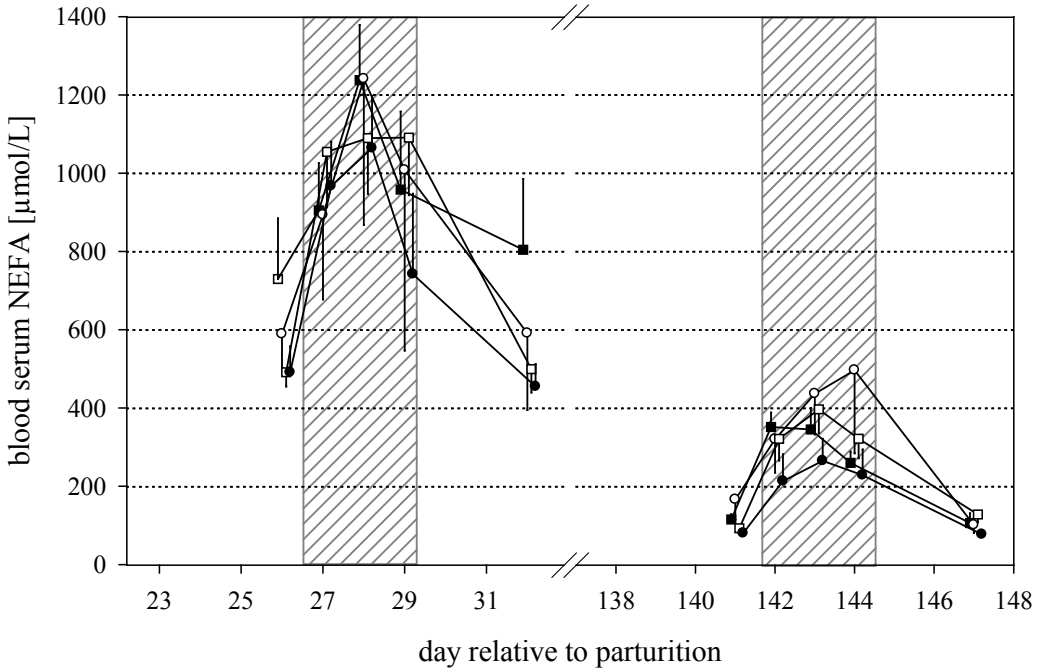

Figure 34: Blood serum NEFA (µmol/L) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model NEFA: time $P < 0.001$, group $P = 0.70$ and time × group $P = 0.97$.

BHBA

Compared to respective days of FR in mid-lactation, all cows showed higher levels of blood serum BHBA at last day of restricted feeding in early lactation ($P < 0.001$) and high yielding cows showed still higher levels after FR in early lactation ($P < 0.05$; table 14, figure 35).

At all days of FR in early lactation, levels of blood serum BHBA were comparable amongst cows. From d 26 to 29 pp, concentrations of BHBA increased markedly in all cows above threshold values of 1.0 mmol/L (by 1.95, 1.50, 2.17 and 1.78 mmol/L in MP-, mp-, Mp- and mP-cows; $P < 0.001$). Afterwards, BHBA levels decreased in all cows, but only in mP-cows below threshold value of 1.0 mmol/L ($P < 0.01$).

During FR in mid-lactation, no increase of blood serum BHBA levels was measured.

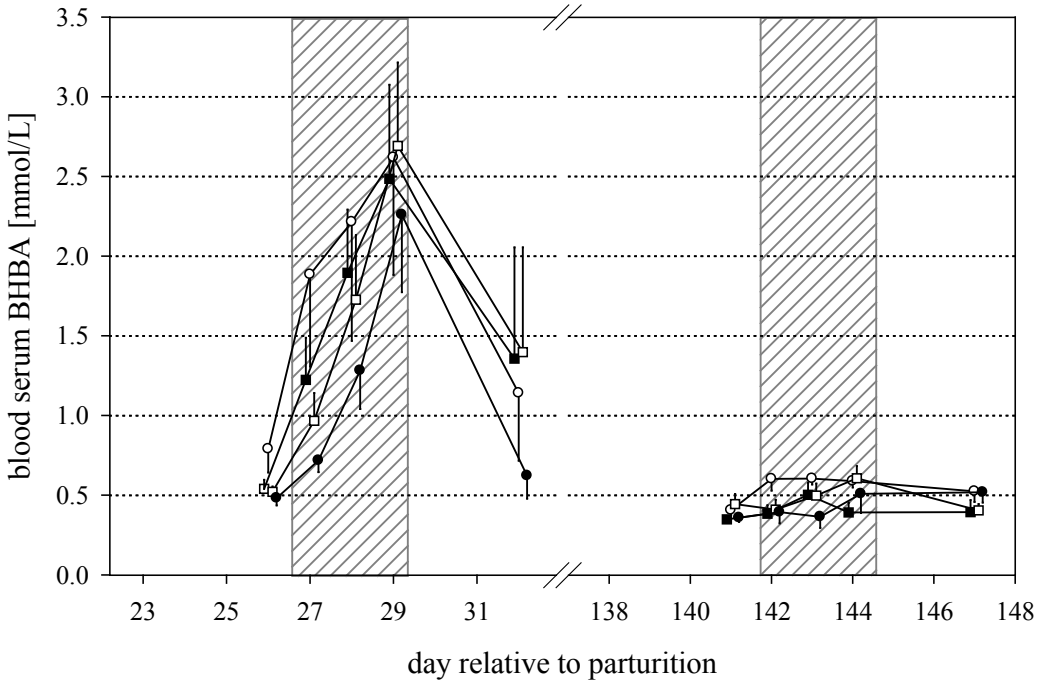

Figure 35: Blood serum BHBA (mmol/L) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model BHBA: time $P < 0.001$, group $P = 0.56$ and time × group $P = 0.99$.

Cholesterol

Blood serum cholesterol concentrations in high yielding cows were lower during early FR compared to second FR ($P < 0.05$), whereas mp-cows showed lower levels of blood serum cholesterol concentrations at d 26 and 29 pp ($P < 0.05$) and mP-cows at d 26 pp ($P < 0.05$; table 14, figure 36).

At all days of FR in early lactation, cholesterol levels were similar amongst cows. During restricted feeding, levels of blood serum cholesterol increased in MP-cows by 0.40 mmol/L ($P < 0.05$) and remained on similar levels afterwards.

Regarding FR in mid-lactation, one Mp-cow showed extremely high blood cholesterol concentrations at d 141 pp (12.30 mmol/L), resulting in significant higher values of Mp-cows (6.49 ± 1.04 mmol/L; $P < 0.01$). During days of restricted feeding, cholesterol levels remained on similar levels and afterwards, Mp-cows showed higher levels at d 144 pp compared to mp- and mP-cows ($P < 0.05$). After days of restricted feeding, blood serum cholesterol concentrations decreased in low protein cows ($P < 0.05$). At d 147 pp, concentrations of blood serum cholesterol were higher in Mp-cows compared to mp- and mP-cows ($P < 0.05$). Additionally MP-cows showed higher blood serum cholesterol levels compared to mp-cows ($P < 0.05$).

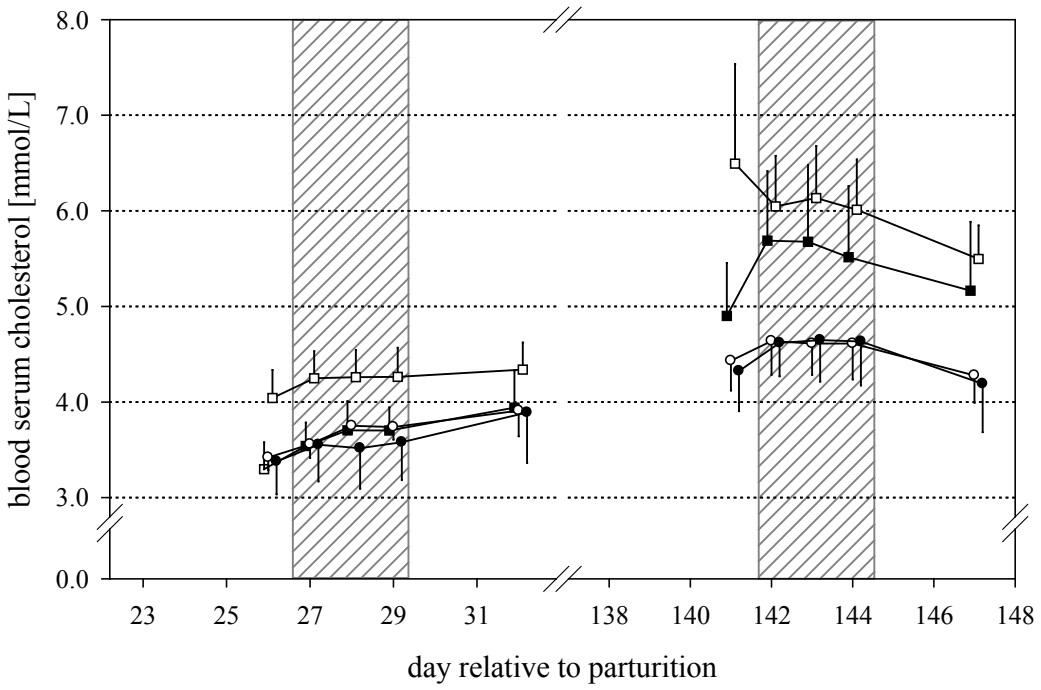

Figure 36: Blood serum cholesterol (mmol/L) during feed restrictions in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey shaded areas show days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model cholesterol: time $P < 0.001$, group $P < 0.05$ and time × group $P < 0.05$.

Total bilirubin

Levels of blood serum tBR showed no distinguishable pattern and no differences between groups (table 14).

Table 14: Mean blood parameters (LSM ± SE) before, during and after three days of feed restriction in early and mid-lactation.

	MP	mp	Mp	mP
blood serum glucose, mmol/L				
d 26 pp	3.63 ± 0.11*[1]	3.34 ± 0.24*	3.50 ± 0.30*	3.81 ± 0.14*
d 29 pp	2.93 ± 0.24°[1]	2.49 ± 0.52°[1]	2.51 ± 0.18°[1]	3.16 ± 0.45°
d 32 pp	3.41 ± 0.26*°[1]	3.84 ± 0.42*	3.27 ± 0.25*	3.92 ± 0.17*
d 141 pp	4.28 ± 0.23	3.77 ± 0.16	3.73 ± 0.15	3.81 ± 0.14
d 144 pp	3.96 ± 0.05	3.53 ± 0.21	3.48 ± 0.09	3.73 ± 0.23
d 147 pp	4.28 ± 0.20[a]	3.81 ± 0.16[ab]	3.61 ± 0.11[b]	3.97 ± 0.07[ab]
blood serum NEFA, µmol/L				
d 26 pp	730 ± 155[1]	746 ± 238[1]	491 ± 37*[1]	491 ± 66*°[1]
d 29 pp	958 ± 199[1]	1,008 ± 461[1]	1,091 ± 147°[1]	742 ± 206*[1]
d 32 pp	804 ± 181[1]	591 ± 196[1]	499 ± 60*[1]	455 ± 57°[1]
d 141 pp	115 ± 13	166 ± 51	93 ± 11	80 ± 9
d 144 pp	260 ± 28	496 ± 212	322 ± 50	228 ± 66
d 147 pp	106 ± 25	101 ± 20	128 ± 20	77 ± 5

	MP	mp	Mp	mP
blood serum BHBA, mmol/L				
d 26 pp	0.54 ± 0.06*	1.12 ± 0.42*	0.52 ± 0.03*	0.48 ± 0.05*
d 29 pp	2.49 ± 0.59°[1]	2.62 ± 0.74°[1]	2.69 ± 0.52°[1]	2.26 ± 0.49°[1]
d 32 pp	1.47 ± 0.67#[1]	1.14 ± 0.42*	1.40 ± 0.66#[1]	0.62 ± 0.14*
d 141 pp	0.35 ± 0.02	0.41 ± 0.03	0.44 ± 0.06	0.36 ± 0.03
d 144 pp	0.39 ± 0.06	0.59 ± 0.04	0.61 ± 0.08	0.51 ± 0.12
d 147 pp	0.39 ± 0.07	0.52 ± 0.07	0.41 ± 0.04	0.52 ± 0.06
blood serum cholesterol, mmol/L				
d 26 pp	3.30 ± 0.28*[1]	3.41 ± 0.13[1]	4.04 ± 0.29[1]	3.38 ± 0.34[1]
d 29 pp	3.70 ± 0.24°[1]	3.74 ± 0.13[1]	4.26 ± 0.31[1]	3.58 ± 0.40
d 32 pp	3.88 ± 0.38°[1]	3.91 ± 0.27	4.34 ± 0.28[1]	3.89 ± 0.53
d 141 pp	4.90 ± 0.55[a]	4.43 ± 0.31[a]*°	6.49 ± 1.04[b]*	4.32 ± 0.42[a]
d 144 pp	5.51 ± 0.75[ab]	4.61 ± 0.38[a]*	6.01 ± 0.53[b]*	4.63 ± 0.46[a]
d 147 pp	5.16 ± 0.72[ac]	4.28 ± 0.28[b]°	5.50 ± 0.35[c]°	4.19 ± 0.51[ab]
blood serum total bilirubin, µmol/L				
d 26 pp	5.60 ± 0.98	6.46 ± 1.77	4.97 ± 0.78	3.99 ± 0.48
d 29 pp	4.18 ± 0.59	7.56 ± 1.97	6.03 ± 0.66	5.73 ± 1.03
d 32 pp	5.57 ± 0.83	3.20 ± 0.38	4.46 ± 0.63	4.06 ± 0.76
d 141 pp	2.54 ± 0.29	3.22 ± 0.83	2.10 ± 0.31	3.11 ± 0.32
d 144 pp	3.49 ± 0.32	5.64 ± 1.42	2.97 ± 0.45	3.57 ± 0.55
d 147 pp	3.23 ± 0.61	2.39 ± 0.40	2.83 ± 0.39	3.30 ± 0.50

[abcd]Alphabetic superscripts indicate differences between groups ($P < 0.05$).
*°#Symbolic superscripts indicate differences between days within a feed restriction ($P < 0.05$).
[1]Superscripted 1 indicates difference ($P < 0.05$) of day in early lactation feed restriction (d 25, 28 and 31 pp) compared to day in mid-lactation feed restriction (d 140, 143 and 146 pp, respectively).
Fixed effects in model bilirubin: time $P < 0.05$, group $P = 0.77$ and time × group $P = 0.74$.

3.4. Hepatic mRNA profiles

No differences between FR in early and mid-lactation or between groups within a FR were found in mRNA abundance of *ACACA*, *CPT1A*, *INSR*, *HMGCS2*, *EIF4B*, *PPARA*, *SREBF1*, *CS* and *TNFA* (table 15).

Lipid metabolism

Concerning β-oxidation of fatty acids, hepatic mRNA levels of *ACADVL* were higher in MP- and Mp-cows after early FR (14.13 ± 0.21 and 14.09 ± 0.21 in MP- and Mp-cows) compared to second FR (in MP-cows 13.36 ± 0.17, $P < 0.05$; in Mp-cows 13.57 ± 0.13, $P < 0.05$). Furthermore, transcript abundance increased in mp-cows from d 15 to 29 pp (13.09 ± 0.19 to 13.63 ± 0.15; $P < 0.05$) and decreased from d 29 to 57 pp in Mp- (14.09 ± 0.21 to 13.59 ± 0.17; $P < 0.05$) and mP-cows (14.05 ± 0.23 to 13.00 ± 0.21; $P < 0.001$).

Although in β-oxidation no differences were observed for *CPT1A* transcripts, mp-cows showed higher levels in early FR compared to d 15 pp (12.71 ± 0.25 and 13.62 ± 0.16 at d 15 and 29 pp; $P < 0.05$).

Furthermore Mp-cows showed in β-oxidation higher mRNA abundance of *ECHS1* at d 29 pp compared to mp-cows (15.93 ± 0.19 and 15.22 ± 0.28 in Mp- and mp-cows; $P < 0.05$), whereas at

d 144 pp MP- (15.74 ± 0.30) and Mp-cows (15.64 ± 0.11) expressed higher hepatic mRNA levels compared to mp-cows (14.63 ± 0.56; $P < 0.05$). Moreover, Mp-cows showed elevated transcript abundance after early FR compared to d 15 pp (15.47 ± 0.17; $P < 0.05$). Second FR led in mp-cows to increased mRNA levels of *ECHS1* compared to d 57 pp (15.51 ± 0.13; $P < 0.01$). Additionally in Mp- and mP-cows, transcript abundance decreased after FR in mid-lactation to day of slaughtering (14.60 ± 0.16 and 14.93 ± 0.19 in Mp- and mP-cows; $P < 0.05$).

Regarding fatty acid synthesis, transcript abundance of *ACACA* was lower in MP- (8.68 ± 0.90) and mp-cows (8.98 ± 0.31) in early FR compared to d 57 pp (9.41 ± 0.74 and 10.48 ± 0.74 in MP- and mp-cows; $P < 0.05$; table 15). In MP-cows, mRNA levels declined during FR in mid-lactation (9.41 ± 0.74 at d 57 pp and 8.77 ± 1.38 at d 144 pp; $P < 0.05$). Furthermore, Mp-cows showed higher transcript levels during second FR compared to day of slaughtering (9.39 ± 0.50 and 6.54 ± 0.61; $P < 0.01$).

Furthermore, mRNA levels of *GPAM*, encoding for the enzyme of triacylglycerol synthesis' initial step, were higher in early FR in Mp-cows (12.67 ± 0.23) compared to MP- (11.41 ± 0.64; $P < 0.05$) and mp-cows (11.88 ± 0.26; $P < 0.05$). Furthermore, mRNA levels were higher in Mp- and mP-cows in second FR compared to day of slaughtering (10.93 ± 0.30 and 10.34 ± 0.31 at d 155 pp in Mp- and mP-cows; $P < 0.001$)

Protein metabolism

Regarding protein catabolism, hepatic transcript abundance of *CTSL* was lowest at d 144 pp in MP-cows (15.01 ± 0.62; $P < 0.05$; table 15). Furthermore, Mp-cows showed higher mRNA levels during early FR compared to d 15 pp (15.41 ± 0.19; $P < 0.01$) and in all cows except of MP-cows, hepatic transcript abundance of *CTSL* decreased after second FR to 14.44 ± 0.28 in mp- ($P < 0.001$), 14.95 ± 0.24 in Mp- ($P < 0.01$) and 14.93 ± 0.25 in mP-cows ($P < 0.001$) at day of slaughtering.

Furthermore hepatic mRNA levels of *TAT* were higher in Mp-cows compared to mp-cows at d 29 pp (16.54 ± 0.23 and 15.68 ± 0.31 in Mp- and mp-cows; $P < 0.05$) and Mp-cows showed higher mRNA abundance after early FR compared to second FR (15.60 ± 0.24; $P < 0.05$). In Mp-cows, mRNA encoding for *TAT* increased from d 15 to 29 pp (by 1.00; $P < 0.01$) and decreased to d 57 pp (15.81 ± 0.26 $P < 0.05$). Additionally, mP-cows showed lower mRNA levels during FR in mid-lactation compared to day of slaughtering (16.59 ± 0.32; $P < 0.05$).

Carbohydrate metabolism

Regarding gluconeogenesis, Mp- and mP-cows expressed higher hepatic mRNA abundance of *PC* at d 29 pp (11.24 ± 0.27 and 10.47 ± 0.80 in Mp- and mP-cows) compared to d 144 pp (9.84 ± 0.69 and 8.72 ± 0.85 in Mp- and mP-cows; $P < 0.05$; table 15). In high yielding and in mP-cows, mRNA levels of *PC* were higher in early FR compared to d 57 (7.61 ± 0.76, 9.96 ± 0.63 and 8.31 ± 0.77 in MP-, Mp- and mP-cows; $P < 0.05$).

Moreover transcript abundance of *PCK1* at d 144 pp was higher in Mp- (17.19 ± 0.41) compared to MP-cows (15.51 ± 0.60; $P < 0.05$). Furthermore, lower mRNA levels were observed in MP-cows during early FR compared to d 57 pp (17.40 ± 0.62; $P < 0.05$) and higher transcript abundance occurred in second FR compared to day of slaughtering in Mp- (15.15 ± 0.72) and mP-cows (15.11 ± 0.62; $P < 0.05$).

Glucose metabolism and ketogenesis

Hepatic transcript abundance of *SLC2A2*, encoding for glucose transporter, decreased in mp-cows from d 57 to 144 pp (from 14.21 ± 0.40 to 12.84 ± 0.57; $P < 0.05$) and then was higher compared

to Mp-cows (13.98 ± 0.16; P < 0.05; table 15). Afterwards, mRNA levels declined in Mp-cows and lower levels were observed at day of slaughtering (P < 0.001).

Hepatic mRNA levels of *INSR* increased in Mp-cows from d 15 pp to FR in early lactation and declined afterwards (11.84 ± 0.16, 12.45 ± 0.33 and 11.90 ± 0.17 at d 15, 29 and 57 pp; P < 0.05). Also FR in mid-lactation led to decreasing mRNA levels in mp-cows compared to d 57 pp (12.11 ± 0.21; P < 0.05).

For ketogenesis, mRNA levels of *HMGCS2* were measured and showed higher levels in Mp-cows during FRs (16.10 ± 0.57 and 15.07 ± 0.75 at d 29 and 144 pp) compared to following sampling time points (14.40 ± 0.49 at d 57 pp and 13.21 ± 0.58 at d 155 pp; P < 0.01).

Translation and transcription factors

Transcript abundance of translation factor *EIF4B* showed in MP-cows higher levels at d 144 pp (15.14 ± 0.57) compared to d 155 pp (14.23 ± 0.33; P < 0.05; table 15). Furthermore Mp-cows showed higher mRNA abundance of transcription factor *HNF4A* at d 29 pp (14.15 ± 0.37) compared to d 144 pp (12.96 ± 0.32; P < 0.05).

Abundance of mRNA encoding for transcription factor *PPARA* showed in MP-cows lower levels during FR in mid-lactation (12.99 ± 0.65) compared to d 57 (13.90 ± 0.20; P < 0.01) and 155 pp (13.80 ± 0.20; P < 0.05). Also hepatic mRNA levels of *SREBF1* showed in mP-cows lower amounts during FR in early lactation (7.05 ± 0.49) compared to d 15 and 57 pp (8.19 ± 0.53 and 7.99 ± 0.53; P < 0.05). In mp-cows, transcript abundance was lower during FR in early lactation compared to d 57 pp (8.95 ± 0.52; P < 0.01). After FR in mid-lactation, hepatic mRNA abundance of *SREBF1* increased in Mp- and mP-cows to d 155 pp (to 9.24 ± 0.50 and 8.72 ± 0.52; P < 0.01).

Common hepatic metabolism

In mP-cows, hepatic mRNA abundance of *CS* showed lower levels during FR in mid-lactation compared to d 57 and 155 pp (10.53 ± 0.34, 7.90 ± 2.47 and 10.07 ± 0.34 at d 57, 144 and 155 pp; P < 0.05; table 15).

Hepatic levels of mRNA encoding for *TNFA* showed no differences amongst cows, FRs or days of lactation.

Hepatic mRNA abundance of *IGF1* was lower at d 29 compared to 144 pp in low yielding cows (10.83 ± 0.25 and 12.45 ± 0.50 in mp-cows, P < 0.05; 11.29 ± 0.90 and 13.12 ± 0.58 in mP-cows, P < 0.05). Furthermore, mRNA levels of *IGF1* declined in low yielding cows from d 15 to 29 pp (by 1.05 and 1.22 to 10.83 ± 0.25 and 11.29 ± 0.90 in mp- and mP-cows; P < 0.05) and increased again to d 57 pp (13.00 ± 0.41 and 13.32 ± 0.42 in mp- and mP-cows; P < 0.001). In Mp-cows, transcript abundance of IGF1 decreased after FR in mid-lactation to 11.72 ± 0.41 at day of slaughtering (P < 0.05).

Table 15: mRNA abundance (LSM ± SE of 15-ΔCt) after three days of feed restriction in early (d 29 pp) and mid-lactation (d 144 pp).

	MP	mp	Mp	mP
Lipid metabolism				
ACACA				
d 29 pp	8.68 ± 0.90	8.98 ± 0.31	9.48 ± 0.46	8.91 ± 0.68
d 144 pp	8.77 ± 1.38	9.76 ± 0.80	9.39 ± 0.50	9.12 ± 0.24

	MP	mp	Mp	mP
ACADVL				
d 29 pp	14.13 ± 0.21*	13.63 ± 0.15	14.09 ± 0.21*	14.05 ± 0.23
d 144 pp	13.36 ± 0.17	13.31 ± 0.12	13.57 ± 0.13	13.51 ± 0.19
CPT1A				
d 29 pp	13.52 ± 0.36	13.62 ± 0.16	14.04 ± 0.18	13.76 ± 0.41
d 144 pp	12.97 ± 0.65	13.07 ± 0.34	13.53 ± 0.10	12.89 ± 0.28
ECHS1				
d 29 pp	15.84 ± 0.25^{ab}	15.22 ± 0.28^{a}	15.93 ± 0.19^{b}	15.50 ± 0.22^{ab}
d 144 pp	15.74 ± 0.30^{b}	14.63 ± 0.56^{a}	15.64 ± 0.11^{b}	15.47 ± 0.14^{ab}
GPAM				
d 29 pp	11.41 ± 0.64^{a}	11.88 ± 0.26^{a}	12.67 ± 0.23^{b}	12.27 ± 0.22^{ab}
d 144 pp	11.91 ± 0.15	11.79 ± 0.44	12.29 ± 0.16	12.42 ± 0.33
Protein metabolism				
CTSL				
d 29 pp	15.40 ± 0.29	16.09 ± 0.24	16.07 ± 0.21	15.73 ± 0.07
d 144 pp	15.01 ± 0.62^{a}	16.31 ± 0.12^{b}	16.03 ± 0.25^{b}	16.11 ± 0.35^{b}
TAT				
d 29 pp	15.99 ± 0.69^{ab}	15.68 ± 0.31^{a}	16.54 ± 0.23^{b}*	15.92 ± 0.41^{ab}
d 144 pp	16.02 ± 0.24	15.16 ± 0.16	15.60 ± 0.24	15.56 ± 0.28
Carbohydrate metabolism				
PC				
d 29 pp	10.32 ± 0.37	10.35 ± 0.47	11.24 ± 0.27*	10.47 ± 0.80*
d 144 pp	8.54 ± 0.69	8.83 ± 0.38	9.84 ± 0.69	8.72 ± 0.85
PCK1				
d 29 pp	16.53 ± 0.59	16.56 ± 0.47	16.58 ± 0.46	16.03 ± 0.29
d 144 pp	15.51 ± 0.60^{a}	15.98 ± 0.70^{ab}	17.19 ± 0.41^{b}	17.03 ± 0.21^{ab}
Glucose transport				
SLC2A2				
d 29 pp	13.22 ± 0.42	13.25 ± 0.30	13.70 ± 0.19	13.10 ± 0.63
d 144 pp	12.85 ± 0.13^{ab}	12.84 ± 0.57^{a}	13.98 ± 0.16^{b}	13.62 ± 0.24^{ab}
Hormone receptor				
INSR				
d 29 pp	11.71 ± 0.19	11.93 ± 0.13	12.45 ± 0.33	12.10 ± 0.31
d 144 pp	11.85 ± 0.24	11.45 ± 0.42	11.87 ± 0.13	11.90 ± 0.21
Ketogenesis				
HMGCS2				
d 29 pp	14.86 ± 1.63	15.57 ± 0.56	16.10 ± 0.57	16.56 ± 1.03
d 144 pp	14.84 ± 1.20	15.78 ± 0.92	15.07 ± 0.75	14.65 ± 0.64
Translation				
EIF4B				
d 29 pp	14.75 ± 0.57	14.25 ± 0.21	14.92 ± 0.51	14.58 ± 0.56
d 144 pp	15.14 ± 0.57	13.76 ± 0.11	14.33 ± 0.14	14.70 ± 0.42

	MP	mp	Mp	mP
Transcription regulation				
HNF4A				
d 29 pp	13.58 ± 0.27	13.26 ± 0.24	14.15 ± 0.37*	13.91 ± 0.70
d 144 pp	13.46 ± 0.32	12.74 ± 0.07	12.96 ± 0.32	13.04 ± 0.29
PPARA				
d 29 pp	13.75 ± 0.24	13.58 ± 0.20	13.99 ± 0.28	13.74 ± 0.30
d 144 pp	12.99 ± 0.65	13.31 ± 0.32	13.71 ± 0.22	13.99 ± 0.11
SREBF1				
d 29 pp	7.78 ± 0.14	7.10 ± 0.53	8.28 ± 0.29	7.05 ± 0.49
d 144 pp	8.15 ± 0.14	8.04 ± 0.44	7.64 ± 0.57	7.22 ± 0.33
Final metabolism				
CS				
d 29 pp	10.32 ± 0.07	9.73 ± 0.65	10.60 ± 0.17	11.08 ± 0.25
d 144 pp	10.32 ± 0.47	10.02 ± 0.76	9.82 ± 0.86	7.90 ± 2.47
Inflammation				
TNFA				
d 29 pp	8.35 ± 0.41	7.63 ± 0.16	8.21 ± 0.35	7.80 ± 0.51
d 144 pp	7.66 ± 0.53	7.45 ± 0.11	7.55 ± 0.18	7.42 ± 0.10
Anabolism				
IGF1				
d 29 pp	11.59 ± 0.48	10.83 ± 0.25*	11.87 ± 0.30	11.29 ± 0.90*
d 144 pp	13.35 ± 0.09	12.45 ± 0.50	12.91 ± 0.26	13.12 ± 0.58

[ab]Means with alphabetic superscripts indicate differences between groups ($P < 0.05$).
*Asterisks indicate differences between FR in early and mid-lactation ($P < 0.05$).
Fixed effects in model *ACACA*: time $P = 0.58$, group $P = 0.61$, time × group $P = 0.92$. Fixed effects in model *ACADVL*: time $P < 0.01$, group $P = 0.27$, time × group $P = 0.75$. Fixed effects in model *CPT1A*: time $P < 0.05$, group $P = 0.19$, time × group $P = 0.92$. Fixed effects in model *CS*: time $P = 0.30$, group $P = 0.89$, time × group $P = 0.47$. Fixed effects in model *CTSL*: time $P = 0.84$, group $P < 0.05$, time × group $P = 0.67$. Fixed effects in model *ECHS1*: time $P = 0.24$, group $P < 0.05$, time × group $P = 0.79$. Fixed effects in model *EIF4B*: time $P = 0.58$, group $P = 0.09$, time × group $P = 0.96$. Fixed effects in model *GPAM*: time $P = 0.78$, group $P = 0.21$, time × group $P = 0.42$. Fixed effects in model *HMGCS2*: time $P = 0.18$, group $P = 0.98$, time × group $P = 0.30$. Fixed effects in model *HNF4A*: time $P = 0.03$, group $P = 0.41$, time × group $P = 0.58$. Fixed effects in model *IGF1*: time $P < 0.001$, group $P = 0.39$, time × group $P = 0.78$. Fixed effects in model *INSR*: time $P = 0.16$, group $P = 0.71$, time × group $P = 0.79$. Fixed effects in model *PC*: time $P < 0.01$, group $P = 0.26$, time × group $P = 0.98$. Fixed effects in model *PCK1*: time $P = 0.99$, group $P = 0.37$, time × group $P = 0.20$. Fixed effects in model *PPARA*: time $P = 0.27$, group $P = 0.22$, time × group $P = 0.59$. Fixed effects in model *SLC2A2*: time $P = 0.98$, group $P = 0.13$, time × group $P = 0.42$. Fixed effects in model *SREBF1*: time $P = 0.52$, group $P = 0.31$, time × group $P = 0.29$. Fixed effects in model *TAT*: time $P = 0.07$, group $P = 0.18$, time × group $P = 0.50$. Fixed effects in model *TNFA*: time $P = 0.09$, group $P = 0.30$, time × group $P = 0.89$.

4. Intravenous glucose tolerance tests

Because of technical and biological reasons, cows were subjected to ivGTTs at d 13.8 ± 2.1 ap, d 19.7 ± 0.2 and 127.1 ± 0.6 pp (table 16). Furthermore, mP-cows had first ivGTT earlier than other cows (d 12.6 ± 1.3, 12.2 ± 1.9, 10.3 ± 1.0 and 20.0 ± 3.0 ap for MP-, mp-, Mp- and mP-cows; $P < 0.01$) due to longer gestation length compared to other cows (278.2 ± 1.0, 278.2 ± 2.6, 277.4 ± 1.6 and 284.8 ± 1.0 days in MP-, mp-, Mp- and mP-cows; $P < 0.05$). Amounts of infused

glucose depended on actual body weight and differed between all time points in MP-cows ($P < 0.01$) and between ivGTT 1 and 2 in mp-, Mp- and mP-cows ($P < 0.001$), but not between groups at the same time point (table 16). Time of glucose infusion averaged 4.4 ± 0.2 minutes.

Table 16: Test day, blood glucose and blood plasma insulin parameters (LSM ± SEM) of ivGTTs at wk 2 ap, wk 3 and 19 pp.

	MP	mp	Mp	mP
Day of test, pp				
ivGTT 1	-12.6 ± 1.3^{a}*	-12.2 ± 1.9^{a}*	-10.3 ± 1.0^{a}*	-20.0 ± 3.0^{b}*
ivGTT 2	20.2 ± 0.3°	19.6 ± 0.5°	19.1 ± 0.3°	20.0 ± 0.3°
ivGTT 3	127.8 ± 1.3$^{\#}$	125.2 ± 2.6$^{\#}$	127.4 ± 0.4$^{\#}$	127.8 ± 1.2$^{\#}$
Body weight, kg				
ivGTT 1	802.4 ± 30.0*	768.0 ± 52.4*	761.0 ± 23.7*	773.5 ± 46.3*
ivGTT 2	709.0 ± 20.8°	646.8 ± 33.0°	654.0 ± 24.7°	704.2 ± 24.3°
ivGTT 3	648.4 ± 10.4$^{\#}$	630.4 ± 46.4°	626.6 ± 20.6°	702.8 ± 26.2°
Infused glucose, g				
ivGTT 1	150.7 ± 4.2*	145.6 ± 7.5*	144.8 ± 3.4*	146.5 ± 6.6*
ivGTT 2	137.3 ± 3.0°	128.1 ± 4.9°	129.2 ± 3.7°	136.6 ± 3.5°
ivGTT 3	128.5 ± 1.5$^{\#}$	125.6 ± 7.0°	125.2 ± 3.1°	136.4 ± 3.8°
Basal blood glucose, mmol/L				
ivGTT 1	2.81 ± 0.19*	2.57 ± 0.06*	2.57 ± 0.12*	2.73 ± 0.13*
ivGTT 2	2.29 ± 0.13^{a}°	1.81 ± 0.17^{b}°	1.68 ± 0.11^{b}°	2.38 ± 0.14^{a}°
ivGTT 3	2.58 ± 0.12*°	2.48 ± 0.12*	2.39 ± 0.08*	2.55 ± 0.10*°
Maximum blood glucose, mmol/L				
ivGTT 1	11.42 ± 0.61*	10.15 ± 0.42*	10.32 ± 0.49*	10.10 ± 0.62*°
ivGTT 2	9.98 ± 0.37^{a}°	8.43 ± 0.26^{b}°	8.72 ± 0.22^{b}°	9.10 ± 0.29^{ab}*
ivGTT 3	9.21 ± 0.25$^{\#}$	9.96 ± 0.47*	9.60 ± 0.29*	9.96 ± 0.39°
Increase of blood glucose, mmol/L				
ivGTT 1	8.67 ± 0.52*	7.48 ± 0.41*°	7.75 ± 0.42	7.55 ± 0.53
ivGTT 2	7.94 ± 0.35^{a}*	6.71 ± 0.27^{b}*	7.20 ± 0.23^{ab}	6.92 ± 0.29^{b}
ivGTT 3	6.79 ± 0.26^{a}°	7.63 ± 0.40^{ab}°	7.22 ± 0.28^{ab}	7.77 ± 0.37^{b}
Reaching basal glucose level again, minute				
ivGTT 1	99.0 ± 9.7^{a}*	73.2 ± 7.1^{b}	84.4 ± 7.6^{ab}*	73.3 ± 13.6^{b}
ivGTT 2	59.2 ± 4.9°	59.8 ± 7.5	68.9 ± 6.7°	56.4 ± 2.9
ivGTT 3	57.4 ± 3.0°	62.8 ± 5.9	56.1 ± 8.1°	53.8 ± 5.7
Steepest decrease of glucose, mmol/L per minute				
ivGTT 1	1.67 ± 0.76	1.00 ± 0.31*	0.97 ± 0.22	1.81 ± 1.00
ivGTT 2	0.93 ± 0.16^{a}	3.16 ± 2.37^{b}°	0.73 ± 0.17^{a}	0.87 ± 0.35^{a}
ivGTT 3	0.59 ± 0.06	0.56 ± 0.06*	0.63 ± 0.08	0.55 ± 0.14
Time point of steepest glucose level decrease, minute				
ivGTT 1	5.20 ± 1.20	4.00 ± 0.00	4.00 ± 0.00*	5.50 ± 0.96
ivGTT 2	4.67 ± 0.42^{ab}	5.60 ± 0.98^{ab}	4.00 ± 0.00^{a}*	6.80 ± 1.96^{b}
ivGTT 3	4.40 ± 0.40^{a}	5.20 ± 0.80^{ab}	6.86 ± 1.06^{b}°	5.60 ± 0.75^{ab}

	MP	mp	Mp	mP
Basal blood plasma insulin, µU/mL				
ivGTT 1	32.29 ± 12.12^{ab}*	29.28 ± 5.66^{ab}*	19.24 ± 3.77^{a}*	39.58 ± 19.97^{b}*
ivGTT 2	4.40 ± 1.42°	2.65 ± 0.38°	2.58 ± 0.23°	6.03 ± 2.36°
ivGTT 3	13.55 ± 2.33°	11.32 ± 2.03°	7.47 ± 1.12*°	12.22 ± 3.19°
Maximum blood plasma insulin, µU/mL				
ivGTT 1	411.7 ± 114.6^{ab}*	443.7 ± 113.3^{ab}*	298.5 ± 99.1^{a}	642.8 ± 388.8^{b}*
ivGTT 2	121.4 ± 16.8°	111.9 ± 17.3°	87.6 ± 18.9	194.3 ± 38.8°
ivGTT 3	180.2 ± 54.4*°	147.7 ± 27.8°	97.1 ± 15.4	175.5 ± 38.1°
Increase of blood plasma insulin, µU/mL				
ivGTT 1	379.4 ± 104.4^{ab}	414.4 ± 111.5^{ab}*	279.2 ± 96.8^{a}	603.2 ± 369.0^{b}*
ivGTT 2	117.0 ± 15.7	109.2 ± 17.3°	85.0 ± 18.9	188.3 ± 37.4°
ivGTT 3	166.6 ± 52.2	136.3 ± 29.0°	89.7 ± 14.4	163.3 ± 36.7°
Reaching basal insulin level again, minute				
ivGTT 1	96.2 ± 6.4*	83.2 ± 7.2	88.0 ± 5.1*	82.0 ± 9.9
ivGTT 2	87.6 ± 9.7*	78.6 ± 5.3	75.6 ± 8.0*°	80.0 ± 5.7
ivGTT 3	64.6 ± 5.6°	78.2 ± 6.4	64.0 ± 4.5°	72.2 ± 7.3
Steepest increase of blood plasma insulin, µU/mL per minute				
ivGTT 1	54.72 ± 17.67^{ab}*	44.69 ± 12.94^{ab}	31.50 ± 15.18^{a}	72.39 ± 36.46^{b}*
ivGTT 2	21.18 ± 5.00°	17.97 ± 2.90	12.27 ± 3.54	28.14 ± 6.87°
ivGTT 3	19.60 ± 7.87°	21.21 ± 3.78	15.27 ± 1.81	25.81 ± 6.65°
Time point of steepest blood plasma insulin level increase, minute				
ivGTT 1	6.0 ± 0.0	6.0 ± 0.0	8.3 ± 1.5*	6.0 ± 0.0
ivGTT 2	6.8 ± 0.8	6.0 ± 0.0	3.0 ± 3.0°	5.0 ± 1.0
ivGTT 3	3.6 ± 3.5	6.0 ± 0.0	3.7 ± 2.3*°	6.0 ± 0.0
Netto AUC of glucose, mmol/L × minute				
ivGTT 1	202.8 ± 17.6*	154.7 ± 27.6*	183.5 ± 24.7*	165.1 ± 26.2*
ivGTT 2	122.2 ± 15.6°	103.8 ± 17.8°	120.8 ± 12.9°	117.4 ± 3.9*°
ivGTT 3	102.8 ± 14.9°	129.5 ± 19.8*°	114.0 ± 22.7°	99.2 ± 17.7°
Netto AUC of insulin, µU/mL × minute				
ivGTT 1	4,160 ± 2,061	4,584 ± 1,765	3,397 ± 1,875	7,081 ± 4,757*
ivGTT 2	1,109 ± 258	1,432 ± 295	1,058 ± 360	2,009 ± 515°
ivGTT 3	2,084 ± 1,028	1,730 ± 696	1,028 ± 138	1,440 ± 282°
Clearance rate of glucose				
ivGTT 1	0.66 ± 0.04*	0.86 ± 0.06*	0.81 ± 0.06*	0.86 ± 0.11*
ivGTT 2	1.00 ± 0.08°	1.20 ± 0.12°	1.13 ± 0.08°	0.99 ± 0.05*°
ivGTT 3	1.01 ± 0.07°	0.97 ± 0.10*	1.04 ± 0.06°	1.06 ± 0.06°
HOMA-IR				
ivGTT 1	4.39 ± 1.92^{b}*	3.33 ± 0.65^{ab}*	2.22 ± 0.48^{a}*	4.85 ± 2.48^{b}*
ivGTT 2	0.49 ± 0.20°	0.22 ± 0.05°	0.20 ± 0.03°	0.63 ± 0.24°
ivGTT 3	1.59 ± 0.35°	1.29 ± 0.28*°	0.78 ± 0.11*°	1.40 ± 0.37°
QUICKI				
ivGTT 1	0.332 ± 0.02*	0.326 ± 0.02*	0.347 ± 0.02*	0.320 ± 0.02*
ivGTT 2	0.470 ± 0.02^{a}°	0.526 ± 0.02^{b}°	0.536 ± 0.02^{b}°	0.444 ± 0.02^{a}°
ivGTT 3	0.360 ± 0.02*	0.374 ± 0.02*	0.406 ± 0.02$^{\#}$	0.374 ± 0.02*

^{ab}Alphabetic superscripts indicate differences between groups within ivGTT ($P < 0.05$).
*°$^{\#}$Symbolic superscripts indicate differences between ivGTTs within group ($P < 0.05$).

4.1. Blood glucose and plasma insulin

Time course of blood glucose and blood plasma insulin of the four groups can be found in figures 37 (ivGTT 1), 38 (ivGTT 2) and 39 (ivGTT 3).

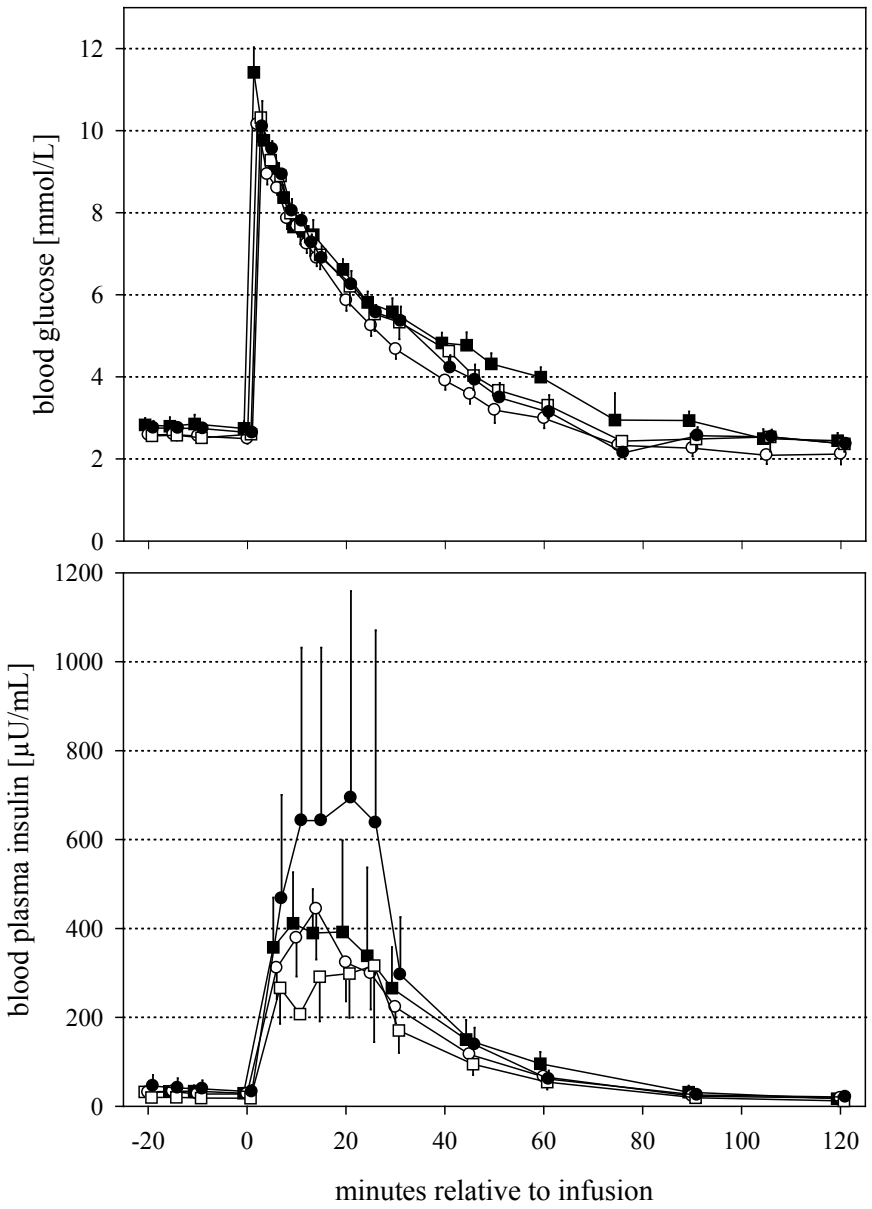

Figure 37: Course of blood glucose (mmol/L) and blood plasma insulin (µU/mL) during ivGTT 1.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Values are presented as LSM ± SEM. Glucose infusion started immediately after blood sampling time point 0, lasted 4.6 ± 0.5 minutes and next sampling was done after end of infusion (2 minutes for glucose and 6 minutes for insulin measurements).

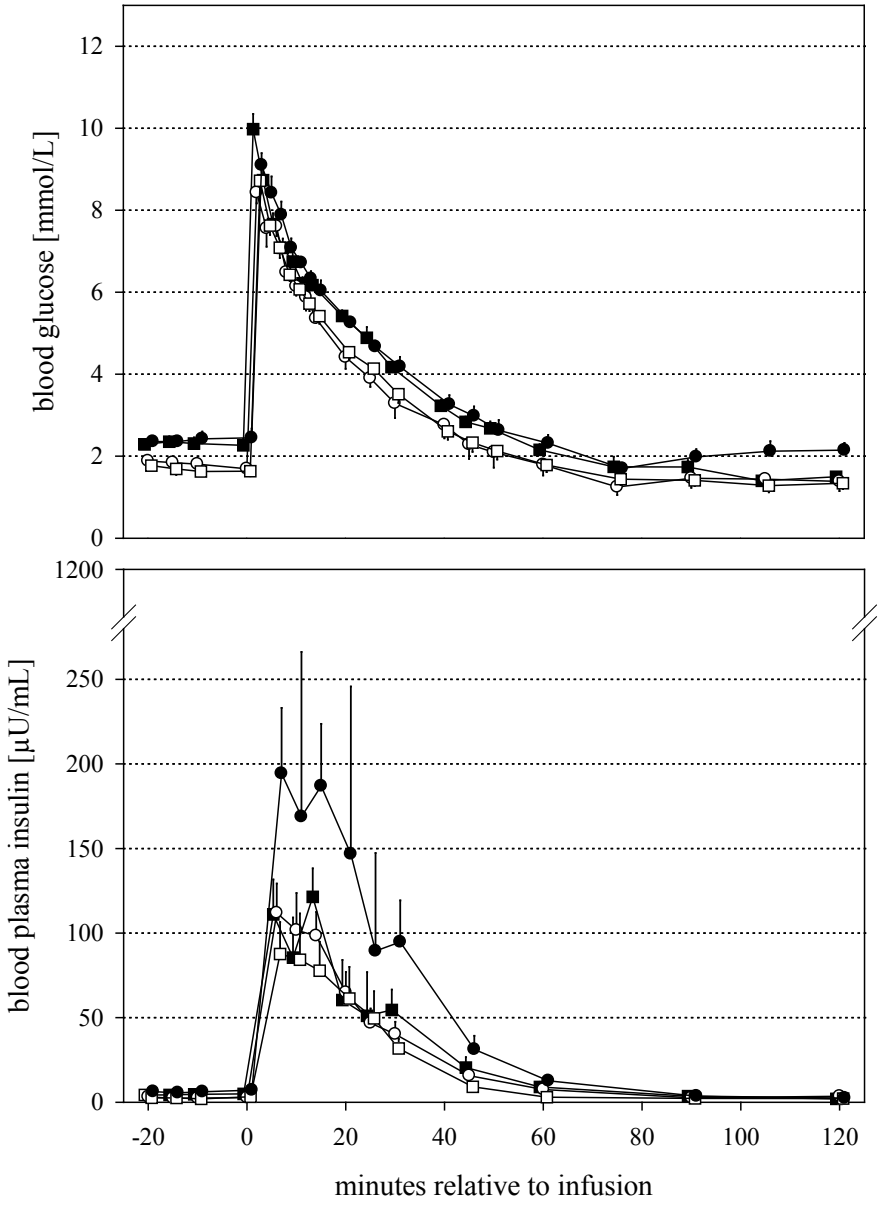

Figure 38: Course of blood glucose (mmol/L) and blood plasma insulin (µU/mL) during ivGTT 2.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Values are presented as LSM ± SEM. Glucose infusion started immediately after blood sampling time point 0, lasted 4.7 ± 0.7 minutes and next sampling was done after end of infusion (2 minutes for glucose and 6 minutes for insulin measurements).

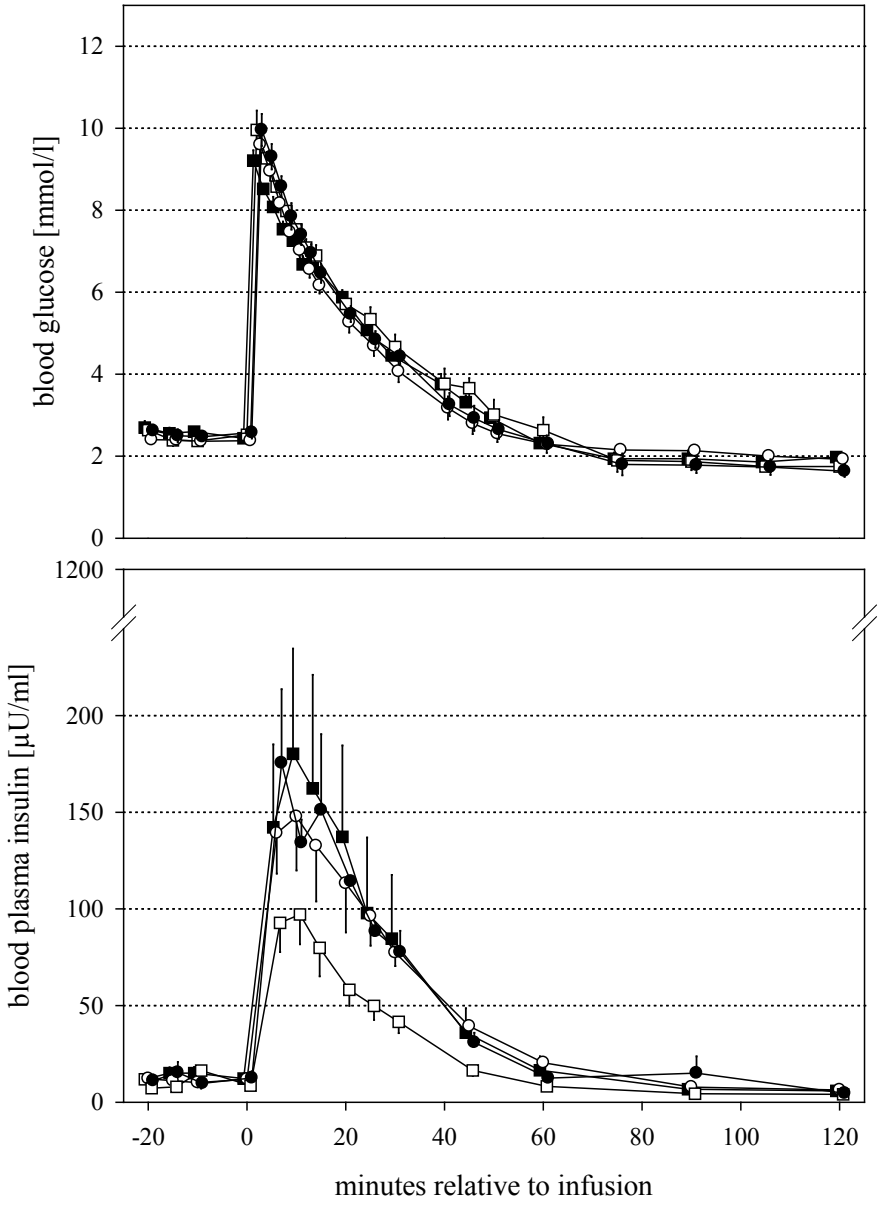

Figure 39: Course of blood glucose (mmol/L) and blood plasma insulin (µU/mL) during ivGTT 3.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Values are presented as LSM ± SEM. Glucose infusion started immediately after blood sampling time point 0, lasted 4.1 ± 0.1 minutes and next sampling was done after end of infusion (2 minutes for glucose and 6 minutes for insulin measurements).

Basal blood glucose

Basal blood glucose was higher at first ivGTT (2.81 ± 0.19, 2.57 ± 0.06, 2.57 ± 0.12 and 2.73 ± 0.13 mmol/L in MP-, mp-, Mp- and mP-cows) compared to second ivGTT in all cows ($P < 0.05$; table 16, figure 40). In mp- and Mp-cows, basal blood glucose was lowest at second ivGTT (1.81 ± 0.17 and 1.68 ± 0.11 mmol/L in mp- and Mp-cows; $P < 0.001$). Moreover mp- and Mp-cows showed lower basal blood glucose levels during second ivGTT compared to MP- (2.29 ± 0.13 mmol/L) and mP-cows (2.38 ± 0.14; $P < 0.01$).

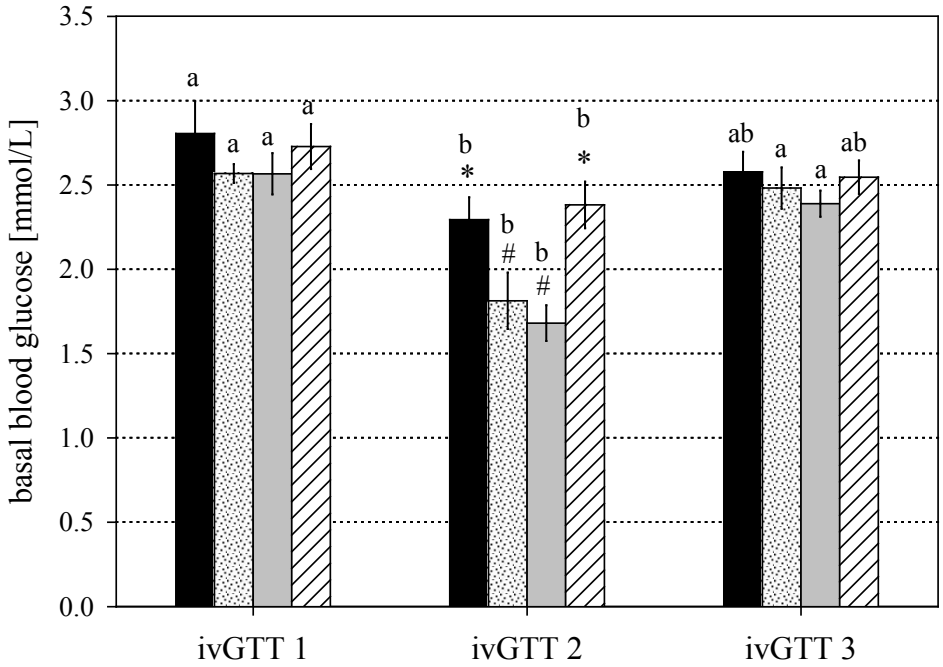

Figure 40: Basal blood glucose (mmol/L) during ivGTT at d 14 ap (ivGTT 1), d 20 (ivGTT 2) and 127 pp (ivGTT 3).

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes.
*[#]Symbolic superscripts indicate differences between groups at the same time point ($P < 0.05$).
[ab]Alphabetic superscripts indicate differences between time points within a group ($P < 0.05$).
Fixed effects in model: time $P < 0.05$, group $P < 0.001$ and time × group $P < 0.1$.

Maximum and increase of blood glucose

Blood glucose levels increased after infusion of glucose, reached higher maximum levels in ivGTT 1 compared to ivGTT 2 in MP-, mp- and Mp-cows ($P < 0.05$) and difference tended to be significant in mP-cows ($P < 0.1$; table 16). In mp-, Mp- and mP-cows, blood glucose peaked higher during ivGTT 2 compared to ivGTT 3 ($P < 0.05$). Only in MP-cows, maximum blood glucose was higher during ivGTT 2 compared to ivGTT 3 ($P < 0.05$). Additionally, MP-cows showed higher maximum blood glucose during second ivGTT compared to low yielding cows (9.98 ± 0.37, 8.43 ± 0.26 and 8.72 ± 0.22 mmol/L in MP-, mp- and mP-cows; $P < 0.01$).

Consequently, increase of blood glucose during ivGTT 3 was lower in MP-cows compared to ivGTT 1 and 2 ($P < 0.01$) and higher in mp-cows compared to ivGTT 2 ($P < 0.05$; table 16, figure 41). Furthermore, MP-cows showed higher increase of blood glucose during second ivGTT compared to low yielding cows (7.94 ± 0.35, 6.71 ± 0.27 and 6.92 ± 0.29 mmol/L in MP-, mp- and mP-cows; $P < 0.05$). During ivGTT 3, increase of blood glucose was higher in mP-cows compared to MP-cows (6.79 ± 0.26 and 7.77 ± 0.37 in MP- and mP-cows; $P < 0.05$).

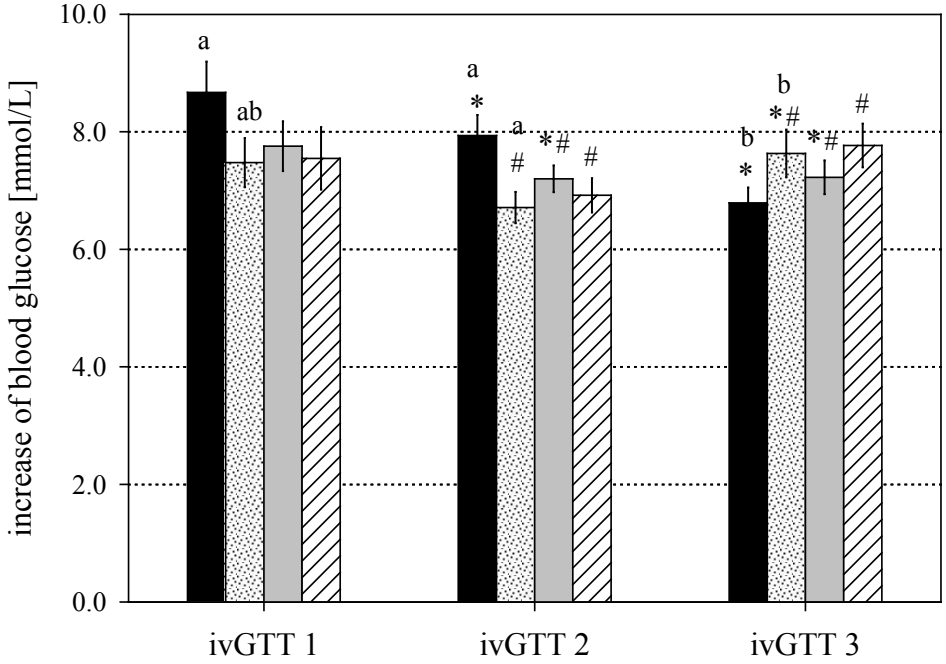

Figure 41: Increase of blood glucose (mmol/L) during ivGTT at d 14 ap (ivGTT 1), d 20 (ivGTT 2) and 127 pp (ivGTT 3).

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes.
*[#]Symbolic superscripts indicate differences between groups at the same time point ($P < 0.05$).
[ab]Alphabetic superscripts indicate differences between time points within a group ($P < 0.05$).
Fixed effects in model: time $P = 0.71$, group $P = 0.22$ and time × group $P = 0.39$.

Time course of blood glucose

During ivGTT at d 14 ap, MP-cows needed more time compared to mp- and mP-cows (73.2 ± 7.1 and 73.3 ± 13.6 minutes; $P < 0.05$) until they reached basal blood glucose level again (table 16). Furthermore, high yielding cows took during ivGTT 1 longest to reach basal blood glucose levels again (99.0 ± 9.7 minutes in MP-cows, $P < 0.001$ and 84.4 ± 7.6 minutes in Mp-cows, $P < 0.05$).

Steepest decrease of glucose during ivGTT 2 was observed in mp-cows at 5.60 ± 0.98 minutes (3.16 ± 2.37 mmol/L per minute; $P < 0.05$; table 16). Furthermore, mp-cows showed lower decreases during ivGTT 1 and 3 (1.00 ± 0.31 and 0.56 ± 0.06 mmol/L per minute) compared to ivGTT 2 ($P < 0.05$).

Steepest decrease of glucose in second ivGTT was observed earlier in Mp-cows (minute 4.00 ± 0.00) compared to mP-cows (minute 6.80 ± 1.96; $P < 0.05$) and during ivGTT 3 earlier in MP- (4.40 ± 0.40) compared to Mp-cows (6.86 ± 1.06; $P < 0.05$; table 16). Furthermore, Mp-cows showed steepest decrease of glucose in ivGTT 3 later compared to earlier ivGTTs ($P < 0.01$).

Basal, maximum and increase of blood plasma insulin

Basal levels of blood plasma insulin during ivGTT 1 were higher in mP-cows (39.58 ± 19.97 µU/mL) compared to Mp-cows (19.24 ± 3.77 µU/mL; $P < 0.05$; table 16).

Furthermore basal blood plasma insulin levels were highest in MP-, mp- and mP-cows during ivGTT 1 (32.29 ± 12.12, 29.28 ± 5.66 and 39.58 ± 19.97 µU/mL, respectively; $P < 0.05$). Basal levels of blood plasma insulin in Mp-cows were higher during ivGTT 1 (19.24 ± 3.77 µU/mL) compared only to ivGTT 2 (2.58 ± 0.23 µU/mL; $P < 0.05$).

Levels of blood plasma insulin peaked higher during ivGTT 1 in mP- compared to Mp-cows (298.5 ± 99.1 and 642.8 ± 388.8 µU/mL in Mp- and mP-cows; $P < 0.05$; table 16). In mp- (443.7 ± 113.3 µU/mL) and mP-cows, blood plasma insulin showed highest peaks during ivGTT 1 ($P < 0.05$), whereas maximum of blood plasma insulin in MP-cows was reached during ivGTT 1 (411.7 ± 114.6 µU/mL) compared to ivGTT 2 (121.4 ± 16.8 µU/mL; $P < 0.05$).

Consequently, increase of blood plasma insulin during ivGTT 1 was higher in mP- compared to Mp-cows (603.2 ± 369.0 and 279.2 ± 96.8 µU/mL; $P < 0.05$; table 16). In low yielding cows, increase of blood plasma insulin was highest during ivGTT 1 (in mp-cows 414.4 ± 111.5 µU/mL, $P < 0.05$ and in mP-cows $P < 0.01$).

Time course of blood plasma insulin

Basal level of blood plasma insulin was reached fastest in MP-cows during ivGTT 3 (64.6 ± 5.6 minutes; $P < 0.05$) and faster in Mp-cows during ivGTT 3 (64.0 ± 4.5 minutes) compared to ivGTT 1 (88.0 ± 5.1, $P < 0.01$; table 16).

Increase of blood plasma insulin levels during ivGTT 1 was strongest in MP- and mP-cows (54.72 ± 17.67 and 72.39 ± 36.46 µU/mL per minute; $P < 0.05$; table 16). Moreover during ivGTT 1, mP-cows showed steeper increase of blood plasma insulin levels compared to Mp-cows (31.50 ± 15.18 µU/mL per minute; $P < 0.05$). Additionally time point of steepest blood plasma insulin increase solely was higher in Mp-cows during ivGTT 1 (minute 8.3 ± 1.5) compared to ivGTT 3 (minute 3.0 ± 3.0; $P < 0.05$).

4.2. AUC of blood glucose and insulin

In high yielding cows, netto area under the curve of glucose ($nAUC_G$) was highest during ivGTT 1 (202.8 ± 17.6 and 183.5 ± 24.7 mmol/L × minute in MP- and Mp-cows; $P < 0.01$), whereas in mp-cows $nAUC_G$ was only higher in ivGTT 1 (154.7 ± 27.6 mmol/L × minute) compared to ivGTT 2 (103.8 ± 17.8 mmol/L × minute; $P < 0.05$; table 16, figure 42). Furthermore in mP-cows $nAUC_G$ was higher in ivGTT 1 (165.1 ± 26.2 mmol/L × minute) compared to ivGTT 3 (99.2 ± 17.7 mmol/L × minute; $P < 0.05$).

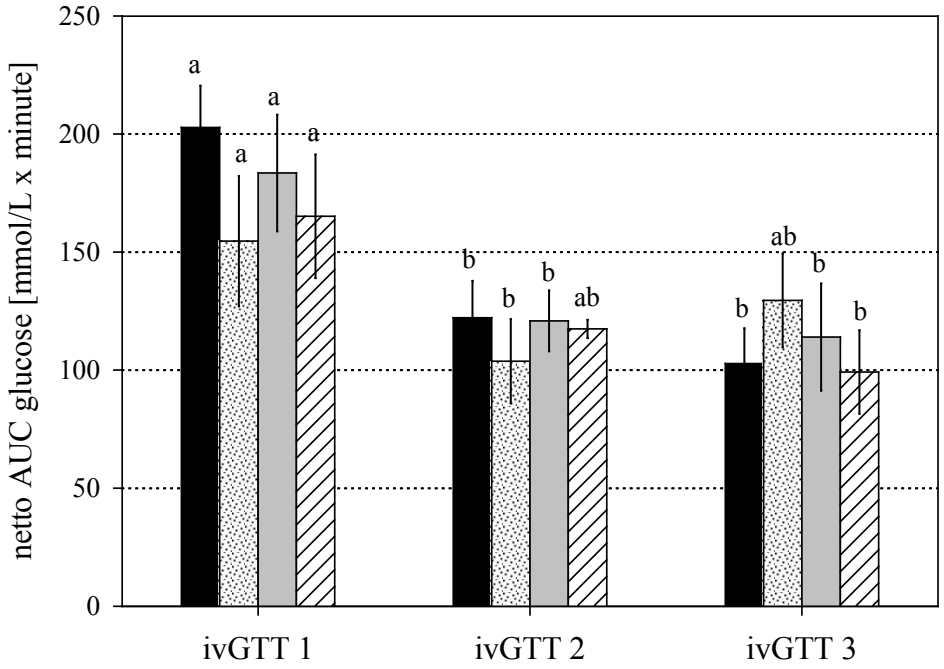

Figure 42: Netto AUC of blood glucose (mmol/L × minute) during ivGTT at d 14 ap (ivGTT 1), d 20 (ivGTT 2) and 127 pp (ivGTT 3).

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes.
*#Symbolic superscripts indicate differences between groups at the same time point ($P < 0.05$).
abAlphabetic superscripts indicate differences between time points within a group ($P < 0.05$).
Fixed effects in model: time $P = 0.90$, group $P < 0.001$ and time × group $P = 0.59$.

Differences in nAUC of blood plasma insulin ($nAUC_I$) could only be observed in mP-cows, where $nAUC_I$ was highest during ivGTT 1 (7,081 ± 4,757 µU/mL × minute; $P < 0.05$; table 16, figure 43) due to one animal in this group (maximum blood plasma insulin 8,901 µU/mL, $nAUC_I$ 34,899 µU/mL × minute).

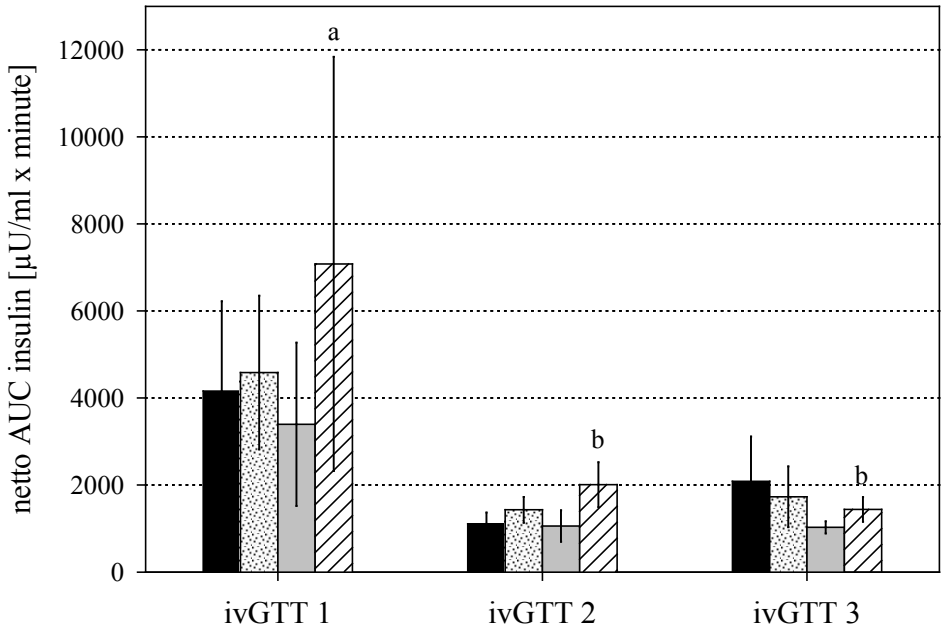

Figure 43: Netto AUC of blood plasma insulin (µU/mL × minute) during ivGTT at d 14 ap (ivGTT 1), d 20 (ivGTT 2) and 127 pp (ivGTT 3).

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes.
*[#]Symbolic superscripts indicate differences between groups at the same time point ($P < 0.05$).
[ab]Alphabetic superscripts indicate differences between time points within a group ($P < 0.05$).
Fixed effects in model: time $P = 0.68$, group $P < 0.01$ and time × group $P = 0.92$.

4.3. Clearance rate of blood glucose

Clearance rate of glucose (CR) was lowest in high yielding cows during ivGTT 1 (0.66 ± 0.04 and 0.81 ± 0.06 in MP- and Mp-cows; $P < 0.01$), whereas mp-cows showed highest CR during ivGTT 2 (1.20 ± 0.12; $P < 0.05$; table 16, figure 44). Furthermore mP-cows showed higher glucose CR during ivGTT 3 (1.06 ± 0.06) compared to ivGTT 1 (0.86 ± 0.11; $P < 0.05$).

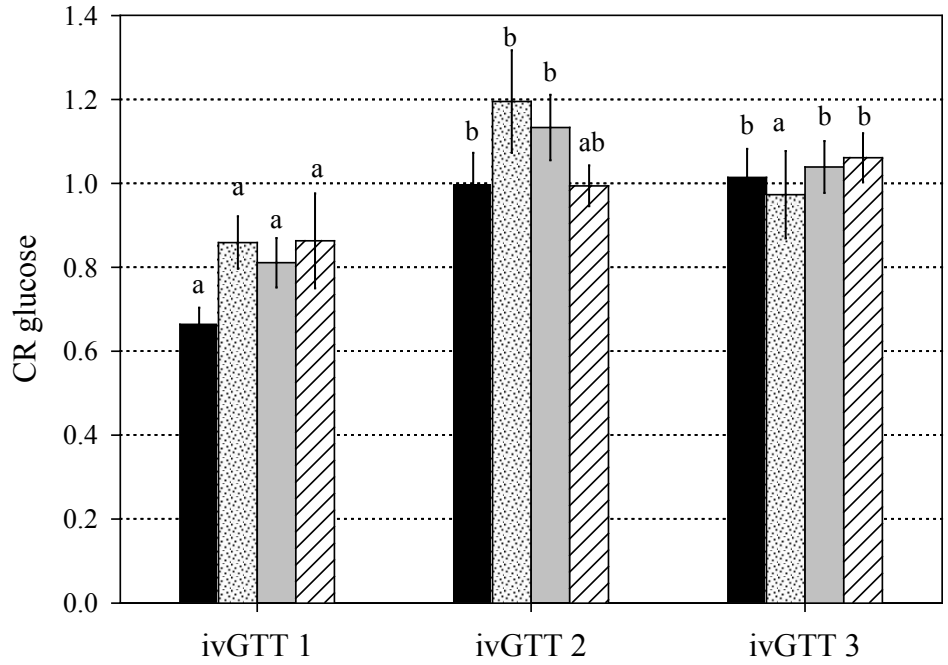

Figure 44: Clearance rate of glucose during ivGTT at d 14 ap (ivGTT 1), d 20 (ivGTT 2) and 127 pp (ivGTT 3).

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes.
*[#]Symbolic superscripts indicate differences between groups at the same time point ($P < 0.05$).
[ab]Alphabetic superscripts indicate differences between time points within a group ($P < 0.05$).
Fixed effects in model: time $P = 0.64$, group $P < 0.001$ and time × group $P = 0.22$.

4.4. Model estimations

HOMA-IR

In high protein cows highest HOMA-IR was observed during ivGTT 1 (4.39 ± 1.92 and 4.85 ± 2.48 in MP- and mP-cows; $P < 0.05$), whereas in low protein cows HOMA-IR was higher during ivGTT 1 (3.33 ± 0.65 and 2.22 ± 0.48 in mp- and Mp-cows) compared to ivGTT 2 (0.22 ± 0.05 and 0.20 ± 0.03 in mp- and Mp-cows; $P < 0.05$; table 16, figure 45). Furthermore Mp-cows showed lowest HOMA-IR during ivGTT 1 compared to MP- ($P < 0.05$) and mP-cows ($P < 0.05$).

QUICKI

The calculated insulin sensitivity index QUICKI was lower in all cows before parturition compared to ivGTT 2 ($P < 0.001$). Furthermore, differences in QUICKI of Mp-cows proved to be significant at all three time points (0.347 ± 0.02, 0.536 ± 0.02 and 0.406 ± 0.02 during ivGTT 1, 2 and 3; $P < 0.05$). During ivGTT 2, high protein cows showed lower QUICKI compared to low protein cows (0.470 ± 0.02, 0.444 ± 0.02, 0.526 ± 0.02 and 0.536 ± 0.02 in MP-, mP-, mp- and Mp-cows; $P < 0.05$).

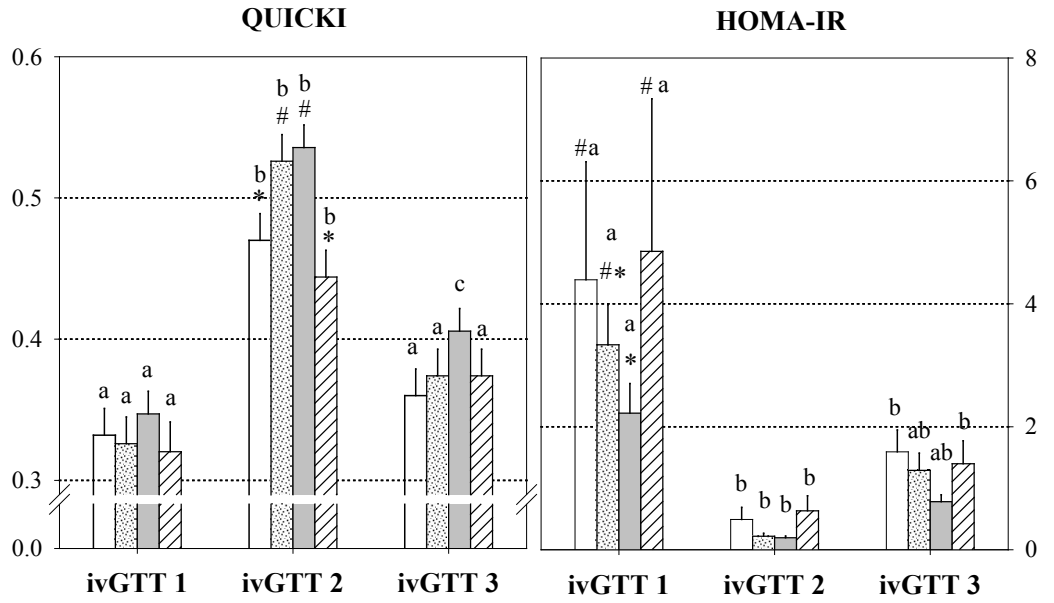

Figure 45: HOMA-IR and QUICKI during ivGTT at d 14 ap (ivGTT 1), d 20 (ivGTT 2) and 127 pp (ivGTT 3).

MP-cows are shown as black boxes, mp-cows as dotted boxes, Mp-cows as grey boxes and mP-cows as shaded boxes.
*[#]Symbolic superscripts indicate differences between groups at the same time point ($P < 0.05$).
[ab]Alphabetic superscripts indicate differences between time points within a group ($P < 0.05$).
Fixed effects in model HOMA-IR: time $P = 0.32$, group $P < 0.001$ and time × group $P = 0.72$. Fixed effects in model QUICKI: time $P < 0.001$, group $P < 0.05$ and time × group $P = 0.13$.

4.5. Milk parameters

Mean milk parameters and differences between days and cows during ivGTT 2 and ivGTT 3 can be observed in table 17.

Table 17: Mean milk parameters (LSM ± SEM) at days of ivGTT at wk 3 and 19 pp.

	MP	mp	Mp	mP
milk yield, kg				
ivGTT 2	37.55 ± 1.29[a]*	34.54 ± 2.46[ab]	40.83 ± 1.68[a]*	30.70 ± 1.36[b]
ivGTT 3	29.65 ± 2.45	30.64 ± 2.19	34.35 ± 2.95	27.39 ± 2.67
FCM, kg				
ivGTT 2	49.57 ± 3.35[a]*	43.45 ± 4.60[ab]*	51.20 ± 2.83[a]*	39.54 ± 1.19[b]
ivGTT 3	34.12 ± 2.97	32.82 ± 3.41	35.65 ± 2.56	32.19 ± 4.11
ECM, kg				
ivGTT 2	51.11 ± 3.16[a]*	44.38 ± 4.37[ab]*	52.45 ± 2.58[a]*	41.52 ± 1.41[b]
ivGTT 3	36.51 ± 2.89	35.32 ± 3.50	38.08 ± 2.74	35.36 ± 4.20
milk protein content, %				
ivGTT 2	3.20 ± 0.10[ac]*	2.90 ± 0.06[b]*	2.95 ± 0.03[bc]	3.44 ± 0.08[a]*
ivGTT 3	3.46 ± 0.18[a]	3.27 ± 0.06[ab]	3.08 ± 0.07[b]	3.94 ± 0.19[c]

	MP	mp	Mp	mP
milk protein yield, g				
ivGTT 2	1204 ± 70	1003 ± 73	1203 ± 47	1060 ± 63
ivGTT 3	1011 ± 60	1004 ± 82	1054 ± 88	1071 ± 94
milk fat content, %				
ivGTT 2	6.12 ± 0.44*	5.68 ± 0.47*	5.72 ± 0.46*	5.95 ± 0.20
ivGTT 3	5.02 ± 0.24	4.41 ± 0.31	4.37 ± 0.36	5.15 ± 0.55
milk fat yield, g				
ivGTT 2	2,303 ± 200^{ab}*	1,976 ± 251^{ab}*	2,325 ± 174^{a}*	1,818 ± 49^{b}
ivGTT 3	1,484 ± 140	1,371 ± 171	1,461 ± 115	1,416 ± 221
milk lactose content, %				
ivGTT 2	4.77 ± 0.04^{ab}	4.67 ± 0.06^{a}	4.73 ± 0.06^{ab}	4.86 ± 0.02^{b}
ivGTT 3	4.76 ± 0.09	4.73 ± 0.07	4.80 ± 0.05	4.79 ± 0.08
milk lactose yield, g				
ivGTT 2	1,794 ± 70^{ab}*	1,617 ± 137^{ab}	1,932 ± 89^{a}*	1,491 ± 64^{b}
ivGTT 3	1,416 ± 130	1,453 ± 121	1,654 ± 149	1,310 ± 126
FPR				
ivGTT 2	1.93 ± 0.18*	1.96 ± 0.17*	1.94 ± 0.16*	1.74 ± 0.10*
ivGTT 3	1.46 ± 0.09	1.35 ± 0.08	1.42 ± 0.11	1.30 ± 0.09
SCC, × 1,000/mL				
ivGTT 2	40.0 ± 11.8	65.6 ± 19.0	35.0 ± 6.0	931.8 ± 791.5
ivGTT 3	73.4 ± 33.8^{a}	87.4 ± 29.3^{ab}	69.6 ± 18.4^{a}	388.5 ± 217.1^{b}
milk urea, mg/L				
ivGTT 2	240.3 ± 30.1	191.8 ± 24.0	245.0 ± 26.8	239.0 ± 37.2
ivGTT 3	237.8 ± 13.4	248.8 ± 23.3	263.1 ± 16.0	238.5 ± 35.8
milk hydrocortisone, nmol/L				
ivGTT 2	2.82 ± 0.39*	3.82 ± 0.67	3.21 ± 0.46	3.00 ± 0.81
ivGTT 3	4.12 ± 0.57	3.36 ± 0.22	3.12 ± 0.47	3.24 ± 0.70

^{abc}Alphabetic superscripts indicate differences between groups within ivGTT ($P < 0.05$).

*Asterisks indicate differences between ivGTTs within group ($P < 0.05$).

5. Milk protein fractions

Altogether, 457 skim milk samples were measured, thereof 325 during lactation and 132 at days of FR. Intra- and inter-assay CVs were 1.7 and 6.5% (α-LA), 4.3 and 6.1% (β-LG), 1.4 and 5.8% (α-CN), 0.0 and 8.0% (β-CN) and 0.0 and 10.8% (κ-CN).

5.1. Composition of milk protein during 22 weeks of lactation

Measurement of protein fractions was performed after all samples were taken. After freezing and thawing, 37 milk samples were unfortunately clotted. Of finally 288 milk samples were 56, 61, 58, 42, 59 and 12 samples from period 1, 2, 3, 4, 5 and 6, respectively.

α-lactalbumin

Content of α-LA was similar amongst groups, except during period 6, when mP-cows showed overall highest concentrations (2.53 ± 0.19%; $P < 0.05$; table 18, figure 46). During lactation, all cows showed similar contents of α-LA with slightly declining amounts after period 3 and increasing concentrations of α-LA in mP-cows from period 5 to 6 ($P < 0.001$).

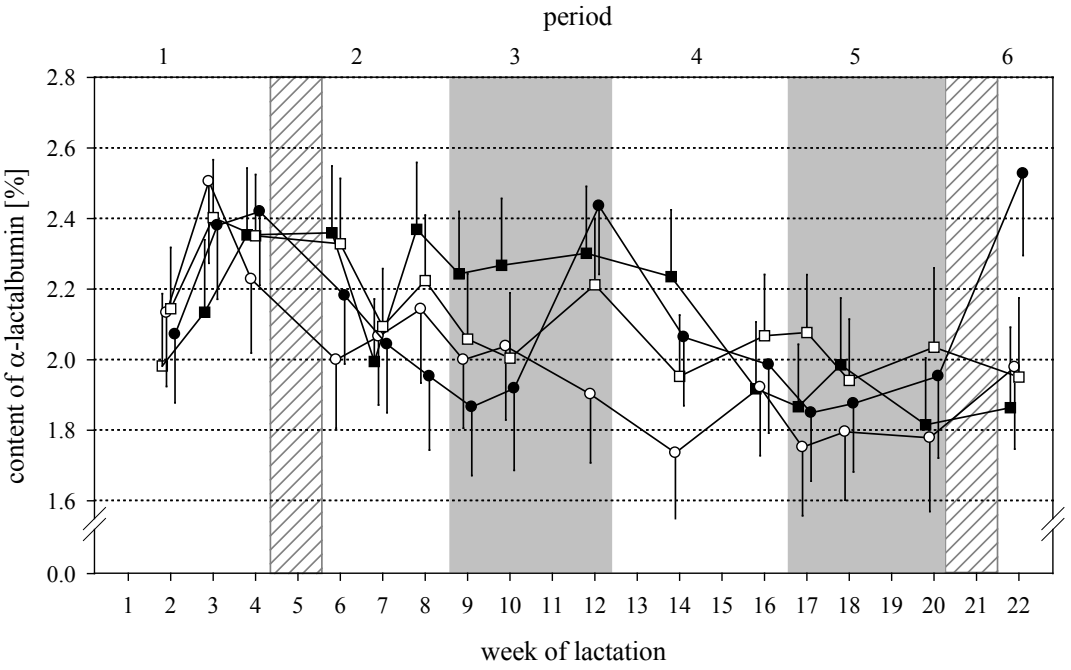

Figure 46: Concentration of α-lactalbumin (% of total protein) in skim milk during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.83$ and time × group $P = 0.24$. Differences between periods and between groups can be found in table 18.

β-lactoglobulin

Mean concentration of β-LG was higher in Mp-cows during period 1 compared to period 2 (10.34 ± 0.74 and 9.37 ± 0.74%; $P < 0.05$) and increased in low yielding cows from period 5 to 6 (9.00 ± 0.88 and 10.44 ± 0.94% in mp-cows, $P < 0.05$; 9.74 ± 0.88 and 12.42 ± 0.94%, $P < 0.001$; table 18, figure 47). Furthermore, concentrations of β-LG were higher at d 36 compared to d 43 pp in high yielding and mP-cows (8.24 ± 0.89, 8.88 ± 0.82 and 10.10 ± 0.98% in MP-, Mp- and mP-cows; $P < 0.05$). In mP-cows, β-LG concentration increased from d 64 to 78 pp by 1.40%-points ($P < 0.01$). Afterwards, β-LG contents decreased in Mp-cows by 1.23%-points ($P < 0.1$) and in mP-cows by 1.98%-points ($P < 0.01$) from d 78 to 92 pp. At d 106 pp, β-LG concentrations were higher in mP-cows compared to d 113 pp (9.08 ± 0.98%; $P < 0.05$). During period 6, mP-cows showed higher concentrations of β-LG compared to high yielding cows (9.42 ± 0.88, 9.79 ± 0.84 and 12.42 ± 0.94% in MP-, Mp- and mP-cows; $P < 0.05$).

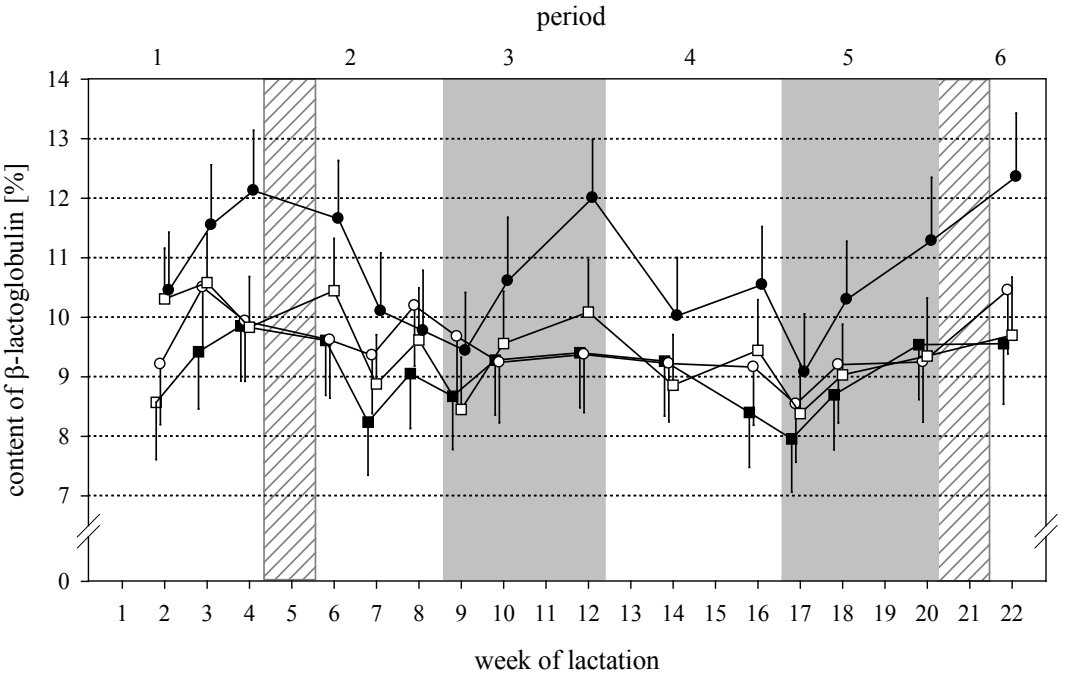

Figure 47: Concentration of β-lactoglobulin (% of total protein) in skim milk during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.41$ and time × group $P = 0.87$. Differences between periods and between groups can be found in table 18.

α-casein

Mean concentration of α-casein was in higher in Mp-cows compared to mP-cows in period 1, compared to low yielding cows in period 2, 3, 4 and 5 and compared to high protein cows in period 6 ($P < 0.05$; table 18, figure 48). Furthermore, MP-cows showed higher mean amounts of α-CN during period 5 compared to period 6 (33.9 ± 0.8 and 32.0 ± 1.0%; $P < 0.05$). From d 8 to 15 pp and from d 57 to 64 pp, concentrations of α-CN decreased in mp- cows by 3.92 and 3.24%-points ($P < 0.05$). At the end of experiment, α-CN concentrations in mp-cows increased to 33.9 ± 1.33% ($P < 0.05$).

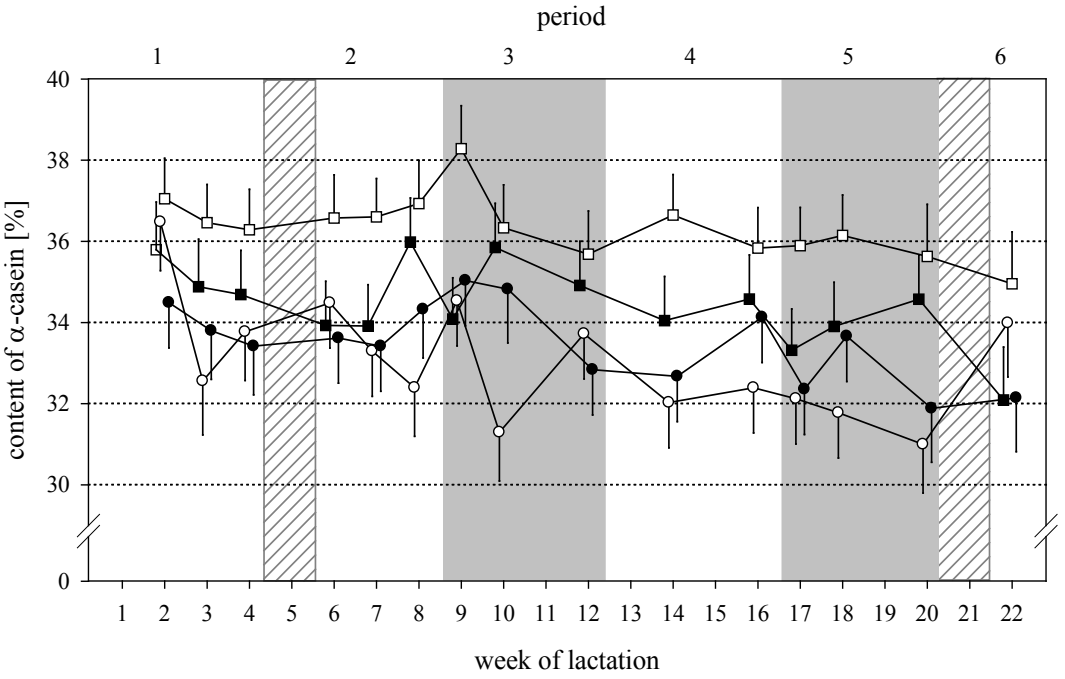

Figure 48: Concentration of α-casein (% of total protein) in skim milk during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.01$, group $P < 0.05$ and time × group $P = 0.71$. Differences between periods and between groups can be found in table 18.

β-casein

All cows showed increasing mean concentrations of β-CN from period 1 to 2 ($P < 0.05$), resulting in higher levels of Mp-cows compared to mP-cows (34.9 ± 0.8 and 31.2 ± 1.0%; $P < 0.01$; table 18, figure 49). From period 3 to 4, Mp- and mP-cows showed decreasing mean contents of β-CN ($P < 0.05$). During period 4, overall lowest mean concentration of β-CN was observed in mP-cows (28.9 ± 1.0%; $P < 0.05$). Afterwards, mean amounts of β-CN increased in Mp-cows to period 5 (34.2 ± 0.8%; $P < 0.05$) and mP-cows showed still overall lowest concentration (29.6 ± 1.0%; $P < 0.01$). In Mp- and mp-cows, mean concentration of β-CN decreased to period 6 (29.1 ± 1.2 and 30.7 ± 1.1% in Mp- and mp-cows; $P < 0.001$). During period 6, MP-cows showed higher mean β-CN content compared to mP-cows ($P < 0.05$). Furthermore, all cows showed higher concentrations of β-CN at d 43, 57 and 113 pp compared to days before and after (d 36, 50, 64, 106 and 120 pp; $P < 0.05$).

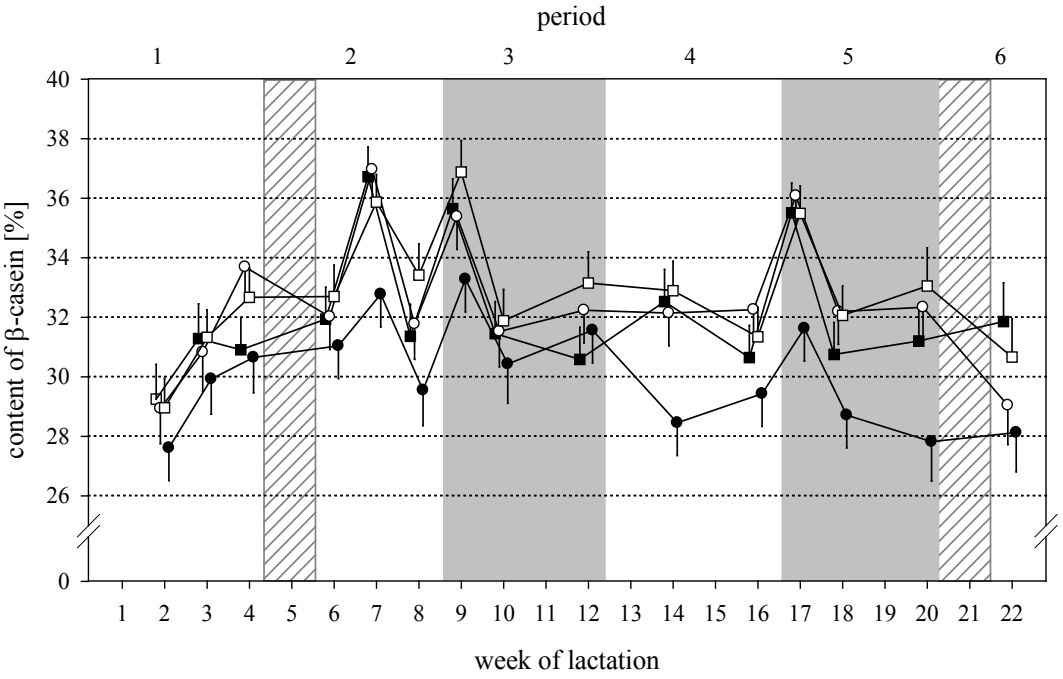

Figure 49: Concentration of β-casein (% of total protein) in skim milk during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.1$ and time × group $P = 0.27$. Differences between periods and between groups can be found in table 18.

κ-casein

Mean concentration of κ-CN declined from period 1 to 2 in Mp-cows (13.6 ± 1.3 and 11.5 ± 1.3%; $P < 0.01$) and was then lower compared to low yielding cows (16.6 ± 1.6 and 16.4 ± 1.6% in mp- and mP-cows; $P < 0.05$; table 18, figure 50). In period 3, Mp-cows showed lower mean content of κ-CN compared to mp-cows (12.1 ± 1.4 and 17.1 ± 1.6% in Mp- and mp-cows; $P < 0.05$) and amounts increased to period 4 in mp-, Mp- and mP-cows ($P < 0.05$). During period 4, Mp-cows still showed lower mean content of κ-CN compared to mp-cows (13.7 ± 1.4 and 19.3 ± 1.6% in Mp- and mp-cows; $P < 0.05$) and in period 5 compared to low yielding cows (18.3 ± 1.6, 12.6 ± 1.3 and 18.6 ± 1.6% in mp-, Mp- and mP-cows; $P < 0.01$). In mP-cows, mean concentration of κ-CN decreased to period 6 (to 14.9 ± 1.7%; $P < 0.001$). Furthermore, Mp-cows showed lower mean κ-CN concentration compared to mp-cows (19.0 ± 1.7 and 13.9 ± 1.5% in mp- and Mp-cows; $P < 0.05$).

In MP-cows, concentration of κ-CN was lower at d 43 compared to d 50 pp (13.9 ± 1.5 and 16.2 ± 1.6% at d 43 and 50 pp; $P < 0.05$) and at d 113 compared to d 106 and 120 pp (17.7 ± 1.6, 15.4 ± 1.5 and 17.7 ± 1.6% at d 106, 113 and 120 pp; $P < 0.05$). Concentration of κ-CN was lower in mp-cows at d 43 compared to d 36 and 50 pp (18.1 ± 1.7, 13.7 ± 1.7 and 18.7 ± 1.7% at d 36, 43 and 50 pp; $P < 0.001$), at d 57 compared to d 50 and 64 (14.0 ± 1.7 and 19.7 ± 1.7% at d 57 and 64 pp; $P < 0.001$) and at d 113 compared to d 106 and 120 pp (19.2 ± 1.7, 16.2 ± 1.7 and 20.0 ± 1.7% at d 106, 113 and 120 pp; $P < 0.01$). In Mp-cows, κ-CN concentration was lower at d 57 and 113 pp compared to following measurements (10.2 ± 1.5, 13.3 ± 1.5, 11.9 ± 1.4 and 14.0 ± 1.4% at d 57, 64, 113 and 120 pp; $P < 0.05$).

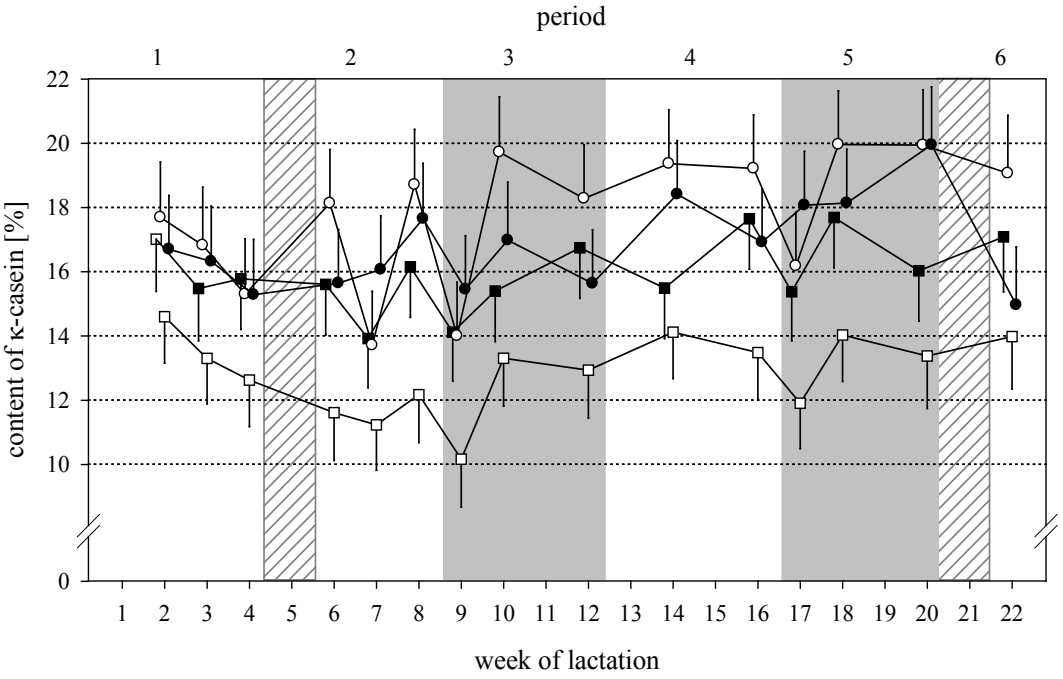

Figure 50: Concentration of κ-casein (% of total protein) in skim milk during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P < 0.1$ and time × group $P < 0.05$. Differences between periods and between groups can be found in table 18.

Unidentified proteins

During period 1, all cows showed similar mean concentrations of unidentified proteins. In mp-cows, mean content decreased to period 2 (from 5.82 ± 0.79 to 4.30 ± 0.75%; $P < 0.05$) and stayed on similar levels throughout period 3 (table 18, figure 51). From period 3 to 4, content of unidentified proteins increased in mP-cows (5.33 ± 0.75 to 7.67 ± 0.75%; $P < 0.001$) and was higher in mP-cows compared to mp-cows (5.28 ± 0.75%; $P < 0.05$). In period 5, mP-cows still showed higher levels of unidentified proteins compared to mp-cows (7.12 ± 0.75 and 4.99 ± 0.75% in mP- and mp-cows; $P < 0.05$). Afterwards, concentration of unidentified proteins increased in mP- (to 9.89 ± 0.84; $P < 0.001$) and Mp-cows (from 6.60 ± 0.64 to 8.64 ± 0.77% in period 6; $P < 0.01$). In period 6, mP-cows showed higher levels of unidentified proteins compared to MP- (7.44 ± 0.80%; $P < 0.05$) and mp-cows (5.55 ± 0.84%; $P < 0.001$) and Mp-cows showed higher levels compared to mp-cows ($P < 0.01$).

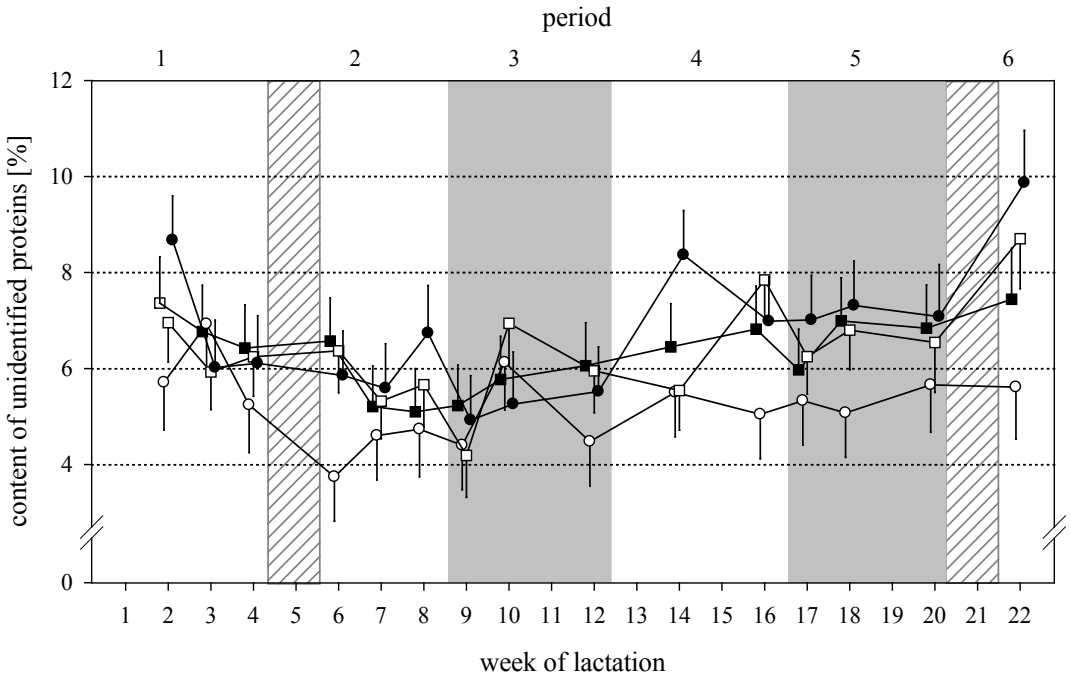

Figure 51: Concentration of unidentified proteins (% of total protein) in skim milk during first 22 weeks of lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model: time $P < 0.001$, group $P = 0.19$ and time × group $P = 0.14$. Differences between periods and between groups can be found in table 18.

Table 18: Mean composition of milk protein (content of major milk proteins in skim milk LSM ± SE) in 6 time periods during 155 days of lactation and mean values of the 6 time periods.

	MP	mp	Mp	mP
α-lactalbumin, % of total protein				
period 1	2.21 ± 0.16	2.25 ± 0.17	2.35 ± 0.14	2.25 ± 0.16
period 2	2.19 ± 0.15	2.07 ± 0.16	2.14 ± 0.14	2.06 ± 0.16
period 3	2.25 ± 0.15	1.97 ± 0.16	2.10 ± 0.15	2.13 ± 0.16
period 4	2.06 ± 0.16	1.83 ± 0.16	2.02 ± 0.14	2.02 ± 0.16
period 5	1.88 ± 0.15	1.78 ± 0.16	2.00 ± 0.14	1.84 ± 0.16
period 6	1.84 ± 0.18[a]	1.97 ± 0.19[a]	1.96 ± 0.18[a]	2.53 ± 0.19[b]*
mean	2.11 ± 0.12	2.00 ± 0.13	2.12 ± 0.11	2.10 ± 0.13
β-lactoglobulin, % of total protein				
period 1	9.40 ± 0.82	9.81 ± 0.90	10.34 ± 0.74	11.20 ± 0.88
period 2	8.83 ± 0.80	9.68 ± 0.88	9.37 ± 0.74*	10.55 ± 0.88
period 3	9.02 ± 0.80	9.45 ± 0.88	9.26 ± 0.77	10.84 ± 0.88
period 4	8.73 ± 0.82	9.19 ± 0.88	9.14 ± 0.75	10.29 ± 0.88
period 5	8.56 ± 0.80	9.00 ± 0.88	8.77 ± 0.74	9.74 ± 0.88
period 6	9.42 ± 0.88[a]	10.44 ± 0.94[ab]*	9.79 ± 0.84[a]	12.42 ± 0.94[b]*
mean	9.03 ± 0.78	9.53 ± 0.85	9.50 ± 0.72	10.75 ± 0.85

	MP	mp	Mp	mP
α-casein, % of total protein				
period 1	35.4 ± 0.8^{ab}	34.5 ± 0.9^{ab}	36.7 ± 0.7^{a}	33.9 ± 0.8^{b}
period 2	34.5 ± 0.8^{ab}	33.9 ± 0.9^{a}	36.7 ± 0.7^{b}	33.4 ± 0.9^{a}
period 3	34.9 ± 0.8^{ab}	33.3 ± 0.9^{a}	36.9 ± 0.8^{b}	34.0 ± 0.9^{a}
period 4	34.2 ± 0.8^{ab}	32.7 ± 0.9^{a}	35.9 ± 0.8^{b}	33.3 ± 0.9^{a}
period 5	33.9 ± 0.8^{ab}	32.7 ± 0.9^{a}	35.3 ± 0.7^{b}	32.7 ± 0.9^{a}
period 6	32.0 ± 1.0^{a}*	34.3 ± 1.0^{ab}	35.4 ± 1.0^{b}	32.4 ± 1.0^{a}
mean	34.4 ± 0.7^{ab}	33.1 ± 0.8^{a}	36.4 ± 0.7^{b}	33.5 ± 0.8^{a}
β-casein, % of total protein				
period 1	30.8 ± 1.0	31.3 ± 1.1	30.9 ± 0.8	29.2 ± 1.0
period 2	33.8 ± 0.9^{ab}*	33.8 ± 1.0^{ab}*	34.9 ± 0.8^{a}*	31.2 ± 1.0^{b}*
period 3	33.2 ± 0.9	33.2 ± 1.0	34.2 ± 0.9	31.8 ± 1.0
period 4	32.0 ± 1.0^{a}	32.2 ± 1.0^{a}	32.3 ± 0.9^{a}*	28.9 ± 1.0^{b}*
period 5	33.3 ± 0.9^{a}	33.7 ± 1.0^{a}	34.2 ± 0.8^{a}*	29.6 ± 1.0^{b}
period 6	32.3 ± 1.1^{a}	29.1 ± 1.2^{ab}*	30.7 ± 1.1^{ab}*	28.2 ± 1.2^{b}
mean	32.1 ± 0.7^{a}	32.5 ± 0.7^{a}	32.8 ± 0.6^{a}	30.1 ± 0.7^{b}
κ-casein, % of total protein				
period 1	15.9 ± 1.5	16.6 ± 1.6	13.6 ± 1.3	16.3 ± 1.6
period 2	15.1 ± 1.5^{ab}	16.6 ± 1.6^{a}	11.5 ± 1.3^{b}*	16.4 ± 1.6^{a}
period 3	15.2 ± 1.5^{ab}	17.1 ± 1.6^{a}	12.1 ± 1.4^{b}	15.9 ± 1.6^{ab}
period 4	16.4 ± 1.5^{ab}	19.3 ± 1.6^{a}*	13.7 ± 1.4^{b}*	17.7 ± 1.6^{ab}*
period 5	16.0 ± 1.5^{ab}	18.3 ± 1.6^{a}	12.6 ± 1.3^{b}	18.6 ± 1.6^{a}
period 6	16.9 ± 1.6^{ab}	19.0 ± 1.7^{a}	13.9 ± 1.5^{b}	14.9 ± 1.7^{ab}*
mean	16.0 ± 1.4^{ab}	17.7 ± 1.5^{a}	12.9 ± 1.3^{b}	16.8 ± 1.5^{ab}
Unidentified proteins, % of total protein				
period 1	6.47 ± 0.71	5.82 ± 0.79	6.28 ± 0.64	6.96 ± 0.75
period 2	5.58 ± 0.69	4.30 ± 0.75*	5.71 ± 0.64	6.05 ± 0.75
period 3	5.64 ± 0.69	4.87 ± 0.75	5.64 ± 0.68	5.33 ± 0.75
period 4	6.57 ± 0.71^{ab}	5.28 ± 0.75^{a}	6.64 ± 0.66^{ab}	7.67 ± 0.75^{b}*
period 5	6.33 ± 0.69^{ab}	4.99 ± 0.75^{a}	6.60 ± 0.64^{ab}	7.12 ± 0.75^{b}
period 6	7.44 ± 0.80^{ab}	5.55 ± 0.84^{a}	8.64 ± 0.77^{bc}*	9.89 ± 0.84^{c}*
mean	6.33 ± 0.62	5.21 ± 0.66	6.35 ± 0.57	6.75 ± 0.66

^{abc}Alphabetic superscripts indicate differences between groups within time period ($P < 0.05$).

*Asterisks indicate differences to previous time periods within group ($P < 0.05$).

5.2. Influence of three days feed restriction on milk protein composition

Out of 132 milk samples taken at days of FRs, 17 were clotted after freezing and thawing. Of the remaining 115 milk samples, 19 were each from d 25 and 28, 21 samples from d 31, 140 and 143 pp and 14 samples from d 146 pp.

α-lactalbumin

In MP-cows content of α-LA was higher during all days of early FR ($P < 0.05$) compared to FR in mid-lactation, whereas in low protein cows higher α-LA content was observed at d 25 pp (2.23 ± 0.19 and 2.49 ± 0.16% in mp- and Mp-cows) compared to d 140 pp (1.67 ± 0.18 and 2.06 ± 0.16% in mp- and Mp-cows; $P < 0.01$; table 19, figure 52). During early FR, mP-cows showed higher concentration of α-LA at d 31 pp (2.31 ± 0.18%) compared to d 146 pp (1.88 ± 0.21%; $P < 0.05$). Moreover during second FR, α-LA content was lowest in mp-cows at d 140 pp (1.67 ± 0.18%; $P < 0.05$). Differences between groups were not observed.

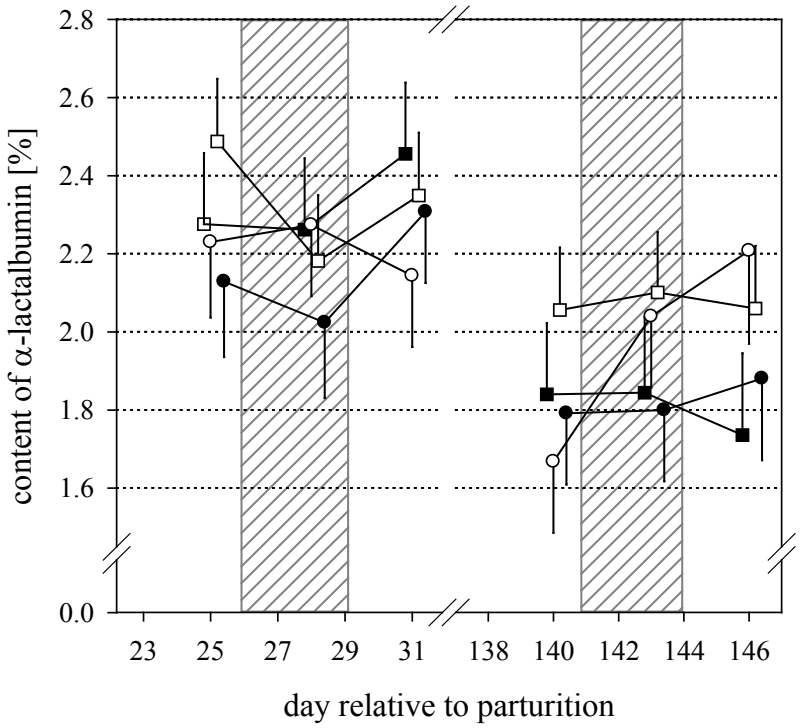

Figure 52: Concentration of α-lactalbumin (% of total protein) in skim milk at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restriction in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model α-lactalbumin: time $P < 0.001$, group $P = 0.74$ and time × group $P = 0.36$. Differences between days and between groups can be found in table 19.

β-lactoglobulin

After both periods of restricted feeding, concentration of β-LG seemed to increase in low protein cows and to decrease in high protein cows. In low yielding cows, content of β-LG was higher at d 25 (10.19 ± 0.90 and 11.18 ± 0.90% in mp- and mP-cows) compared to 140 pp (8.82 ± 0.87 and 9.81 ± 0.87% in mp- and mP-cows; $P < 0.05$; table 19, figure 53). Furthermore during second FR, mp-cows showed lower concentration of β-LG at d 140 (8.82 ± 0.87%) compared to d 146 pp (10.43 ± 1.03%; $P < 0.05$). No differences between groups were observed.

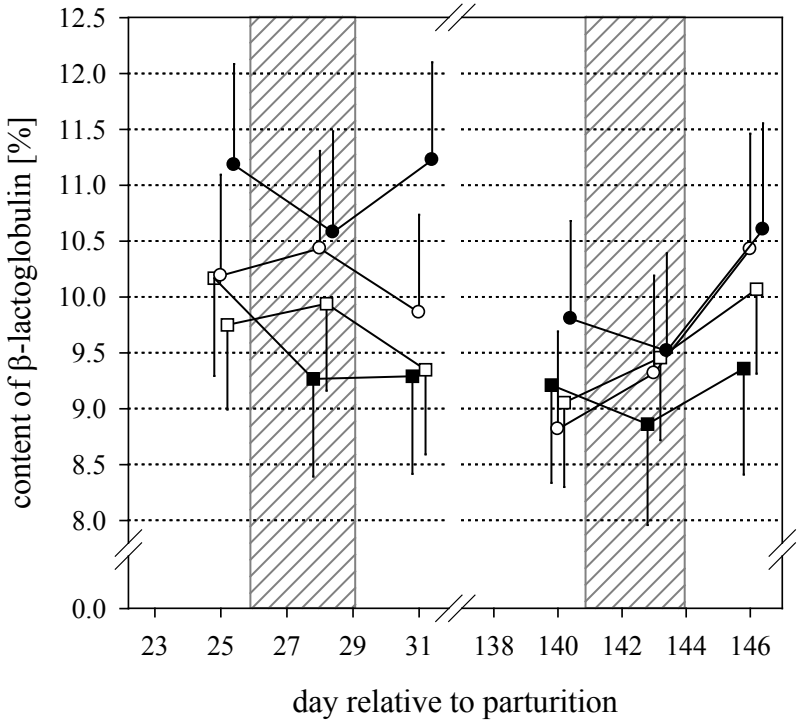

Figure 53: Concentration of β-lactoglobulin (% of total protein) in skim milk at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restriction in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model β-lactoglobulin: time $P < 0.01$, group $P = 0.77$ and time × group $P = 0.68$. Differences between days and between groups can be found in table 19.

α-casein

During FR in early lactation, mp-cows showed lower α-CN contents at d 25 pp compared to Mp-cows (32.9 ± 1.2 and 37.0 ± 1.0% in mp- and Mp-cows; $P < 0.05$) and at d 28 pp compared to Mp- and mP-cows (31.2 ± 1.1, 35.6 ± 1.1 and 34.8 ± 1.2% in mp-, Mp- and mP-cows; $P < 0.05$; table 19, figure 54). At d 31 pp, Mp-cows showed overall highest content of α-CN (37.5 ± 1.0%; $P < 0.05$). Furthermore, α-CN concentration in mp-cows was lower at d 28 compared to d 143 pp ($P < 0.01$).

During FR in mid-lactation, α-CN content increased in mp-cows from d 140 to 143 pp (32.1 ± 1.1 and 35.2 ± 1.1% at d 140 and 143 pp; $P < 0.05$). Furthermore, Mp-cows showed higher content of α-CN at d 140 pp compared to mp-cows (35.7 ± 1.0% in Mp-cows; $P < 0.05$) and at d 143 pp compared to MP- and mP-cows (37.7 ± 1.0, 33.8 ± 1.2 and 32.3 ± 1.1% in Mp-, MP- and mP-cows; $P < 0.05$).

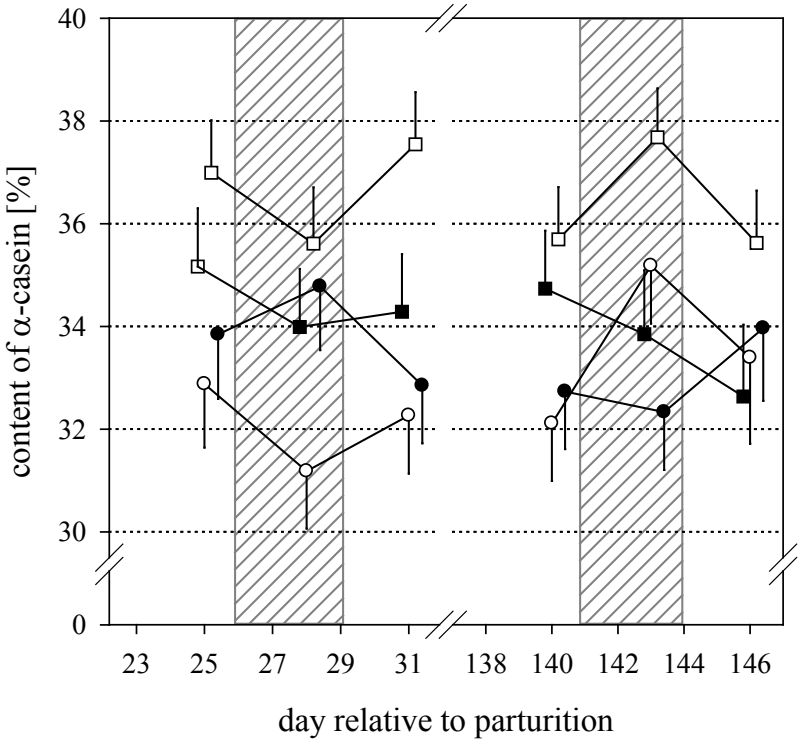

Figure 54: Concentration of α-casein (% of total protein) in skim milk at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restriction in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model α-casein: time $P = 0.63$, group $P < 0.01$ and time × group $P = 0.31$. Differences between days and between groups can be found in table 19.

β-casein

After the period of restricted feeding in early lactation, concentration of β-CN seemed to decrease in low protein cows and to increase in high protein cows, whereas after period of restricted feeding in mid-lactation, β-CN content seemed to increase in all cows.

During FR in early lactation, no differences between groups or days were observed. However content of β-CN was higher in mP-cows at d 25 compared to d 140 pp (31.3 ± 1.1 and 27.6 ± 1.0%; $P < 0.05$; table 19, figure 55).

Moreover, mP-cows showed lower concentrations of β-CN compared to low protein cows at d 140 pp (31.6 ± 1.0 and 31.3 ± 0.9% in mp- and Mp-cows; $P < 0.01$) and d 143 pp (32.4 ± 1.0, 32.1 ± 0.8 and 29.2 ± 1.0% in mp-, Mp- and mP-cows; $P < 0.05$).

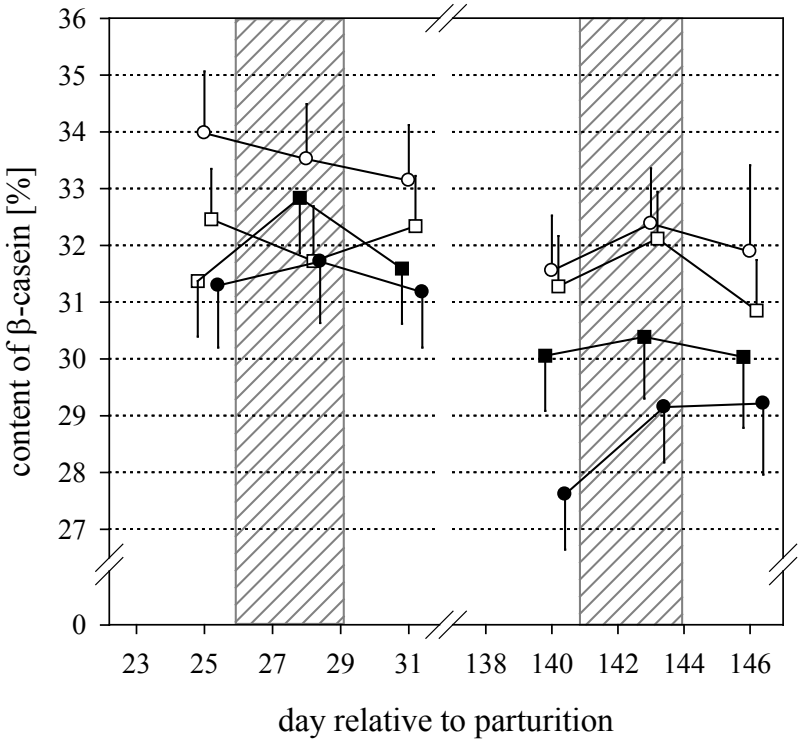

Figure 55: Concentration of β-casein (% of total protein) in skim milk at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restriction in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model β-casein: time $P < 0.01$, group $P < 0.01$ and time × group $P = 0.97$. Differences between days and between groups can be found in table 19.

κ-casein

Concentration of κ-CN was higher in mp- compared to Mp-cows at d 31 pp (18.0 ± 1.7 and 12.8 ± 1.5% in mp- and Mp-cows; $P < 0.05$; table 19, figure 56). Compared to FR in mid-lactation, lower κ-CN contents were observed in mp-cows before (16.8 ± 1.7 and 21.0 ± 1.7 at d 25 and 140 pp; $P < 0.01$) and in mP-cows before and after periods of restricted feeding in early lactation (15.9 ± 1.7, 14.2 ± 1.7, 19.8 ± 1.7 and 19.0 ± 1.7% at d 25, 28, 140 and 143 pp; $P < 0.05$).

During FR in mid-lactation, κ-CN concentration declined in mp-cows from d 140 to 143 pp (21.0 ± 1.7 to 15.4 ± 1.7%; $P < 0.001$). Moreover, content of κ-CN was lower in Mp-cows at d 140 pp compared to low yielding cows (21.0 ± 1.7, 14.8 ± 1.5 and 19.8 ± 1.7% in mp-, Mp- and mP-cows; $P < 0.05$) and at d 143 pp compared to high protein cows (17.5 ± 1.7, 13.0 ± 1.4 and 19.0 ± 1.7% in MP-, Mp- and mP-cows; $P < 0.05$).

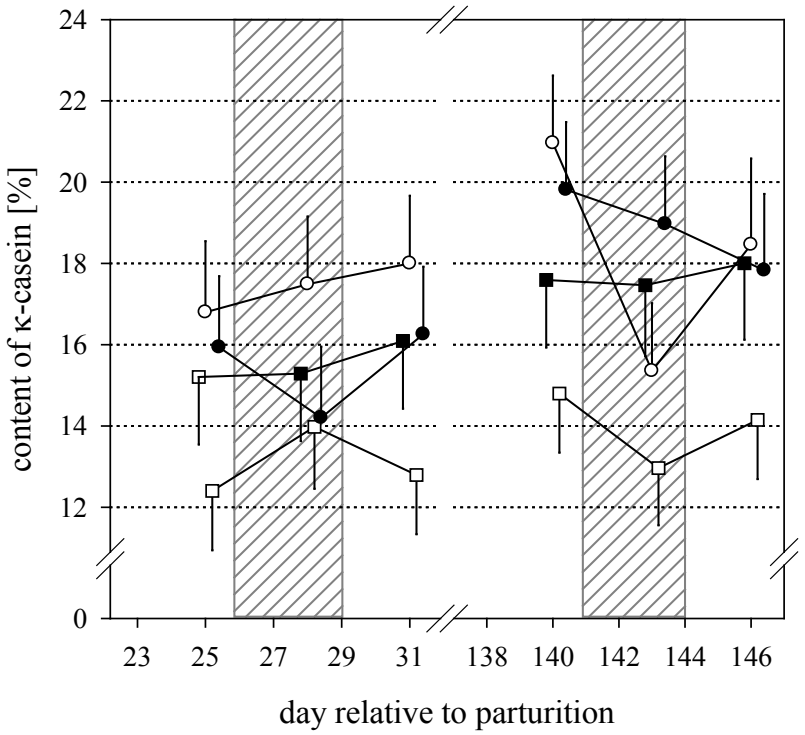

Figure 56: Concentration of κ-casein (% of total protein) in skim milk at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restriction in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model κ-casein: time $P < 0.001$, group $P = 0.11$ and time × group $P = 0.19$. Differences between days and between groups can be found in table 19.

Unidentified proteins

During FR in early lactation, high protein cows seemed to have higher concentrations of unidentified proteins compared to low protein cows.

Differences between days and between groups during FR in early and in mid-lactation proved not to be significant (table 19, figure 57). However, Mp-cows showed lower levels of unidentified proteins at d 31 compared to d 146 pp (5.21 ± 0.88 and 7.35 ± 0.88%; $P < 0.05$).

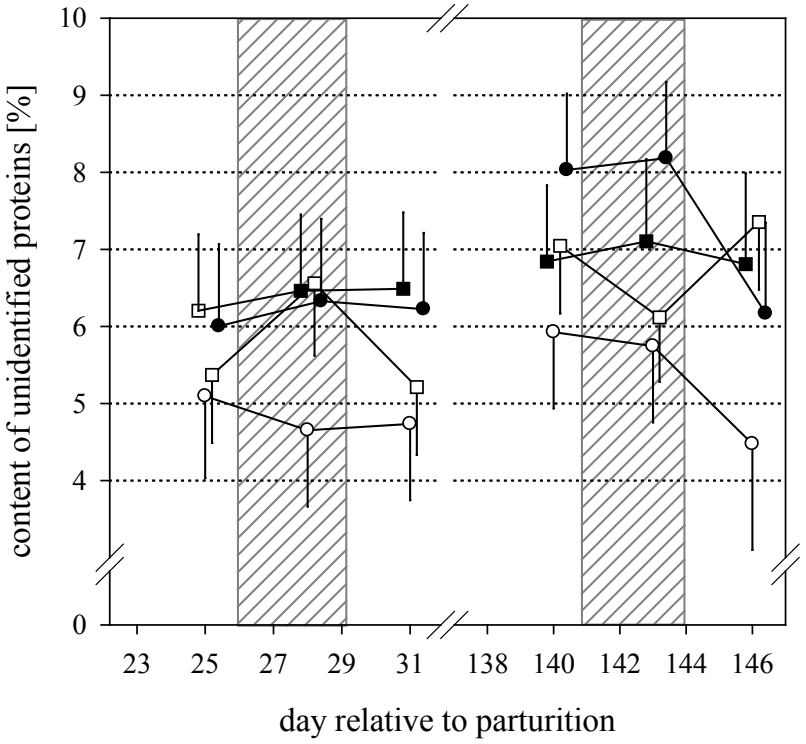

Figure 57: Concentration of unidentified proteins (% of total protein) in skim milk at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restrfiction in early and mid-lactation.

MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. White and grey areas show period 1 to 6. Shaded areas show feed restrictions. Values are presented as LSM ± SEM. Fixed effects in model unidentified proteins: time P = 0.05, group P = 0.40 and time × group P = 0.87. Differences between days and between groups can be found in table 19.

Table 19: Composition of milk protein (content of major milk proteins in skim milk LSM ± SE) at last day before (d 25 and 140 pp), last day during (d 28 and 143 pp) and last day after (d 31 and 146 pp) three days of feed restriction in early and mid-lactation.

	MP	mp	Mp	mP	mean
α-lactalbumin, % of total protein					
d 25 pp	2.28 ± 0.18[1]	2.23 ± 0.19[1]	2.49 ± 0.16[1]	2.13 ± 0.19	2.28 ± 0.09[1]
d 28 pp	2.26 ± 0.18[1]	2.27 ± 0.18	2.18 ± 0.17	2.02 ± 0.19	2.19 ± 0.09[1]
d 31 pp	2.46 ± 0.18[1]	2.14 ± 0.18	2.35 ± 0.16	2.31 ± 0.18[1]	2.31 ± 0.09[1]
d 140 pp	1.84 ± 0.18	1.67 ± 0.18*	2.06 ± 0.16	1.79 ± 0.18	1.84 ± 0.09
d 143 pp	1.84 ± 0.19	2.04 ± 0.18°	2.10 ± 0.15	1.80 ± 0.18	1.95 ± 0.09
d 146 pp	1.74 ± 0.21	2.21 ± 0.24°	2.06 ± 0.16	1.88 ± 0.21	1.97 ± 0.10

	MP	mp	Mp	mP	mean
β-lactoglobulin, % of total protein					
d 25 pp	10.17 ± 0.87	10.19 ± 0.90[1]	9.75 ± 0.76	11.18 ± 0.90[1]	10.32 ± 0.43[1]
d 28 pp	9.27 ± 0.87	10.43 ± 0.87	9.94 ± 0.78	10.58 ± 0.90	10.06 ± 0.43[1]
d 31 pp	9.29 ± 0.87	9.86 ± 0.87	9.35 ± 0.76	11.23 ± 0.87	9.93 ± 0.42
d 140 pp	9.21 ± 0.87	8.82 ± 0.87*	9.06 ± 0.75	9.81 ± 0.87	9.22 ± 0.42°
d 143 pp	8.86 ± 0.90	9.32 ± 0.87*°	9.46 ± 0.74	9.52 ± 0.87	9.29 ± 0.42°
d 146 pp	9.36 ± 0.95	10.43 ± 1.03°	10.07 ± 0.76	10.60 ± 0.95	10.12 ± 0.46*
α-casein, % of total protein					
d 25 pp	35.2 ± 1.1[ab]	32.9 ± 1.2[a]	37.0 ± 1.0[b]	33.8 ± 1.3[ab]	34.7 ± 0.6
d 28 pp	34.0 ± 1.1[ab]	31.2 ± 1.1[a1]	35.6 ± 1.1[b]	34.8 ± 1.2[b]	33.9 ± 0.6
d 31 pp	34.3 ± 1.1[a]	32.3 ± 1.1[a]	37.5 ± 1.0[b]	32.8 ± 1.1[a]	34.2 ± 0.5
d 140 pp	34.7 ± 1.1[ab]	32.1 ± 1.1[a]*	35.7 ± 1.0[b]	32.7 ± 1.1[ab]	33.8 ± 0.5
d 143 pp	33.8 ± 1.2[a]	35.2 ± 1.1[ab]°	37.7 ± 1.0[b]	32.3 ± 1.1[a]	34.8 ± 0.6
d 146 pp	32.6 ± 1.4	33.4 ± 1.7*°	35.6 ± 1.0	34.0 ± 1.4	33.9 ± 0.7
β-casein, % of total protein					
d 25 pp	31.4 ± 1.0	34.0 ± 1.1	32.5 ± 0.9	31.3 ± 1.1[1]	32.3 ± 0.51
d 28 pp	32.8 ± 1.0	33.5 ± 1.0	31.7 ± 1.0	31.7 ± 1.1	32.4 ± 0.51
d 31 pp	31.6 ± 1.0	33.1 ± 1.0	32.3 ± 0.9	31.2 ± 1.0	32.1 ± 0.5
d 140 pp	30.1 ± 1.0[ab]	31.6 ± 1.0[a]	31.3 ± 0.9[a]	27.6 ± 1.0[b]	30.1 ± 0.5
d 143 pp	30.4 ± 1.1[ab]	32.4 ± 1.0[a]	32.1 ± 0.8[a]	29.2 ± 1.0[b]	31.0 ± 0.5
d 146 pp	30.0 ± 1.2	31.9 ± 1.5	30.9 ± 0.9	29.2 ± 1.3	30.5 ± 0.6
κ-casein, % of total protein					
d 25 pp	15.2 ± 1.7	16.8 ± 1.7[1]	12.4 ± 1.5	15.9 ± 1.7[1]	15.1 ± 0.81
d 28 pp	15.3 ± 1.7	17.5 ± 1.7	14.0 ± 1.5	14.2 ± 1.7[1]	15.2 ± 0.8
d 31 pp	16.1 ± 1.7[ab]	18.0 ± 1.7[a]	12.8 ± 1.5[b]	16.3 ± 1.7[ab]	15.8 ± 0.8
d 140 pp	17.6 ± 1.7[ab]	21.0 ± 1.7[a]*	14.8 ± 1.5[b]	19.8 ± 1.7[a]	18.3 ± 0.8°
d 143 pp	17.5 ± 1.7[a]	15.4 ± 1.7[ab]°	13.0 ± 1.4[b]	19.0 ± 1.7[a]	16.2 ± 0.8*
d 146 pp	18.0 ± 1.9	18.5 ± 2.1*°	14.1 ± 1.5	17.8 ± 1.9	17.1 ± 0.9°*
Unidentified proteins, % of total protein					
d 25 pp	6.20 ± 0.99	5.10 ± 1.06	5.37 ± 0.88	6.00 ± 1.07	5.67 ± 0.50[1]
d 28 pp	6.46 ± 0.99	4.65 ± 0.99	6.56 ± 0.94	6.33 ± 1.07	6.00 ± 0.50
d 31 pp	6.49 ± 0.99	4.73 ± 0.99	5.21 ± 0.88[1]	6.22 ± 0.99	5.66 ± 0.48
d 140 pp	6.84 ± 0.99	5.93 ± 0.99	7.05 ± 0.88	8.03 ± 0.99	6.96 ± 0.48
d 143 pp	7.10 ± 1.06	5.74 ± 0.99	6.12 ± 0.84	8.18 ± 0.99	6.79 ± 0.49
d 146 pp	6.81 ± 1.18	4.47 ± 1.37	7.35 ± 0.88	6.17 ± 0.18	6.20 ± 0.58

[ab]Alphabetic superscripts indicate differences between groups within time point ($P < 0.05$).
*°[#]Symbolic superscripts indicate differences between time points within a feed restriction and within groups ($P < 0.05$).
[1]Superscripted 1 indicates difference ($P < 0.05$) of day in FR during early lactation (d 25, 28 and 31 pp) compared to day in FR during mid-lactation (d 140, 143 and 146 pp, respectively).

V. DISCUSSION

1. Experimental animals

Grouping according to FCM and milk protein content during d 23 to 25 pp of the 23 cows resulted in groups differing in those parameters, even if single animals seemed not to fit in their respective group (e.g. 14030 03870 with 40.97 kg/d in MP-cows). However after omitting these animals, groups and significant results did not change, so they were further included in the study.

Mean milk and blood parameters

As intended, mean milk yield, FCM and ECM values during 4th week of lactation in high yielding cows surpassed those in low yielding cows as did protein concentration in high protein cows compared to low protein cows.

Over the course of the whole experimental period of 155 d most differences or non-differences between respective groups remained throughout times of improved metabolic and energetic status. This could be explained by the genetic influence on milk yield and milk protein concentration. However, milk yield and milk protein content of some individual animals after 155 d of lactation deviated from respective means of early selected groups. One reason could be found in differences in the metabolic reaction to a negative energy balance during early lactation with concomitant influence on milk yield and milk protein content. Particularly, MP- and mP-cows differed in average milk protein concentration. This agrees with previous findings which show that high milk yield is associated with lower concentrations of constituents (Teepker and Swalve, 1988).

However, milk fat concentration was highest in MP-cows resulting in highest FPR and they showed numerically highest blood serum NEFA throughout first 22 weeks of lactation. It can be concluded that those cows produced the highest amount of milk energy during that time period. Nevertheless, they mobilised only 32% of their back-fat throughout experimental period. Previous studies showed that not only excessive mobilisation of body fat, but rather individual factors contribute to degree of metabolic imbalance (Ingvartsen et al., 2003; Hammon et al., 2009). Most excessive fat mobilisation was observed in mP-cows (-63%). However this did not lead to increased blood serum NEFA levels neither in mean values nor in values during early lactation as reviewed by Drackley (1999), but milk fat concentration was higher compared to low protein cows. Nevertheless mP-cows seemed not to be impaired in metabolism, showing low FPR and numerically lowest blood serum NEFA and BHBA values.

Yields of milk protein (0.92), fat (0.77) and lactose (0.96) are positively correlated to milk yield (Teepker and Swalve, 1988; Shahbazkia et al., 2010) and therefore highest in high yielding cows. Overall highest SCC could be observed in the 5 mP-cows. This is due to the single cow with the only clinical case of mastitis during wk 5 pp and the three cows showing elevated SCC during two consecutive weeks in the study.

Course of milk parameters

Throughout the experiment, all cows showed typical lactation curves with steep increases of milk yield, FCM and ECM after calving. Protein concentration reached a nadir in wk 6 pp and increased constantly afterwards.

In low yielding cows, FCM and ECM did not increase after wk 2 pp although milk yield did. This is explained by the slightly sharper decrease in milk constituents compared with high yielding cows during that time period. As a result of high milk yield, economic valuable milk protein yield was highest in Mp- and MP-cows during the first 3 weeks of lactation and throughout

experimental period. In Mp-cows highest values were observed during the first week of lactation and values decreased thereafter. This is due to the sharp decrease in milk protein concentration which could be a result of ruminal microbial energy shortage (Brun-Lafleur et al., 2010). Contrary to intended similarity of protein yield between Mp- and mP-cows, values were still different at the end of the experimental period due to the persistent influence of milk yield on this parameter. However, milk protein concentration of mP-cows after 22 weeks was at a very high level (4.07%) as compared to Mp-cows (3.19%). A further increase in milk protein concentration of mP-cows could be expected towards the end of lactation which might blur the differences.

In Mp-cows during wk 9 pp, one cow showed exceptional low protein concentration (2.22%) and in wk 10 pp another cow high concentration (4.05%), resulting in lower and higher mean milk protein concentration compared to previous and following week (figure 10). Milk fat concentration shows high fluctuation throughout lactation and from milking to milking (Quist et al., 2008). Furthermore milk fat concentration decreases with increasing amount of feeding rapidly fermented carbohydrates due to diminished synthesis of short chain fatty acids (leading to rumen acidosis) and is increased by enhanced body fat mobilisation due to insufficient energy intake or decreased energy output (e.g. lactation, gravidity; van Knegsel et al., 2005).

Milk hydrocortisone could be used as a diagnostic marker of chronic stress in animals. In wk 2 pp one mP-cow showed a highly increased hydrocortisone level (18.7 nmol/L). This cow was over-conditioned (BCS 4.5) before parturition. Furthermore lameness and inflammation of interdigital space were diagnosed one week pp. Moreover during wk 19 pp, one MP-cow showed unusual high milk hydrocortisone levels due to an inflammation of subcutaneous tissue following muscle biopsy three days earlier. Fisher et al. (2002) showed elevated basal blood levels of hydrocortisone in lying deprivated cows. In contrast to van der Kolk (1990) and consistent with Patel et al. (1996), in our study milk hydrocortisone did not decrease during first 22 weeks of lactation.

Course of blood serum parameters

Remarkable high blood serum glucose levels in mp-cows at day of parturition were observed. Sampling took place within 24 hours after calving and mp-cows showed unintentionally lowest average sampling time (6 h pp). Therefore, results of blood sampling could be influenced by the physiological high blood glucose level during calving (Silva-del-Río et al., 2010).

Massive metabolic transformation processes for preparation of parturition and lactation already take place during prepartal transition period. This is reflected among others by enhanced gluconeogenesis and fat mobilisation (Loor et al., 2005; Ingvartsen, 2006; van Dorland et al., 2009). In the present study, only Mp-cows showed numerically higher blood serum NEFA levels prior to parturition which had been associated recently with higher risks of postpartum diseases (Piechotta et al., 2012). However, most of cows reached critical (1,000 µmol/l; Chapinal et al., 2011) and maximum levels in wk 2 pp and NEFA values declined constantly thereafter (Grummer et al., 2004).

Consequently, blood serum BHBA increased in all cows after parturition above 0.5 mmol/L and reached maximum levels at d 8 pp. In two mp-cows highly increased blood serum BHBA levels were observed from d 15 to 22 pp and one cow showed BHBA levels over 1.5 mmol/L at d 8, 15 and 22 pp. However, no concurrent excessive body fat mobilisation or weight loss nor enhanced ketogenesis as described by Grummer (1993) and Hegardt (1999) could be found in these cows. Graber et al. (2010) also described elevated blood BHBA levels without enhanced ketogenesis due to elevated abundance of *HMGCS2* mRNA. As all cows received the same feed ration, elevation due to uptake of ketogenic substances, e.g. fermentation product butyric acid in wet silages or supplementation with butanediol (Mills et al., 1986) can nearly be excluded.

In accordance with Herdt and Smith (1996), blood serum cholesterol levels of cows in the present study were lowest at day of parturition and increased afterwards. Furthermore high yielding cows showed higher blood serum cholesterol levels from wk 6 pp on. Previous studies showed that blood cholesterol levels are positively correlated to milk yield, but not to milk fat yield or concentration. This is due to the simultaneous transport of triacylglycerols and cholesterol in lipoproteins, which supply mammary gland with fat precursors (Palmquist and Mattos, 1978; Mazur et al., 1992). Moreover low levels of blood cholesterol at the beginning of lactation indicate higher risk of fatty liver disease, due to impairment of hepatic lipoprotein synthesis (Rayssiguier et al., 1988; Mazur et al., 1989).

Blood serum bilirubin is elevated in early lactation due to its diminished hepatic uptake in favour of NEFA uptake (Reid et al., 1977). In the study of Steen (2001) diseased liver was obvious, if total bilirubin exceeded 6.0 µmol/L and one of the liver-specific enzymes aspartate aminotransferase, glutamate dehydrogenase or gamma-glutamyl transferase was elevated. In the present study, all cows exceeded critical values of blood serum tBR up to d 22 pp.

Body condition parameters

Cows showed typical course of body condition parameters with declining weights, BCS and BFT throughout early lactation (Drackley, 1999; Ingvartsen and Andersen, 2000; Grummer et al., 2004). Nevertheless, mP-cows gained more weight from wk 6 pp on and BCS increased from wk 12 pp on. The reason could be that mP-cows reached positive energy balance after parturition faster due to lower energy outputs in milk (ECM) compared with high yielding cows.

Cyclicity and health status

Three cows were excluded from statistical analysis of cyclicity because of ovarial cysts. One mp-cow suffered inflammation of uterus (retained placenta) and the other two Mp-cows showed during first six weeks overall highest FCM yield (48.5 ± 0.41 kg/d) and second highest ECM yield (52.2 ± 1.24 kg/d). According to Walsh et al. (2011) are the two main reasons leading to decreased fertility infectious diseases of genital tract and risk of metabolic imbalances over the course of peri-parturient period due to high milk yields. Dairy cows in good nutritional state ovulate around d 15 pp (Crowe, 2008). In the present study cows ovulated at d 23 ± 2 pp but within the physiological timeframe up to approximately 30 days pp. However, only 40% of Mp-cows ovulated before d 30 pp (average d 31 ± 4 pp) indicating higher fertility problems in those animals.

Incidence of lameness and retained placenta was highest in high yielding dairy cows, whereas mp-cows showed lowest incidence of clinical diseases. Previous studies confirmed that high yielding dairy cows are more susceptible to diseases (Mallard et al., 1998; LeBlanc, 2010), whereas Ingvartsen et al. (2003) found only positive correlation between milk yield and infectious risk of mammary gland and associate degree of increasing milk yield until peak yield with higher incidence of diseases.

100 day performance

For previous lactation, 100 day performance of milk yield, milk protein and fat concentration was calculated according to procedure of Arbeitsgemeinschaft Deutscher Rinderzuechter e.V. (Bonn), which uses milk performance test data of every four weeks. In contrast, 100 day performance of milk yield during experimental lactation was calculated by summing up daily milk yields and weekly means of milk fat and milk protein yield were multiplied by 7 and then summed. Therefore 100 day performance of previous lactation is based on the first three milk performance measurements, whereas performance in current lactation based on 91 measurements for milk yield and 27 measurements of milk fat and protein yield, consequently aggravating comparison.

In Mp-cows 85% of cows were in second lactation during the study and showed higher milk yield, milk fat and protein yield compared to previous lactation. Fleischer et al. (2001) and Sejrsen (1994) showed that development of mammary gland is not completed with onset of first lactation and milk output increases further in second lactation. Yet, due to the high proportion of heifers in the original herd (49%), intended selection of solely multiparous cows fitting to all groups was not feasible.

Recapitulation

Based on milk, blood serum and body condition parameters, high yielding cows seemed to be in severest metabolic stress during early lactation. However mP-cows mobilised more body fat reserves compared to other groups but restored those early in mid-lactation. Furthermore mp-cows showed in part unclear individual metabolic adaptations. Fertility of Mp-cows seemed to be diminished in this study. Considering the fact that MP-cows did not show signs of abnormal fertility further detailed studies with more animals could shed light on reproductive problems of high yielding dairy cows with different milk protein concentration.

2. Hepatic mRNA expression during lactation

Lipid metabolism

Elevated NEFA and BHBA levels in blood due to reduced DMI in peri-parturient period increase expression rates of hepatic transcription regulation factors *HNF4A* and *PPARA*. Therefore they promote β-oxidation of fatty acids and enhance expression of *ACADVL*, *CPT1A* and *ECHS1* mRNA (Mandard et al., 2004; Odom et al., 2004; Loor et al., 2005).

Abundances of mRNA involved in β-oxidation of fatty acids and their transcription regulation factors were diminished in mp-cows at d 15 pp which is reflected by the lowest blood serum BHBA at that time point. This suggests lower body fat mobilisation in these cows during the first two weeks of lactation or delayed enhancement of hepatic fatty acid oxidation until d 57 pp, when mRNA levels were similar amongst cows. Solely mRNA of translation factor *EIF4B*, triggered by decreasing DMI and EB stayed on higher levels in mp-cows, similar to others. Furthermore, Mp-cows showed higher levels of *ACADVL* mRNA at d 57 pp and MP-cows at d 155 pp, both compared to mP-cows. Also, MP-cows had higher levels of *ECHS1* mRNA compared to mp-cows at d 155 pp. This could indicate enhanced fatty acid oxidation in these cows even during mid-lactation.

Hepatic fatty acid synthesis is also positively influenced by elevated NEFA levels in blood through enhanced expression of *SREBF1* mRNA. This is a transcription regulation factor for *GPAM* mRNA, encoding the enzyme of initial step in triacylglycerol synthesis. Furthermore, elevated levels of *PPARA* mRNA diminish *SREBF1* expression (Loor et al., 2005; 2006).

In all cows, *SREBF1* mRNA levels increased until end of study as did *ACACA* and *GPAM* until d 57 pp. These results go in line with Loor et al. (2005), who found increasing transcript abundances for *SREBF1* and *GPAM* throughout early lactation. Kim et al. (2004) and Romics et al. (2004) reported that up-regulation of *SREBF1* in mice was followed by up-regulation of *GPAM*. These modifications were important for adaptation to the greater influx of NEFA into liver. Additionally, Ueki et al. (2004) described increased expression of *SREBF1* leading to fatty liver in mice.

Carbohydrate metabolism

Transcription regulation factors *HNF4A* and *PPARA* are also involved in regulation of hepatic gluconeogenesis, *HNF4A* promotes *PCK1* expression and *PPARA* expression of *PC* (Loor et al.,

2005; 2006).

All cows except of Mp-cows showed highest *PC* mRNA levels at d 1 pp. With onset of lactation, increase in milk yield and demand for lactose is associated with up-regulation of *PC* mRNA levels (Greenfield et al., 2000).

Hepatic levels of *PCK1* mRNA increased steeply in mp-cows from d 1 to 57 pp, whereas Mp- and mP-cows showed only slow increases. In MP-cows, *PCK1* abundance was elevated within two weeks after parturition. Comparable results were reported by Greenfield et al. (2000). Up-regulation of *PCK1* during early lactation in the current study is associated with the large demand of glucose for milk synthesis and a result of increased gluconeogenesis. Nevertheless, high yielding Mp-cows seemed to have diminished gluconeogenesis potential due to slower increasing mRNA abundance of *PCK1* mRNA and lower *PC* mRNA in early lactation.

Protein metabolism

Hepatic mRNA *TAT* and *CTSL* encode for proteinases associated with amino acid catabolism which is diminished during peri-parturient period (Loor et al., 2005). The present data revealed a down-regulation of *CTSL* and up-regulation of *TAT* in all cows on d 155 pp compared to time points in early lactation. Moreover MP-cows showed lower mRNA levels of *CTSL* at d 1 and 15 pp compared to mp-cows. Enhanced activities of TAT and CTSL are associated with increased amino acid fragments, partly used for gluconeogenesis or ketogenesis. Regarding diminished fatty acid oxidation and regular gluconeogenesis in mp-cows, these animals probably met enhanced requirements in early lactation from amino acids.

Glucose metabolism

In mp-cows, abundance of insulin independent glucose transporter *SLC2A2* mRNA was lower at d 15 compared to 57 pp. This glucose transporter is mainly involved in release of hepatic glucose and regulation of insulin secretion from β-cells (Zhao and Keating, 2007). Therefore, mp-cows seemed to show lower hepatic glucose output two weeks after parturition. Furthermore Mp-cows showed lowest *SLC2A2* mRNA abundance at day of slaughtering compared to earlier time points and compared to low yielding cows. However in contrast to Rencurel et al. (1996), Mp-cows showed not significantly lower blood glucose levels at this time.

At day of parturition, hepatic *INSR* mRNA abundance was highest in mP-cows compared to high yielding cows. According to Liu et al. (2010), high yielding cows seemed therefore to show higher risk for fatty liver. Moreover, high levels of insulin lead to a decrease in *INSR* mRNA expression in calf hepatocytes (Zhang et al., 2011). This could be an explanation for mP-cows showing lower *INSR* abundance at day of slaughter compared to Mp-cows and also supporting the fact that basal insulin levels during ivGTT (d 127 pp) were lowest in Mp-cows.

Moreover, hepatic ketogenesis occurs commonly during peri-parturient period reflected in mRNA changes of control enzyme 3-hydroxy-3-methylglutaryl-CoA synthase 2 (Voet and Voet, 2004; Loor et al., 2005). In contrast, van Dorland et al. (2009) and Graber et al. (2010) found no different *HMGCS2* expression during early lactation. In the current study, hepatic ketogenesis seemed to be diminished at day of parturition in all cows except of mP-cows and increased to maximum at d 15 pp. Afterwards, Mp-cows showed lower levels of *HMGCS2* mRNA at d 57 pp compared to MP-cows and at day of slaughter compared to high protein cows. Despite of high milk yield in Mp-cows, these cows either seemed to achieve their energy demands by reaching of maximum feed intake fastest or ketogenesis was diminished due to hepatic dysfunction.

Common metabolism

Hepatic levels of CS mRNA were similar amongst all cows and time points, indicating no impairment of hepatic citric acid cycle.

Abundance of hepatic *TNFA* mRNA was higher in mP-cows at day of parturition compared to d 155 pp and similar results were observed by Loor et al. (2005). Diminished levels of *TNFA* mRNA in MP-cows could be related to decreased body fat mobilisation (Loor et al., 2005), however blood serum NEFA levels and body condition parameters did not reflect diminished lipolysis.

Abundance of *IGF1* mRNA was higher in mp-cows at d 57 pp compared to earlier time points and in mP-cows compared to day of parturition. According to Fenwick et al. (2008), these results indicated enhanced negative EB in mP-cows at parturition and in mp-cows during first two weeks of lactation. They also found elevated IGF-1 blood levels whereas Graber et al. (2010) observed highest plasma levels at wk 3 ap and decreasing levels until wk 4 pp.

Recapitulation

Hepatic mRNA abundances of key enzymes in lipid, protein and carbohydrate metabolism were similar in cows observed here compared to previous studies.

Furthermore, mp-cows seemed to differ in metabolism during early lactation due to enhanced protein catabolism instead of lipolysis. Nonetheless, high yielding cows and especially Mp-cows showed higher risk for fatty liver development and seemed to have diminished gluconeogenesis potential.

3. Feed restrictions

In immediate postpartum period, negative EB leads to a marked decrease in milk protein concentration and therefore to an undesirable loss in average 305-d milk protein yield (Depeters and Cant, 1992; Murphy and Omara, 1993; Walker et al., 2004). However, the base level of the nadir in milk protein concentration during early lactation varies between animals according to individual metabolic and endocrine adaptation capacities to nutritional shortage and to genetic background of cows (Kessel et al., 2008). It was possible in the current experimental trial to evaluate those physiological adaptive responses in cows with significantly different milk protein concentrations during early lactation and concomitant significantly varying FCM yield under same housing and feeding conditions.

DMI intake and energy balance

For individual measurement of feed intake cows were brought to a tie stall with separated feed troughs and with eye contact to the herd. Although cows were accustomed to cubicle housing system, no effects on behaviour such as excessive mooing or restlessness were detected during tied-stall housing. Average DMI of all animals was 17.9 ± 0.59 kg at d 25 pp and 20.5 ± 0.61 kg at d 140 pp, therefore slightly higher compared to previously reported DMI for multiparous cows in wk 4 and 20 pp (approx. 16 and 18 kg; Ingvartsen and Andersen, 2000).

As expected, mp-cows showed lowest *ad libitum* DMI. This could either be associated to endocrine feed intake regulation due to low energy demand for milk production or to individual low feed intake which results in a low milk production (Baile and McLaughlin, 1987). The short-term FR intended to decrease DMI to 70% of average DMI of d 23 to 25 pp and d 138 to 143 pp, which was only roughly met. During FR in early lactation MP-cows showed 65.9, mp-cows 66.5, Mp-cows 71.9 and mP-cows 72.1% of previous DMI, whereas during FR in mid lactation, DMI

was even lower (68.9, 69.0, 65.6 and 68.2% in MP-, mp-, Mp- and mP-cows). Particularly during mid-lactation, cows adopted slowly to straw-supplemented RD. Moreover, hyperketonaemia could have decreased feed intake in early lactation. Nonetheless, this metabolic challenge had to be faced by all animals during FRs. These results are supported by the well-known fact that during early lactation energy intake regularly lags behind milk-production related energy demands leading to a NEB (Bell, 1995; Drackley, 1999).

All *ad libitum* fed cows, regardless of group membership, experienced negative EB at d 25 pp (average -57.1 ± 4.84 MJ NE_L), but negative EB during early lactation was even lower compared to Kessel et al. (2008; approx. -35 MJ NE_L). Energy balance of low protein cows was also negative at d 140 pp (average -15.9 ± 9.48 MJ NE_L).

On the first day of FRs, severe decline of EB was found in all cows. High yielding cows showed most negative values, whereas mP-cows in early and mp-cows in mid-lactation showed only slight decreases. Subsequently, on the second day of FR, EB increased slightly in high yielding cows during early lactation and in all cows during mid-lactation. These findings reflect the fast metabolic adaptation of high yielding animals to increased negative EB in early lactation and the greater potential to cope with metabolic challenges in mid-lactation.

Milk parameters

As expected, milk yield declined during restricted feeding but decrease was not significant in mP-cows. Furthermore, FCM yield decreased only in high yielding cows and only during FR in early lactation, showing disturbance of milk production due to restricted feeding in those cows.

In agreement with Nielsen et al. (2003) and Agenäs et al. (2003), milk protein concentration of low protein cows was unaffected by both FRs, whereas in high protein cows during early lactation and in MP-cows also during mid-lactation protein content of milk decreased. Agenäs et al. (2003) illustrated a distinct decline in milk protein concentration during subsequent first two days of realimentation. In the present study, milk protein concentration also declined to a nadir two days after FR in early lactation, but not during FR in mid-lactation. As expected due to the decreased milk yield and milk protein concentration, milk protein yield also declined over the course of FRs.

Moreover in all cows, milk fat concentration did not vary during FRs and milk fat yield decreased only in MP-cows during FR in early lactation due to decreased milk yield. These results are supported by Guinard-Flament et al. (2007) and Carlson et al. (2006), but differ from other studies with longer FR periods, where FR led to decreasing milk fat yields (Velez and Donkin, 2005).

Throughout the experiment, milk lactose concentration was constant in all cows except of mp-cows in FR during early lactation. Constant milk lactose content can be explained by the osmotic role of lactose and the fact that milk volume is mainly depending on lactose synthesis (Linzell and Peaker, 1971).

As shown before, lowest level of protein content was reached on the second day of refeeding in early lactation, while milk fat and lactose content were not responsive to the reduced feeding level and no influence of FR was observed during mid-lactation. The subsequent minimum of protein content in FR during early lactation is not unusual, as fat and lactose synthesis have top priority due to breeding preferences over the last decades. Also, fat mobilisation is the prior feedback on unsatisfying energy supply in dairy cows. Beside this, if the deficiency lasts, changes in protein metabolism towards catabolism will occur.

Cows displaying FPR more than 1.5 during early lactation are at risk for ketosis or are already affected with it (Heuer et al., 1999). All cows showed higher FPR values during FR in early lactation as well as in mid-lactation reflecting the increase in blood serum BHBA values during

those time periods. Prior to FRs, FPR was elevated only in early lactation.

Although no signs of clinical mastitis could be observed, SCC increased steeply in two mP-cows during FR in early lactation and in one mp-cow after FR in mid-lactation. On the one hand this is certainly due to individual variations in resistance to mammary infections, on the other hand due to higher incidence of mammary gland infections in tie stalls (Valde et al., 1997).

Regarding milk hydrocortisone content, it is well known that high milk yield and concurrently large energy deficit as well as ketosis are correlated to lower hydrocortisone levels (Torres et al., 1997; Beerda et al., 2004; Forslund et al., 2010). Fisher et al. (2002) observed no effects of moderate feed restriction on hydrocortisone levels. As expected, milk BHBA-levels increase during FR, but differences proved to be significant only in mp-cows.

Blood serum parameters

As previously shown, blood serum glucose levels decreased during FR in early lactation in all groups and reached the initial level during subsequent *ad libitum* feeding (Nielsen et al., 2003; Loor et al., 2007). Nonetheless in mid-lactation, differences proved not to be significant due to diminished milk output, higher feed intake and less severe negative energy balance.

Insufficient energy supply results in lipolysis of adipose tissue and circulating NEFAs in blood are supplied to β-oxidation in hepatocytes (Mashek and Grummer, 2003). In agreement with previous results, blood serum NEFA concentrations increased in all groups until second day of FR in early lactation and reached initial levels during subsequent *ad libitum* feeding (Nielsen et al., 2003; Loor et al., 2007). Furthermore, average blood serum NEFA levels rose in all groups above threshold levels of 1,000 µmol/L during restricted feeding, whereas during FR in mid-lactation no significant increases could be observed and levels stayed well below the threshold level. Lower serum NEFA levels in mid-lactation suggested a more stable metabolic status together with a sustained physiological serum glucose concentration.

Due to deficiency of glucose, the product of β-oxidation Acetyl-CoA is not metabolized in citrate-cycle and induces ketogenesis during negative EB (Zammit, 1983). Blood serum BHBA levels increased in all groups drastically up to the third day of FR in early lactation above threshold values for subclinical ketosis of 1.2 - 1.4 mmol/L (LeBlanc, 2010), whereas during mid-lactation, no increases were observed.

Blood serum cholesterol showed only slight increase in MP-cows during FR in early lactation, whereas during mid-lactation levels were lower after refeeding in low protein cows. Drackley et al. (1992) showed that during first week of FR in early lactation and during realimentation, blood cholesterol concentrations increased slightly. Moreover, Gerloff et al. (1986) reported negative correlation of serum cholesterol with hepatic triglyceride content. Therefore, Mp-cows seemed to show higher susceptibility to hepatic steatosis even in mid-lactation.

In contrast to Reid et al. (1977), who found increasing serum bilirubin concentrations during fasting, the present study revealed no distinguishable patterns. Differences proved to be significant only during FR in early lactation in Mp-cows showing declining levels from first to second day of FR and in mp-cows, showing increasing levels during first day of FR and afterwards declining until refeeding.

However, results show large animal-to-animal variation in all measured blood metabolites regardless of group membership. Earlier, Baird et al. (1972) showed that starvation induced different compensatory modifications in individual cows.

Hepatic mRNA profile

Throughout both FRs, several significant changes of hepatic mRNA abundances occurred.

In all cows, abundance of mRNA encoding for enzymes of fatty acid oxidation was enhanced by FR in early lactation, whereas FR in mid-lactation resulted only in mild and variable increases. Moreover, high yielding cows showed higher levels of mRNA compared to mp-cows during both FRs. Patterns of elevation in translation and transcription regulating factors with influence on fatty acid oxidation could be observed similarly in all cows for *EIF4B* influence on *ECHS1* during both FRs as well as during FR in early lactation for *EIF4B* and *HNF4A* on *ACADVL* and *CPT1A* and for *PPARA* on *ACADVL* (Drackley, 1999; Loor et al., 2005; 2006). The only translation or transcription regulating factor differing between FRs was *HNF4A* with higher levels in Mp-cows during early lactation, suggesting enhanced gluconeogenesis and fatty acid oxidation (Loor et al., 2005).

In contrast to Loor et al. (2007), who reported down-regulation of *ACACA* associated with FR in high yielding cows, *ACACA* and also *GPAM* mRNA levels were decreased in all cows except of Mp-cows by FR in early lactation. During FR in mid-lactation, MP-cows showed decreased and Mp-cows increased mRNA levels. Levels of transcription regulation factor *SREBF1* decreased at least numerically during FR in early lactation in all cows, except of Mp-cows (Loor et al., 2005; 2006). In Mp-cows, higher levels of hepatic mRNA encoding for enzymes of fatty acid synthesis might suggest a higher risk of hepatic steatosis (Romics et al., 2004; Ueki et al., 2004).

Hepatic proteinases *TAT* and *CTSL* showed higher mRNA levels in all cows after FR in early lactation and *CTSL* mRNA was higher after FR in mid-lactation in all cows except of MP-cows. Therefore FR in early lactation resulted in enhanced amino acid catabolism, whereas during FR in mid-lactation only *CTSL* was induced, producing precursors for gluconeogenesis or ketogenesis.

Although *PPARA* and *HNF4A* regulate transcription of *PC* and *PCK1* mRNA (Loor et al., 2005; 2006), their expression patterns were not similar to those of *PC* and *PCK1*. Unlike FR in mid-lactation, FR in early lactation increased *PC* mRNA abundance. In contrast, *PCK1* abundance was elevated during FR in mid-lactation and highest in Mp- and mP-cows. Therefore, hepatic gluconeogenesis was enhanced by both FRs and Mp-cows showed highest *PC* levels in early lactation and highest *PCK1* levels in mid-lactation.

Transcript abundance of *SLC2A2* was not influenced by FR in early lactation, whereas during mid-lactation, Mp-cows showed higher and mp-cows lower abundances. Due to increased gluconeogenesis in Mp-cows during FR in mid-lactation, hepatic output and therefore expression of *SLC2A2* mRNA seemed to increase according to Zhao et al. (1993).

Both FRs altered mRNA abundance of *INSR*: during early lactation, Mp-cows showed higher levels and during mid-lactation, mp-cows showed reduced levels. Selection for milk yield results in low circulating insulin concentrations (Bonczek et al., 1988) and thus in higher hepatic *INSR* mRNA (Zhang et al., 2011). The opposite could happen during FR in mid-lactation in mp-cows. If they showed high insulin concentrations, which is supportable due to less milk output and restoring of body reserves, hepatic INSR abundance would have decreased.

Elevated levels of *HMGCS2* mRNA in Mp-cows during both FRs show clearly that also during mid-lactation, metabolic impairment can be simulated in high yielding cows. Also Hegardt et al. (1999) observed increased activity of *HMGCS2* in fasting rats, whereas van Dorland et al. (2009) and Graber et al. (2010) found no changes in *HMGCS2* expression in cows during early lactation.

Baird et al. (1972) found slight decreases of citrate synthase activity after starvation and Heitzman et al. (1972) found significant correlation between concentration and activity of citrate synthase.

Therefore, reduced *CS* mRNA levels in mP-cows after FR in mid-lactation might contribute to lower enzyme activity. Reasons for this phenomenon remain to be elucidated.

As previously shown, blood levels of IGF1 declined during FR (McGuire et al., 1992; Thissen et al., 1994). In the present study, similar results were observed after FR in early lactation: abundance of hepatic *IGF1* mRNA decreased numerically in all cows and differences proved to be significant in low yielding cows, whereas no effect of FR in mid-lactation could be observed.

Recapitulation

Feed restriction during early lactation exacerbated metabolic imbalance, especially in high yielding cows. Physiological adaptation seemed to be in part diminished in cows with high milk yield and low milk protein concentration (Mp-cows). Although FR during mid-lactation provoked only lighter changes in metabolic adaptation, also Mp-cows seemed to be more susceptible.

4. Intravenous glucose tolerance tests

Due to technical reasons, cows were only subjected to ivGTTs, although HECs are considered to be the gold standard (Holtenius et al., 2000). Nevertheless, previous studies showed that ivGTTs are suited to characterise insulin response and glucose metabolism in lactating cows (Cummins and Sartin, 1987; Stangassinger, 2006; Chagas et al., 2009; Grünberg et al., 2011). Body weight and therefore amount of infused glucose decreased in all cows from ivGTT before parturition to ivGTT 2 and in MP-cows also to ivGTT 3, reflecting firstly a loss of total weight due to parturition, but secondly also mobilisation of body reserves to maintain lactation (Grummer et al., 2004; Schröder and Staufenbiel, 2006).

Blood glucose and blood plasma insulin

Basal blood glucose was lower during ivGTT 2 (2.04 ± 0.06 mmol/L) in all cows due to glucose distribution to mammary gland and milk synthesis (Bell and Bauman, 1997). Basal blood glucose showed higher levels during ivGTT 2 in high protein cows (table 16), but milk yield in MP-cows was higher compared to mP-cows. This suggests better gluconeogenesis capacity in MP-cows, compared also to Mp-cows with similar milk yield but lower basal blood glucose at this day (Aschenbach et al., 2010; Loor, 2010). After infusion of glucose, all cows showed lower maximum blood glucose levels in ivGTT 2 compared to ivGTT 1 due to previously mentioned redirection of glucose to mammary gland. In ivGTT 2 compared to ivGTT 3, solely MP-cows showed higher maximum levels of blood glucose and also higher compared to low protein cows. This might lead to the conclusion that glucose withdrawal by mammary gland was lower compared to that of other cows due to lower expression of GLUT1. Also hepatic gluconeogenesis could exceed needs for milk production and therefore amounts of infused glucose elevated blood glucose more compared to other cows (Zhao and Keating, 2007; Graber et al., 2010). The latter is supported by the patterns of blood glucose increase: MP-cows showed higher increase of blood glucose and Mp-cows numerically higher increase during ivGTT 2 compared to low yielding cows.

Basal glucose levels were reached again earlier in low yielding cows during ivGTT 1 compared to other cows and later in high yielding cows compared to other time points. This suggests reduced insulin responsiveness to glucose or reduced insulin sensitivity in high yielding cows before parturition. Sano et al. (1993) reported that insulin responsiveness to glucose is higher in dry cows compared to lactating cows, whereas tissue responsiveness to insulin (insulin sensitivity) remains unchanged. Furthermore, steepest decrease of blood glucose was found in mp-cows during ivGTT 2, probably due to exceeding renal glucose threshold (Gould and Holman, 1993).

According to Blum et al. (1973) and Stangassinger (2011), basal blood plasma insulin levels were higher before parturition compared to levels during early and mid-lactation. Furthermore, insulin levels increase with energy balance of cows (Hart et al., 1979; Vasilatos and Wangsness, 1981), suggesting higher energy balance in mP-cows before parturition and in early lactation. Maximum as well as increase of blood plasma insulin (insulin response) were higher during ivGTT 1 in low yielding cows compared to ivGTT 2 and 3, similar to results of Sano et al. (1993), which also supports faster glucose clearance during ivGTT 1 in those cows at this time point. In contrast, insulin response proved not to be different between experimental days in high yielding cows. Moreover before parturition, insulin response in mP-cows was twice higher compared to Mp-cows due to one animal with exceptional high values (insulin increased by 1,709.7 µU/mL). Furthermore increase of blood plasma insulin was steeper, providing support that high yielding dairy cows experience considerable metabolic changes and also diminished tissue responsiveness to insulin prior to parturition (Prior and Christenson, 1978; Petterson et al., 1994; Drackley, 1999; Hayirli, 2006).

It is well known that AUC of insulin and glucose, representing bioavailability of respective metabolites, declines from before parturition to early lactation (Subiyatno et al., 1996; Stangassinger, 2006; Grünberg et al., 2011). Low and high yielding cows show similar AUC of insulin and glucose during early lactation (Chagas et al., 2009) and also in the present study. As observed before (Bell, 1995; Subiyatno et al., 1996), CR of glucose was higher during lactation due to high secretion of glucose as milk lactose, but differences between cows proved not to be significant.

Indices

Regarding the IR estimation models HOMA-IR and QUICKI, one has to keep in mind that they are derived from human analyses. The denominator in calculation of HOMA-IR derives from basal levels of blood glucose and plasma insulin in humans. In the current study the product would be 80.7 (2.68 mmol/L and 30.11 µU/mL, mean of all cows during ivGTT 1). According to Muniyappa et al. (2008) in healthy humans, HOMA-IR equals 1 and correlates positively to IR. With a denominator being 3.6 fold higher in cows at d 14 ap compared to healthy humans, HOMA-IR of 3.6 shows physiological IR in wk 2 ap and the other time points are compared to this time point. However MP- and mP-cows showed enhanced IR before parturition compared to low protein cows. In all cows, HOMA-IR decreased to ivGTT 2 and showed similar levels during ivGTT 3 in high protein cows. Nevertheless, in humans with severely impaired or absent pancreatic β-cell function, HOMA-IR provides no appropriate results (Muniyappa et al., 2008). However regarding QUICKI, similar patterns were observed: QUICKI was overall highest during ivGTT 2, suggesting decreased IR (Holtenius and Holtenius, 2007; Muniyappa et al., 2008). Moreover, high protein cows showed lower QUICKI during ivGTT 2 compared to other cows.

These results go in line with Hayirli (2006), Baird (1981), Sano et al. (1993) and others showing that reduced tissue sensitivity to insulin is a complex regulated physiological process to ensure nutrition of the offspring. It starts in late pregnancy due to insulin independent glucose distribution to uterus and foetus and leads further in early lactation to insulin independent mammary glucose uptake and lactose synthesis. Therefore it was expected that high yielding cows showed also highest IR (Chagas et al., 2009), whereas in the actual study highest IR was observed in high protein cows during ivGTT 1 and 2.

Recapitulation

The present study supports the well known facts of metabolism in dry and lactating cows. Furthermore, differences in cows grouped for milk yield and milk protein content can be observed: high yielding cows seemed to show gluconeogenesis activity meeting demands of milk synthesis

in early lactation and MP-cows seemed even to exceed the needs of mammary gland. Insulin sensitivity was reduced in high yielding dairy cows prior to parturition and mP-cows seemed to be in better energetic situation compared to other cows. Furthermore, no differences in bioavailability of glucose or insulin and glucose CR in early lactation were found amongst the cows.

During ivGTT before parturition and in early lactation, high protein cows showed highest IR, distributing most of glucose to foetus or mammary gland. Therefore during early lactation, cows with lower milk yield (mP-cows) show higher body tissue utilisation of glucose, which is according to Rose et al. (1997) mainly insulin-independent.

5. Milk protein fractions

For the first time, high reproducible and high throughput capillary electrophoresis was used to determine composition of milk protein during course of lactation.

Composition of milk protein during 22 weeks of lactation

Regarding the concentrations of individual milk proteins, the present study showed higher values of α- (34.3 compared to 19.6%) and β-CN (31.9 compared to 28.2%) compared to Bobe et al. (2007). This resulted in lower total concentrations of whey proteins (17.7%) and higher concentrations of total caseins (82.3%) compared to Mackle et al. (20.8 and 79.2%; 1999), whereas Bobe et al. (2009) observed lower whey protein concentrations (14.2%) and higher casein concentrations (85.8%). Partly these differences are due to the different determination methods (HPLC and PAGE) as seen for other proteins by Goetz et al. (2004).

Throughout the study, α-CN was higher in Mp-cows compared to low yielding cows. As was shown previously, high yielding dairy cows produce milk with higher concentrations of α_{S1}-CN (Bobe et al., 2007), which represents about 75% of α-CN (De Marchi et al., 2009; Demeter et al., 2010).

Concentrations of β-CN were lowest in mP-cows from wk 14 pp on. It is well known that cows with β-LG genotype BB show higher relative amounts of all caseins (Ng-Kwai-Hang et al., 1987; Heck et al., 2009). Nevertheless, composite β-LG genotype AB was found in all low yielding cows, whereas in 66% of MP-cows genotype AB and 34% genotype AA was determined. In 57% of Mp-cows genotype AA, in 29% genotype BB and in 14% genotype AB was observed. Therefore it was expected that high yielding cows had lower casein concentrations compared to low yielding cows. Regarding concentrations of κ-CN, this could be confirmed comparing concentrations of Mp- with those of mp-cows. Moreover all Mp-cows expressed κ-CN genotype AA, whereas in mp-cows 20% genotype BB and 20% genotype AB were observed. Additionally, κ-CN genotype BB is associated with higher concentrations of α- and κ-CN, resulting in higher curd yield and firmness (Ng-Kwai-Hang et al., 1987; Heck et al., 2009).

Further it has to be taken into account that concentrations of whey proteins increase during inflammations of mammary gland due to increased permeability of blood mammary barrier (Hogarth et al., 2004) and milk SCC is positive correlated to concentrations of α-LA, β-LG and α-CN (Ng-Kwai-Hang et al., 1987). This could explain that concentrations of α-LA and β-LG and also SCC were higher in mP-cows during period 6.

Concentrations of unidentified proteins reflect all proteins detectable in capillary electrophoresis except of above mentioned major proteins (e.g. BSA, IgG; Anema and Lloyd, 1999), but also proteolysis products developed by endogenous proteases like plasmin (Eigel et al., 1979; Reimerdes, 1983) or by microbial proteolytic activity (Haddadi et al., 2005). Furthermore, elevated SCC is associated with higher proteolysis in milk (Wedholm et al., 2008). Due to higher levels of

unidentified proteins in mP-cows during wk 14 pp and lower levels of β-LG and β-CN, these cows seemed to show higher proteolytic activity of β-LG and β-CN in milk. Similar results can be observed during wk 22 pp regarding concentrations of κ-CN and unidentified proteins in mP-cows.

At time points of biopsies (muscle biopsy d 43 and 113 pp, liver biopsy d 57 pp), concentrations of whey proteins and κ-CN declined, whereas β-CN content increased and α-CN stayed on similar levels. Although tissue of cows was anesthetised, the biopsy procedure seemed to be a stressor for cows. Acute stress situations lead to increased values of adrenaline and noradrenalin and those diminish milk yield and alter nutritive blood flow to the mammary gland (Linzell, 1960; Prosser et al., 1996).

Influence of FR on milk protein composition

Although Auldist et al. (2000) found significant changes of all individual milk proteins during restricted pasture allowance in dairy cows having both β-LG genotype AA and BB. Bobe et al. (2009) found slightly decreased whey protein concentrations with declining α-LA and increasing β-LG and constant casein concentrations with slightly increased α_{S1}-CN and decreased α_{S2}-, β- and κ-CN concentrations. In the present study, no obvious differences amongst cows were observed in concentrations of individual proteins during both FRs. During FR in mid-lactation, whey proteins increased in mp-cows, probably due to elevated SCC at d 146 pp in those cows. Furthermore levels of α-CN were higher and levels of κ-CN lower in Mp-cows compared to low yielding cows, as seen during lactation.

Regarding mean concentrations of individual milk proteins (table 19), levels of whey proteins and β-CN were higher and those of κ-CN were lower in FR during early lactation. Interestingly, restricted feeding influenced solely economically valuable κ-CN during mid-lactation, resulting in a decrease which was compensated after the three days of realimentation.

Recapitulation

Over the first 22 weeks of lactation, differences in concentrations of individual milk proteins could be observed in an expected manner. Furthermore the cows showed no obvious pattern of changed protein concentrations after a short term FR, neither during early lactation nor during mid-lactation. Solely after FR in mid-lactation, mean concentration of κ-CN decreased. Therefore composition of major milk proteins seems to be influenced mainly by individual factors like genotype and health and is rather stable.

VI. CONCLUSION

For the first time, two easy conductible and reproducible analytical methods were used: an EIA for hydrocortisone in skim milk and determination of milk protein composition during the course of lactation by capillary electrophoresis on a chip. Furthermore, cows grouped for FCM and milk protein concentration were characterised in metabolic and productive parameters.

Regarding the presented results, it can be concluded that high yielding cows were in severest metabolic stress during early lactation, showed higher risk for fatty liver development and seemed to have diminished gluconeogenesis potential. In Mp-cows, these impairments were most obvious. Also an enhancement of the metabolic imbalance in early lactation by feed restrictions affected the high yielding cows and especially those with low milk protein concentration in the severest manner. Fertility of MP-cows seemed to be diminished. These results indicate that MP-cows are less susceptible to metabolic imbalances compared to those with low milk protein content (Mp-cows).

Furthermore mP-cows showed higher insulin resistance prior to parturition compared to MP-cows. Therefore mP-cows showed higher utilisation of glucose in body tissues during early lactation. Moreover, these cows lost more body fat reserves compared to other cows and solely these cows gained body weight and back-fat-thickness in mid-lactation. Regarding percentage amount of milk constituents in total energy output during experimental period, MP-cows showed highest values accounting for milk fat (57.9 ± 0.7%) and mP-cows highest amounts accounting for milk protein yields (19.4 ± 0.3%; table 20). Even though protein yield of mP-cows tended to be lower compared to MP-cows (P = 0.06), this shift in nutrient distribution results in higher nutrient density of protein in mP-cows (10.8 ± 0.2 g/MJ).

In summary, it can be said that a possible way to reduce metabolic stress of high yielding dairy cows in early lactation, besides optimised feeding regime, could be selection of cows and bulls inheriting higher milk protein concentration and lower milk yield. This results in similar yields of milk protein, which is the most rewarded output of dairy cows, and concurrently improves health status of dairy cows in early lactation, a common aim of farmers and veterinarians.

Table 20: Nutrient density of milk constituents during experimental period.

		MP	mp	Mp	mP
milk constituent	**kJ/g**	**total yield, kg**			
protein	18.0	167.2 ± 6.2^{ac}	143.9 ± 10.2^{bc}	170.9 ± 3.9^{a}	148.5 ± 4.9^{c}
fat	39.7	248.3 ± 9.6^{a}	192.2 ± 14.1^{b}	232.2 ± 6.9^{a}	193.1 ± 8.8^{b}
carbohydrates	17.6	236.7 ± 9.9^{a}	215.7 ± 13.4^{b}	264.0 ± 7.9^{a}	195.7 ± 6.8^{b}
		total energy output, GJ			
protein		3.0 ± 0.1^{ac}	2.6 ± 0.2^{bc}	3.1 ± 0.1^{a}	2.7 ± 0.1^{c}
fat		9.9 ± 0.4^{a}	7.6 ± 0.6^{b}	9.2 ± 0.3^{a}	7.7 ± 0.3^{b}
carbohydrates		4.2 ± 0.2^{a}	3.8 ± 0.2^{ac}	4.6 ± 0.1^{b}	3.4 ± 0.1^{c}
		total energy output, %			
protein		17.7 ± 0.5^{a}	18.5 ± 0.2^{ab}	18.2 ± 0.3^{a}	19.4 ± 0.3^{b}
fat		57.9 ± 0.7^{a}	54.4 ± 0.6^{b}	54.4 ± 1.0^{b}	55.6 ± 0.9^{ab}
carbohydrates		24.4 ± 0.4^{a}	27.1 ± 0.4^{b}	27.4 ± 0.6^{b}	25.0 ± 0.8^{a}
		nutrient density, g/MJ			
protein		9.8 ± 0.3^{a}	10.3 ± 0.1^{ab}	10.1 ± 0.2^{a}	10.8 ± 0.2^{b}
fat		14.6 ± 0.2^{a}	13.7 ± 0.1^{b}	13.7 ± 0.2^{b}	14.0 ± 0.2^{ab}
carbohydrates		13.9 ± 0.2^{a}	15.4 ± 0.2^{b}	15.6 ± 0.4^{b}	14.2 ± 0.4^{a}

^{abc}Alphabetic superscripts indicate differences between groups ($P < 0.05$).

VII. SUMMARY

Metabolic and productive characterisation of multiparous cows grouped for fat-corrected milk yield and milk protein concentration

Objective of this study was to determine health and productivity of cows grouped for fat corrected milk yield and protein concentration. Therefore 23 cows (6 cows with high FCM and high protein content, 5 with low FCM and low protein, 7 with high FCM and low protein and 5 cows with low FCM and high protein concentration) were examined from two weeks before parturition throughout the first 22 weeks of lactation. Regularly, milk and blood samples were collected and body condition observed. Cows were subjected to 30% feed restriction in early (d 26 to 28 pp) and mid-lactation (d 141 to 143 pp) to evaluate metabolic reaction. Three intravenous glucose tolerance tests (ivGTT; d -14, 20 and 127 pp) were conducted to assess differences in insulin response. Furthermore hepatic gene expression profiles were examined at day of parturition, d 15, 57 and 155 pp as well as at the last day of both feed restriction periods. Measured target genes (20) play key roles in glucose transport, lipid, protein and carbohydrate metabolism as well as in ketogenesis.

Milk, blood serum and body condition parameters revealed highest risk for metabolic imbalances of high yielding dairy cows during early lactation. Gene expression profiles indicated that high yielding and especially cows with high FCM and low protein concentration had higher risk for fatty liver development and diminished gluconeogenesis potential. Also, during feed restriction in early lactation, these cows seemed to have reduced physiological adaptation capacities to the exacerbated energetic imbalance. Nevertheless, results of ivGTTs suggest that high yielding cows show gluconeogenesis activity meeting demands of milk synthesis in early lactation and cows with high FCM and protein concentration even seemed to exceed the needs of mammary gland. Insulin sensitivity was reduced in high yielding dairy cows prior to parturition.

Furthermore only cows with low FCM and high protein content were able to restore body reserves in mid-lactation. Regarding results of hepatic mRNA abundance and ivGTTs, those cows seemed to be in an improved energetic situation compared to other cows. Moreover, high protein cows showed highest insulin resistance in early lactation. Property of cows with low FCM and low protein concentration was a difference in metabolism due to seemingly enhanced protein catabolism instead of lipolysis.

Additionally, protein composition of milk was analysed throughout experimental period with capillary electrophoresis on a chip (Agilent Protein 80 Chip for Bioanalyzer). Cows grouped for FCM yield and protein concentration showed differences in concentrations of major milk proteins α-lactalbumin, β-lactoglobulin, α-, β- and κ-casein, which could be explained by their different genotypes of β-LG and κ-CN: high yielding cows showed lower casein concentrations compared to low yielding cows. Furthermore, low yielding cows had lower contents of α-CN.

Composition of milk protein was almost not altered by restricted feeding. Solely restricted feeding in mid-lactation resulted in decreased mean contents of κ-CN and therefore potentially diminished processing quality of milk for cheese making.

VIII. ZUSAMMENFASSUNG

Metabolische und produktive Charakterisierung von mehrkalbigen Kühen gruppiert anhand fettkorrigierter Milchmenge und Milchproteinkonzentration

Ziel dieser Studie war die Beschreibung von metabolischer Anpassungsfähigkeit und Produktivität von Kühen, die nach fettkorrigierter Milchmenge und Proteingehalt der Milch eingeteilt wurden. Darum wurden 23 Kühe (sechs davon mit hohem FCM und hohem Milchproteingehalt, fünf mit niedrigem FCM und niedrigem Proteingehalt, sieben mit hohem FCM und niedrigem Proteingehalt und fünf Tiere mit niedrigen FCM und hohem Proteingehalt der Milch) im Zeitraum von zwei Wochen vor der Abkalbung bis zur 22. Woche der Laktation untersucht. Regelmäßig wurden Blut- und Milchproben gesammelt und die Körperkondition überwacht. Die Kühe wurden einer 30%igen Futterrestriktion in der frühen (Tag 26 bis 28 der Laktation) und der mittleren Laktation (Tag 141 bis 143 der Laktation) unterzogen, um die metabolische Reaktion zu untersuchen. Drei intravenöse Glukose Toleranz Tests (ivGTT, Tag -14, 20 und 127 der Laktation) wurden durchgeführt, um Unterschiede in der Insulinantwort zu beleuchten. Des Weiteren wurden Genexpressionsprofile der Leber erstellt am Tag der Kalbung, Tag 15, 57 und 155 der Laktation sowie am letzten Tag beider Futterrestriktionen. Die untersuchten Zielgene (20) spielen Schlüsselrollen im Glukosetransport, Fett-, Protein- und Kohlenhydratstoffwechsel sowie in der Ketogenese.

Milch, Blutserum und Körperkonditionsparameter der hochleistenden Tiere deuteten auf ein erhöhtes Risiko an metabolischer Imbalance während der frühen Laktation hin. Genexpressionsprofile zeigten, dass hochleistende und vor allem die Kühe mit hohem FCM und niedrigem Proteingehalt ein höheres Risiko hatten, an Fettleber zu erkranken, und ein verringertes Glukoneogenese Potential aufwiesen. Während der Futterrestriktion in der Frühlaktation schienen diese Kühe reduzierte physiologische Anpassungsfähigkeiten an die gesteigerte energetische Belastung zu zeigen. Nichtsdestotrotz lassen die Ergebnisse der ivGTTs darauf schließen, dass hochleistende Kühe Glukoneogenese Aktivität zeigen, welche den Anforderungen an die Milchsynthese in der Frühlaktation genügt. Bei Kühen mit hohem FCM und hohem Milchproteingehalt schien die Glukoneogenese Kapazität diese Ansprüche sogar zu übertreffen. Insulin Sensitivität war in hochleistenden Kühen vor der Kalbung reduziert.

Des Weiteren waren nur Kühe mit niedrigem FCM und hohem Proteingehalt in der Lage, Körperreserven in der mittleren Laktation wieder aufzubauen. Verglichen mit den anderen Kühen schienen sich diese Tiere auch hinsichtlich der Genexpressionsprofile und der ivGTTs in einer besseren energetischen Situation zu befinden. Ferner zeigten die hoch-Protein-Kühe die höchste Insulinresistenz in der frühen Laktation. Eigenheit der Tiere mit niedrigem FCM und Proteingehalt war ein Unterschied im Stoffwechsel aufgrund des scheinbar verstärkten Proteinabbaus anstatt der Lipolyse.

Zusätzlich wurde die Proteinzusammensetzung der Milch während des Versuchs mithilfe der Kapillarelektrophorese auf einem Chip gemessen (Agilent Protein 80 Chip für den Bioanalyzer). Nach FCM und Proteingehalt gruppierte Kühe zeigten Unterschiede in den Hauptmilchproteinfraktionen α-Laktalbumin, β-Laktoglobulin, α-, β- und κ-Casein. Diese können mit den verschiedenen Genotypen der Tiere hinsichtlich β-LG und κ-CN erklärt werden. Hochleistende Kühe zeigten geringere Konzentrationen an Caseinen verglichen mit den niedrigleistenden Kühen. Des Weiteren hatten niedrigleistende Tiere niedrigere Konzentrationen von α-CN.

Die Zusammensetzung des Milchproteins änderte sich fast nicht durch die restriktive Fütterung. Nur die Futterrestriktion in der Mitte der Laktation resultierte in gesunkenen κ-CN Konzentrationen, die wiederum zu verringerter Prozesssierungsqualität im Käsereiprozess führen kann.

IX. REFERENCE LIST

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