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Transcript regulation in primary bovine mammary epithelial cells purified from milk and effects of restricted feeding on mammary protein biosynthesis pathways, hepatic regulation, milk yield and composition in dairy cows selected for different milk protein content

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ABBREVIATIONS

AA = amino acid
ACACA = acetyl-CoA carboxylase α
ACADvl = acyl-CoA dehydrogenase, very long chain
ACTB = actin β
ad lib = ad libitum
AKT1 = v-akt murine thymoma viral oncogene homolog 1
BCS = body condition score
BE = primary magnetic bead-isolated mammary epithelial cell
BHBA = β-hydroxybutyric acid
BMEC = bovine mammary epithelial cells
bp = base pairs
BSA = bovine serum albumine
BW = body weight
c = *cis*
cDNA = complementary deoxyribonucleic acid
CE = mammary epithelial cell harvested from cell culture
CEBPB = CCAAT/enhancer binding protein beta
CLA = conjugated linoleic acid
CN = casein
CPT1A = carnitine palmitoyltransferase 1A
Cq = quantitative cycle
CSN1S1 = α_{S1}-casein
CSN1S2 = α_{S2}-casein
CSN2 = β-casein
CSN3 = κ-casein
CTSL = cathepsin L
CV = coefficient of variation
DIM = days in milk
DM = dry matter
DMI = dry matter intake
EAA = essential amino acid
EB = energy balance
ECHS1 = enoyl CoA hydratase
EIF4B = eukaryotic translation initation factor 4B
EIF4EBP1 = eukaryotic translation initiation factor 4E binding protein 1
ELF5 = E74-like factor 5
FPR = fat-protein ratio
FR = feed restriction
GAPD = glyceraldehyde 3-phosphate dehydrogenase
GH = growth hormone
GPAM = glycerol-3-phosphate acyltransferase
GR = glucocorticoid receptor
H3F3A = H3 histone family 3A
HMGCS2 = 3-hydroxy-3-methylglutary-coenzyme A synthase 2
HNF4A = hepatocyte nuclear factor-4A
HP = cow with high milk protein content
IGF1 = insulin-like growth factor 1
IGF1R = insulin-like growth factor 1 receptor
IgG = immunoglobulin G
INSR = insulin receptor

JAK2 = janus kinase 2
KRT8 = keratin 8
LA = lactalbumin
LALBA = α-lactalbumin
LD = lactational diet
LG = lactoglobulin
LP = cow with low milk protein content
LSM = least squares means
ME = metabolized energy
MEC = mammary epithelial cell
MJ_{NEL} = mega joule net energy lactation
MP = cow with high milk yield and high milk protein content
Mp = cow with high milk yield and low milk protein content
mP = cow with low milk yield and high milk protein content
mp = cow with low milk yield and low milk protein content
mRNA = messenger ribonucleic acid
mTOR = mammalian target of rapamycin
NEB = negative energy balance
NEFA = non-esterified fatty acids
NR3C1 = nuclear receptor subfamily 3, group C, member 1, known as glucocorticoid receptor
OAT = ornithine δ-aminotransferase
PAEP = progestagen-associated endometrial protein, known as β-lactoglobulin
pbMEC = primary bovine mammary epithelial cell
PBS = phophate buffered saline
PC = pyruvate carboxylase
PCK1 = phosphoenolpyruvate carboxykinase, cytosolic
PCK2 = phosphoenolpyruvate carboxykinase, mitochondrial
PCR = polymerase chain reaction
pp = post partum
PPARA = peroxisome proliferator activated receptor-α
PPARG = peroxisome proliferator activated receptor-γ
qPCR = quantitative polymerase chain reaction
R = correlation coefficient
RD = restricted diet
RNA = ribonucleic acid
RPS6KB1 = ribosomal protein S6 kinase
RPS9 = ribosomal protein 9
RUNX2 = runt-related transcription factor 2
SAS = Statistical Analysis System
SCC = somatic cell count
SEM = standard error of mean
SLC2A1 = solute carrier family 2, member 1
SLC2A2 = facilitated glucose transporter, member 2
SLC5A1 = solute carrier family 5, member 1
SOCS2 = suppressor of cytokine signaling 2

SREBF1 = sterol regulatory element binding transcription factor 1

STAT5A = signal transducer and activator of transcription 5A

t = *trans*

TAT = tyrosine aminotransferase

TNFA = tumor necrosis factor α

UBB = polyubiquitin

YY1 = yin yang 1

ABSTRACT

The objective of the study was to investigate the milk protein biosynthesis in primary bovine mammary epithelial cells (pBMEC) of dairy cows with different milk protein content. For this purpose, a method for isolating pBMEC from raw milk was modified and applied. An indirect immunomagnetic bead based method was appropriate to isolate desquamated pBMEC directly from 1.8 L raw milk for further quantitative polymerase chain reaction (qPCR) analysis. The percentage of shed pBMEC in relation to somatic milk cells was highly correlated to milk yield. Furthermore, cell cultures with pBMEC from milk were performed and expression profiles of several genes were compared between pBMEC from raw milk and pBMEC harvested from cell culture. Finally, varying physiological and metabolic ambiance in cell culture demanded a high range of morphological and functional adjustment strategies from the pBMEC and lead to different expression profiles in pBMEC harvested from cell culture compared to pBMEC from raw milk. Due to this, further studies concerning milk protein biosynthesis were performed with immunomagnetic isolated pBMEC from raw milk.

To investigate differences and changes in milk protein content during lactation cycle, 26 multiparous Holstein Friesian cows from a dairy farm in Saxony (Germany, 800 dairy cows) were selected for different milk protein content and transferred to the Versuchsstation Veitshof in Freising (Bavaria, Germany) during their dry-off period. With the onset of lactation, cows were sampled for milk composition, metabolic performance (blood serum haemogram, gene expression in liver and skeletal muscle) and body condition (body weight, body condition score, backfat thickness) during the first 155 days of lactation. Additionally, the effect of a 3-day feed restriction (FR; -30% of previously ingested dry matter) on metabolic situation, milk production and composition, and on hepatic key performance indicators during early lactation was determined. Restricted feed intake resulted in decreased blood serum glucose concentrations, increased non-esterified fatty acid (NEFA) and β-hydroxybutyric acid (BHBA) levels in blood serum, calculated negative energy balance (NEB) and was accompanied by milk yield depression. Additionally, poorer metabolic status was reflected by marked changes of transcript abundance of regulating factors of lipid and protein metabolism, gluconeogenesis and ketogenesis, for example, increased levels of transcripts for carnitine palmitoyltransferase 1A (CPT1A) and acyl-CoA dehydrogenase, very long chain (ACADVL), cathepsin L (CTSL),

phosphoenolpyruvate carboxykinase, cytosolic (PCK1) and mitochondrial (PCK2) and 3-hydroxy-3-methylglutary-coenzyme A synthase 2 (HMGCS2). However, in cows with high milk yield and low milk protein content (Mp), physiological adaptation seemed to be in part diminished by the enormous metabolic effort for milk synthesis.

Moreover, to study mammary protein biosynthesis pathways, pBMEC were extracted from 10 morning milk samples from each experimental cow. Purified pBMEC from raw milk were used for qPCR analysis. Transcripts of all six major milk protein genes were found to peak during the first two weeks of lactation and to decline continuously towards mid lactation. In addition, transcript abundances encoding for E74-like factor 5 (ELF5) decreased with increased day of lactation and might explain the decrease of all major milk protein gene expression observed during the first half of lactation. Especially after FR, a simultaneous increase of messenger ribonucleic acid (mRNA) levels for ELF5 and of all milk protein genes was determined. Considering the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, amino acid transfer and glucose transporter and the β-casein promoters, an overall increase in transcript abundances could be observed during mid lactation. This might explain the maintenance of relative proportions of the different caseins and whey proteins in milk during lactation despite the decrease in their expression and possibly the increasing milk protein content during mid lactation.

In conclusion, the immunomagnetic bead based method was appropriate to isolate pBMEC directly from raw milk for further qPCR assays. Transcripts of the six milk protein genes were found to be similar in dairy cows selected for different milk protein content, but levels of transcripts for solute carrier family 2, member 1 (SLC2A1) were higher in cows with high milk protein content compared to cows with low milk protein content. Our results showed that short-time FR in early lactation succeeded in enhancing energy deficit of cows with different milk protein content. Therefore, physiological adaptation to a metabolic challenge seemed to be in part reduced in Mp-cows. Furthermore, our findings suggest a pivotal role of the transcription factor ELF5 for milk protein mRNA expression and support the central role of SLC2A1 and the mammalian target of rapamycin (mTOR) and JAK/STAT pathway for the regulation of protein biosynthesis in the bovine mammary gland.

ZUSAMMENFASSUNG

Ziel der Studie war es, die Milcheiweißbiosynthese in pBMEC von Milchkühen mit unterschiedlichem Milcheiweißgehalt zu untersuchen. Für diese Untersuchungen wurde eine Methode zur Isolierung von pBMEC aus Rohmilch verändert und angewandt. Die indirekte immunomagnetische auf Kügelchen basierte Methode erwies sich als geeignet abgeschilferte pBMEC direkt aus 1,8 Liter Rohmilch zu extrahieren und die pBMEC für weitere qPCR Studien zu verwenden. Der Anteil an abgeschilferten pBMEC an den gesamt-somatischen Zellen korrelierte mit der Milchmenge. Des Weiteren wurden pBMEC aus Rohmilch in Zellkultur angezüchtet und die Expressionsprofile einiger Gene in den pBMEC aus Milch und in denen aus Kultur gewonnen Zellen verglichen. Schlussendlich erforderte die veränderte physiologische und metabolische Umgebung in der Zellkultur einen hohen Grad an morphologischen und funktionellen Anpassungsstrategien der pBMEC und führte zu unterschiedlichen Expressionsprofilen in den pBMEC aus Milch und in denen aus Kultur gewonnenen pBMEC. Folglich wurden weitere Studien bezüglich der Milcheiweißbiosynthese mit den aus Rohmilch immunomagnetisch isolierten pBMEC durchgeführt.

Um Unterschiede und Veränderungen im Milcheiweißgehalt während der Laktationsphase zu untersuchen, wurden 26 mehrkalbige Kühe der Rasse Holstein Friesian an Hand ihres Milcheiweißgehaltes ausgewählt und während ihrer Trockenstehphase von einer 800er Milchviehanlagein Sachsen auf die Versuchsstation Veitshof in Freising (Bayern, Deutschland) gebracht. Beginnend mit dem Einsetzen der Laktation wurde während der ersten 155 Laktationstage die Milchzusammensetzung der Tiere analysiert, die Stoffwechselsituation (Blutbild, Genexpression in Leber- und Skelettmuskelgewebe) und die Körperkondition (Körpergewicht, Körperkonditionsbewertung, Rückenfettdickemessung) erfasst. Zusätzlich wurden die Auswirkungen einer dreitägigen Futterrestriktion (-30 % der vorher aufgenommenen Trockenmasse) während der Frühlaktation auf die metabolische Situation, die Milchproduktion und -zusammensetzung sowie auf die zentralen Leistungsindikatoren in der Leber untersucht. Hierzu wurden die Versuchskühe an Hand ihrer Milchleistung und ihres Milcheiweißgehaltes in vier Gruppen eingeteilt. Die restriktive Fütterung während der Frühlaktation bewirkte eine negative Energiebilanz, einen abfallenden Blutglukosespiegel und ansteigende Konzentrationen an nicht-veresterten Fettsäuren und β-Hydroxybuttersäure im

Blutserum sowie einen Rückgang der Milchleistung. Außerdem spiegelte sich der schlechte metabolische Zustand in einer markanten Veränderung der Transkripte der regulierenden Faktoren des Fett- und Eiweißstoffwechsels sowie der Glukoneo- und Ketogenese wider. Indes schien die physiologische Anpassung der Kühe mit einer hohen Milchleistung bei einem gleichzeitig geringen Milcheiweißgehalt teilweise verringert zu sein, wohl ausgelöst durch den enormen metabolischen Aufwand für die Milchsynthese.

Fernerhin wurden für die molekularbiologischen Untersuchungen der Milcheiweißsynthese von jeder Versuchskuh zehn Milchproben vom Morgengemelk während der ersten Laktationshälfte genommen. Die aus der Rohmilch extrahierten pBMEC wurden für qPCR Studien verwendet. Die Transkripte aller sechs majoren Milcheiweißgene erreichten ihren Höchstwert in der zweiten Laktationswoche und fielen kontinuierlich bis zur Mittlaktation ab. Weiterhin sanken die Transkripte für ELF5 mit zunehmendem Laktationstag und sind möglicherweise eine Erklärung für den beobachteten Abfall der Transkripte der Milcheiweißgene während der ersten Laktationshälfte. Besonders nach der Futterrestriktion wurde ein synchroner Anstieg der Transkripte für ELF5 und der Milcheiweißgene gemessen. Weiterhin wurde ein Anstieg der Transkripte des JAK/STAT Pfads, des Aminosäurentransfers und des Glukosetransportes sowie der β-Kasein-Promoter bis zur Mittlaktation gemessen. Dies könnte die Aufrechterhaltung der relativen Verhältnisse der verschiedenen Kaseine und Molkenproteine, unabhängig von dem Abfall ihrer Transkripte, in der Milch während der Laktation erklären und somit auch der Grund für den ansteigenden Milcheiweißgehalt während der Mittlaktation sein.

Zusammenfassend lässt sich festhalten, dass die immunomagnetische auf Kügelchen basierte Methode geeignet war um pBMEC aus Rohmilch zu isolieren und diese für weitere qPCR Studien zu verwenden. Die Transkripte der sechs Milchproteingene waren in den Milchkühen mit unterschiedlichem Eiweißgehalt vergleichbar, wobei die Transkripte für SLC2A1 in den Kühen mit hohem Milcheiweißgehalt verglichen mit den Kühen mit niedrigem Milcheiweißgehalt höher waren. Die Ergebnisse zeigten dass die kurzzeitige Futterrestriktion während der Frühlaktation in ein gesteigertes Energiedefizit der Kühe mit unterschiedlichem Milcheiweißgehalt resultierte. Weiterhin schien die physiologische Anpassung der Mp-Kühe auf die metabolische Herausforderung teilweise vermindert gewesen zu sein. Des Weiteren deuten die Ergebnisse auf die grundlegende Rolle des

Transkriptionsfaktors ELF5 für die Expression der Milcheiweiß-mRNA hin und verdeutlichen die zentrale Rolle von SLC2A1, dem mTOR- und JAK/STAT-Pfad bei der Regulation der Eiweißbiosynthese in der bovinen Milchdrüse.

1 INTRODUCTION

1.1 Functionality of primary bovine mammary epithelial cells (pBMEC)

The mammary gland is made up of the secreting tissue, the tubulo-alveolar epithelium, and a variety of support tissues, like adipose tissue and blood and lymph vessels. The milk is synthesized in the pBMEC and bovine milk contains between 2.5 and 3.7% of protein, 3.5 and 5.0% of fat and 4.7 and 5.0% of lactose as the major milk components (Cerbulis and Farrell, 1975). During the last decades, it was a main approach in dairy science to manipulate milk composition whereas milk protein content has received less attention.

The milk protein content is influenced by breeding, nutrition and management factors. Breeding with bulls with over-average heredity for milk protein or crossing with breeds noted for high milk protein content, such as Brown Swiss (Cerbulis and Farrell, 1975) may enhance milk protein yield in the dairy cow. Besides genetic improvement, feeding regime can elevate protein yield in individual herds. One approach is to increase the amount of amino acids (AA) in small intestine, and therefore the uptake in blood, by elevation the amount of rumen-undegradable protein in lactational diets, e.g. by feeding fish meal (Santos et al., 1998). Additionally, it is essential to stabilize the microbiological flora in rumen by adequate fractions of roughage (Jouany, 1994; Pop et al., 2001). Within physiological limits, dairy cows are also able to compensate insufficient supply of AA by mobilization of body reserves (Botts et al., 1979). Furthermore, conjugated linoleic acid (CLA) supplementation is known for milk fat depression and simultaneously in some cases responsible for the increase of milk protein content. Previous studies reported increased milk protein content after abomasal infusion of *trans*(*t*)10,*cis*(*c*)12-CLA (Baumgard et al., 2002; Bell and Kennelly, 2003) whereas in an own previous study no effects of feeding *t*10,*c*12-CLA on milk protein and either milk fat content were measured in primiparous cows during early lactation (Sigl et al., 2010). Moreover, management regimes, like milking without drying-off or once daily milking were reported as useful tools to produce milk with higher milk protein content. Patton et al. (2006) demonstrated higher milk protein and fat concentration during the first 28 d of lactation for cows milked once daily compared to cows milked thrice daily. In addition, Schlamberger et al. (2010) reported higher milk protein content in continuously milked cows compared to cows with a traditional dry period of 56 d.

However, daily produced amount and composition of milk is even more influenced by the number of mammary secretory cells and their secretory activity (Boutinaud et al., 2004). During the milking process, some pBMEC detach from the alveolar epithelium and are continuously shed into milk during the entire lactational period comprising approximately 2% of total somatic cells (Boutinaud and Jammes, 2002; Figure 1). After peak lactation, numbers of pBMEC decline gradually by 8% between d 90 and 240 of lactation accounting for lower milk yields at the end of lactation (Capuco et al., 2003).

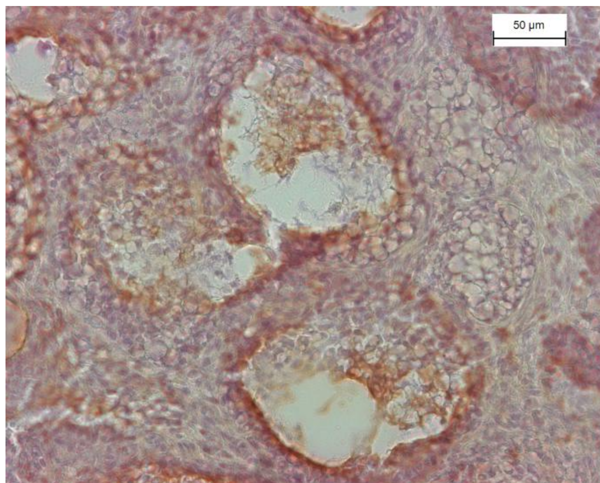

Figure 1. Cryosection through a lactating bovine mammary gland. Cells are counterstained in blue and cytokeratin filaments stained in brown. Brown areas show mammary alveolar epithelial cells around alveolar lumen. Source: Own illustration

To study the cellular mechanism responsible for milk constituents synthesis, especially protein synthesis, and to better understand these molecular events, pBMEC samples need to be harvested either by invasive mammary gland biopsies or by purification from milk. In numerous previous studies, mammary gland tissue was obtained by biopsies (Farr et al., 1996; Finucane et al., 2007) or after slaughter (Capuco et al., 1997; Capuco et al., 2001; Colitti et al., 2010) and used for RNA extraction and PCR analysis. Another possibility to obtain pMEC is to culture cells from mammary tissue after biopsy or slaughter (Talhouk et al., 1990; Rabot et al., 2007; Griesbeck-Zilch et al., 2008; Stiening et al., 2008) or directly from milk (Buehring 1990). These sampling methods often resulted in samples that include a large fraction of other non-MEC, like fibroblasts and adipocytes. Unfavorable, slaughtering implies the bereavement of precious experimental animals and allows sampling only at one point of time. Earlier studies reported isolation of pBMEC from mammary gland tissue using immunomagnetic separation in order to avoid receiving non-MEC (Gomm et al., 1995). Recent studies established even lactating MEC

culture models to study milk and milk protein synthesis (Hu et al., 2009; Zhao et al., 2010). Boutinaud et al. (2008) refined the isolation of viable pBMEC directly from fresh milk. Therefore, gene expression studies in pBMEC are frequently repeatable during lactation cycle but are not feasible during mammogenesis and involution.

1.2 Composition and structural organization of bovine milk protein

The milk proteins that are synthesized in the bovine mammary gland are composed of AA either derived from the blood stream or from the AA synthesized by the pBMEC. There are two fractions of milk proteins synthesized in the pBMEC, comprising 95% of the total protein, namely the caseins (CN; 80%) and whey proteins (20%). The four CN were previously been classified into α_{S1}, α_{S2}, β and κ, and the two major whey proteins into α-lactalbumin (LA) and β-lactoglobulin (LG; Threadgill and Womack, 1990).

Milk protein fractions vary considerably depending on stage of lactation, age and health status of the cow as well as on nutritional regimen and season (Ng-Kwai-Hang et al., 1987). Particularly, a deficit in energy supply might be detrimental for protein synthesis and even casein proportion of total milk protein (Reichardt et al., 1995). A decreased casein proportion leads to lower cheese yield (Melilli et al., 2002) and may also modify the processes of coagulation and cheese ripening. Caroli et al. (2009) emphasized in their review the major effect of milk protein on cheese yield and quality. As a result, milk protein content varies during course of lactation and therefore stage of lactation has an influence on cheesemaking properties. Waite et al. (1956) reported increasing age of cow correlated with poorer quality of milk, especially in lower amounts of casein. Moreover, diseases of the mammary gland, like mastitis, cause a decrease of casein content and modify the sensorial quality of cheeses (Munro et al., 1984). Furthermore, casein and its fractions can be affected by environmental factors such as season and ambient temperature (Kroeker et al., 1985; Lacroix et al., 1994). Although genetic variants of milk protein have no influence on milk yield, milk fat and protein content, different casein genotypes are known to affect casein concentrations. Consequently, typing of different protein variants as well as knowledge about the regulation of expression of the different milk protein genes during lactation is crucial for the genetic improvement of milk composition and milk yield (Groenen and van der Poel, 1994).

The genes encoding for the milk proteins are α_{S1}-, α_{S2}-, β- and κ-CN (CSN1S1, CSN1S2, CSN2 and CSN3, respectively) and α-LA (LALBA) and β-LG (progestagen-associated endometrial protein, PAEP). The four casein genes are tightly linked in a 250-kb cluster (Ferretti et al., 1990; Threadgill and Womack, 1990) and mapped on chromosome 6 (Hayes et al., 1993; Popescu et al., 1996), whereas the two main whey protein genes, LALBA and PAEP, are mapped on chromosome 5 (Hayes et al., 1993) and 11 (Hayes and Petit, 1993), respectively (Table 1).

Table 1. Lengths and loci of the bovine milk protein genes

Gene[1]	Protein	Length (kb)[2]	Locus (Chromosome)[2]
CSN1S1	α_{S1}-CN	17.5	6
CSN1S2	α_{S2}-CN	18.5	6
CSN2	β-CN	8.5	6
CSN3	κ-CN	13.0	6
LALBA	α-LA	2.0	5
PAEP	β-LG	4.0	11

[1]CSN1S1 = α_{S1}-casein; CSN1S2 = α_{S2}-casein; CSN2 = β-casein; CSN3 = κ-casein; LALBA = α-lactalbumin; PAEP = progestagen-associated endometrial protein, known as β-lactoglobulin
[2]Caroli et al., 2009

1.3 Regulation of the milk protein biosynthesis

Milk protein synthesis is controlled at multiple levels within the MEC including transcription, post-transcription, translation and AA supply (Menzies et al., 2009). The genes encoding these proteins are regulated by the complex interplay of peptide and steroid hormones, and cell-cell and cell-substratum interactions. Furthermore, interactions between MEC and the extracellular matrix seem to play a crucial role in the expression of the milk protein genes (Aggeler et al., 1988; Jolivet et al., 2001; Figure 2).

The expression of milk protein genes occurs during late pregnancy, lactation and early involution during the lactation cycle. However, Rosen et al. (1999) reported that the expression of each milk protein gene varies at different physiological stages. Furthermore, the milk protein genes contain composite response elements, which are clustered of transcription factor binding sites that contain both the positive and negative regulatory elements that integrate the signal transduction pathways (Jiang and Levine, 1993). It is well established, that peptide and steroid hormones,

predominantly the lactogenic hormones - prolactin, insulin and hydrocortisone - affect the expression of the milk protein genes through phosphorylation control of transcription factors. Moreover, the response to each hormone varies with different milk protein genes. The translation of mRNA is a fundamental process in all living organisms. Bevilacqua et al. (2006) investigated the translational efficiency of the bovine CN transcripts and remarked that α_{S1}- and β-CN transcripts are translated about 3- to 4-fold more efficiently than α_{S2}- and κ-CN transcripts. Moreover, the availability of glucose in the bovine mammary gland is predominantly important for the synthesis of the milk constituents (Reynolds et al., 1994) whereas the availability of AA is not only important for the regulation of translation whereby the transport rate of AA seemed to be a major limiting factor for milk protein synthesis (Bionaz and Loor, 2011).

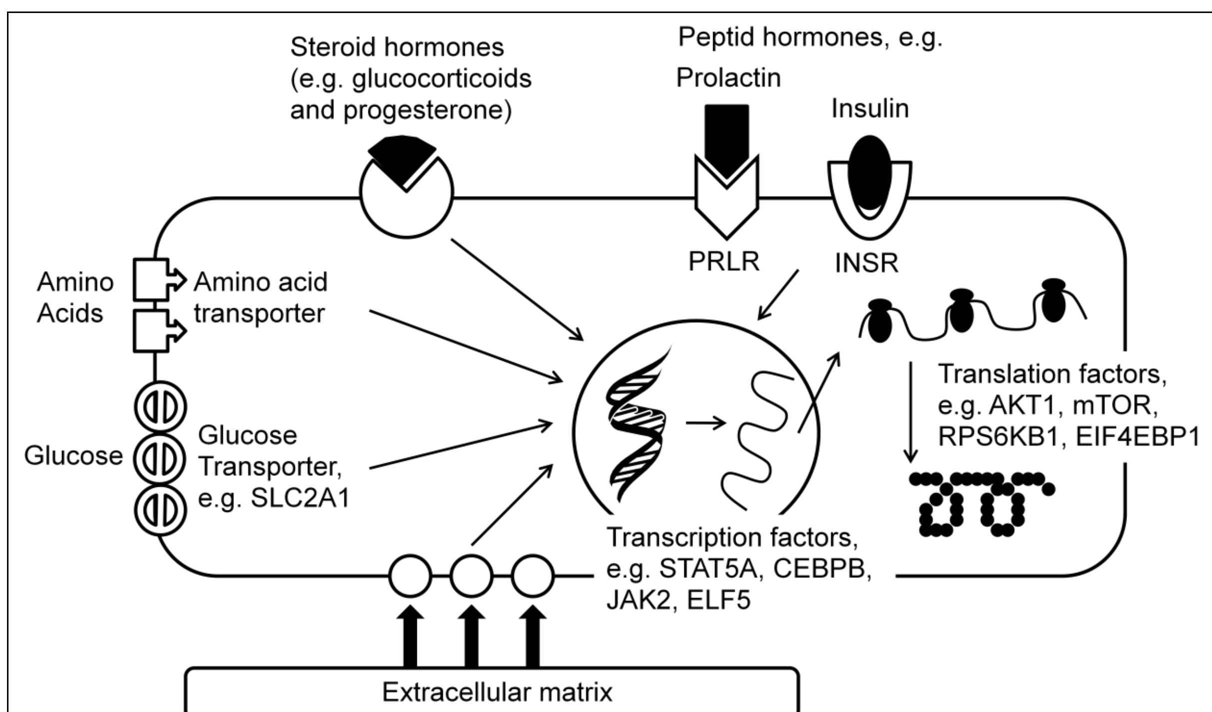

Figure 2. Model of the factors (genes, hormones and metabolites) involved in the regulation of milk protein biosynthesis in the pBMEC; Source: Own illustration

1.4 Aim of the study

The objective of this thesis was to study the main regulating factors responsible for milk protein biosynthesis in pBMEC. In order to evaluate the role of gene network, central gene expression pathways for milk protein biosynthesis were studied in pBMEC from dairy cows with low or high milk protein content. The hypothesis to be tested was that hepatic key parameters and gene expression profiles in pBMEC vary

among cows with low or high milk protein content during early lactation and particularly during restricted feeding.

2 MATERIAL AND METHODS

2.1 Animal trial

The study started in August 2009 and ended in January 2011. A total of 26 multiparous Holstein Friesian dairy cows were assigned to their 100-d performance of previous lactation. Four groups were classified (Table 2): cows with

a) high milk yield and high milk protein content (MP),
b) low milk yield and low milk protein content (mp),
c) high milk yield and low milk protein content (Mp), and
d) low milk yield and high milk protein content (mP).

Table 2. 100-d performance of cows selected for study during previous lactation

Cow-ID	Group[1]	Parity	Milk yield, kg	Milk protein, %	Milk protein, kg	Milk fat, %	Milk fat, kg
15263	MP	1	3721	3.57	133	3.82	142
25564	MP	2	4478	3.34	149	4.11	184
34439	MP	2	4082	3.32	136	4.40	180
34460	MP	2	4153	3.48	144	4.15	172
63660	MP	2	4766	3.41	163	4.55	217
15265	mp	1	3111	2.84	88	3.62	113
15366	mp	1	3904	2.90	113	3.71	145
15625	mp	1	2880	2.84	82	3.95	114
15662	mp	1	3182	2.71	86	4.85	154
20073	mp	1	3250	2.77	90	4.42	144
20330	mp	1	2910	2.98	87	4.06	118
34303	mp	2	3642	2.96	108	3.73	136
03827	Mp	1	4021	2.84	114	3.24	130
03863	Mp	1	3740	2.86	107	3.63	136
25242	Mp	3	5009	2.92	146	3.67	184
34456	Mp	2	4959	2.88	143	4.10	203
63689	Mp	1	4562	2.61	119	3.65	166
03463	mP	2	2904	3.65	106	4.93	143
03642	mP	1	2650	3.33	88	4.68	124

03870	mP	1	3094	3.35	104	3.80	118
15582	mP	1	2618	3.38	89	4.44	116
24232	mP	4	3594	3.35	120	3.29	118
34230	mP	3	2977	3.60	107	4.93	147
34311	mP	2	3356	3.74	125	4.28	144
34346	mP	2	3966	3.30	131	4.19	166
57758	mP	3	4449	3.28	146	4.78	213

[1]M represents cows with high milk yield, m represents cows with low milk yield, P represents cows with high milk protein concentration, and p represents cows with low milk protein concentration.

Two cows were removed from the study and another two cows were euthanized about 100 d after parturition; the reasons for animals leaving the study were not associated to any of treatments and are presented in the following table (Table 3).

Table 3. Cows removed from experiment, day of and reasons for removal

Cow	Days after calving	Reason for removal
24232	During calving	Calf to big, died suddenly
34456	3	Downer cow syndrome, euthanized
63660	108	Foreign body, pericarditis, euthanized
34439	114	E.coli mastitis, euthanized

Exclusively cows with recordings over 100 d of lactation were considered for statistical analysis of data. The study was performed according to strict federal and international guidelines on animal experimentation. The experiment was set up according to the requirements of the Regierungsbezirk Oberbayern animal welfare committee (Munich, Germany).

2.1.1 Animal housing and feeding

Cows were housed in a freestall barn with rubber-coated slatted floors and cubicles bedded with straw powder. During calving, cows were kept in a single calving box bedded with barley straw. Starting two weeks before expected calving and continued after calving, cows were fed the lactational diet (LD; Table 4). 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 (ad lib) intake (residual feed >5%). Additional concentrates (7 MJ NE_L/kg; Raiffeisen Kraftfutterwerke Sued, Wuerzburg, Germany) were fed in automated feeding stations, after parturition 2 kg and increasing up to 6

kg at d 14 post partum (pp) with daily increase of 0.286 kg. 6 kg concentrates were fed until d 100 pp. To underline cows individually different milk yield and milk composition and to eliminate effects of performance-related feeding, additional concentrates were just fed depending on day of lactation. Water was available all time. Milking was done in a 2 × 2 tandem milking parlour (GEA WestfaliaSurge GmbH, Boenen, Germany) twice daily at 0420 and 1540 h.

Table 4. Components and nutritional values[1] of lactational (LD) and restrictional (RD) diet

Variable	LD[2]	RD[3]
Components, %		
corn silage	60.0	56.4
grass silage	23.0	21.6
hay	4.0	3.8
concentrates[4]	12.0	11.3
mineral mix[5]	1.0	0.9
straw		6.0
dry matter (DM)	45.2	47.6
Nutritional values, % of DM		
crude ash	6.3	6.3
crude protein	16.7	15.9
crude fiber	17.2	18.7
crude fat	3.4	3.2
non-fibre carbohydrates	56.4	55.7
neutral detergent fiber	37.4	39.9
acid detergent fiber	22.4	23.8
available crude protein	15.7	15.2
ruminal nitrogen balance	0.1	-0.2
ME MJ/kg DM	11.2	11.0
NE_L MJ/kg DM	6.8	6.6

[1]Nutritional values and composition of partial mixed ration was determined by enhanced Weender-analysis, done at the Bayerische Landesanstalt für Landwirtschaft, Zentrallabor Grub (Poing, Germany).
[2]Lactation diet received all cows ad libitum beginning one week before parturition and during first 21 weeks of lactation.
[3]Restrictional diet received cows only during restrictional phases from DIM 26 to 28 and DIM 141 to 144.
[4] Composition: 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, 0.40% plant oil (palm coconut) (Raiffeisen Kraftfutterwerke Sued GmbH, Wuerzburg, Germany)
[5] Ingredients: 14% Ca, 10% Na, 5% P, 5% Mg (Josera, Kleinheubach, Germany)

From d 23 until 31 pp and d 138 until 147 pp, cows were moved to a tie-stall with separated feed troughs and free access to water. During the first three days (d 23 to 25 and 138 to 140 pp), cows were fed ad lib with LD and additional concentrates (6 and 5 kg during early and mid lactation, respectively). From d 26 to 28 and 141 to 143 pp, cows received restrictional diet (RD, 70% of ad lib; Table 4) but received no additional concentrates. Fresh feed was mixed daily and cows were fed half of their daily allotment of RD at 0700 and 1700 h. The following three days (d 29 to 31 and 144 to 147 pp) they were fed again with ad lib LD and defined amounts of additional concentrates. The amount of feed offered and refused was weighed and recorded daily for calculation of dry matter intake (DMI).

2.1.2 Performance data

Milk yield

During each milking, milk yield was recorded with electronic milk meters (Metatron P21, GEA WestfaliaSurge GmbH) and stored electronically (DairyPlan C21, GEA WestfaliaSurge GmbH).

Body weight, back fat thickness and body condition

All animals were weighed biweekly, using weighing elements underneath the claw stand (FX1, Texas Trading, Windach, Germany). At the same point of time, subcutaneous adipose tissue (backfat thickness) was assessed with ultrasonography (Sonovet 2000, Universal Ultrasound, NY, USA) near the pelvic region (Schroeder and Staufenbiel, 2006) and the body condition score (BCS) was determined by the same person using a scale from 1 to 5 (1 = emaciated, 5 = obese), in increments of 0.25 (Edmonson et al., 1989).

Reproduction and health check

Transrectal examinations were done approximately at d 20 and 40 after parturition. Estrus activity was monitored by measurement of milk progesterone. Milk samples for analysis of progesterone were obtained twice a week throughout the experimental timeframe beginning at d 8 pp and stored at -20°C until analysis. Progesterone was determined in the laboratories of Physiology Weihenstephan (Freising, Germany) with an enzyme immunoassay as described by Prakash et al. (1988). Day of first ovulation was defined as three days before first progesterone concentration was ≥ 0.5 ng/mL. In case of anestrus and to synchronize estrus cycle, Prid-spiral® (Ceva Santé Animale SA, Libourne, France) was administered for seven days followed by

administration of prostaglandin F2α analog or gonadotropin-releasing hormone to therapy of dysfunction and to enable slaughtering on d 12th of estrus cycle. Cows were monitored daily concerning their health status and disease was defined as necessary veterinary intervention. During this study, retained placenta, endometritis, ketosis, lameness and mastitis were diagnosed.

2.2 Collection of biological sample material

2.2.1 Milk and blood

Milk

Proportional subsamples of total milk (~ 1 L) were obtained during morning and evening milking, controlled by total amount of milk and milk flow rate (Metatron P21, GEA WestfaliaSurge, Boenen, Germany). Milk samples for analysis of milk components were taken at d 1 to 10, 12, 15, 17, 20 to 32, 36, 43, 50, 57, 64, 71, 78, 85, 92, 99, 106, 113, 120, 127, 134, 137 to 146, and 155 pp. To achieve a representative sample, aliquots of morning and evening milk were composited according to the morning and evening milk yield and stored at 4°C (maximum seven days) with a preservative (acidiol) until analysis. Milk samples were analyzed for components (protein, fat, lactose and urea content, somatic cell count (SCC) in the laboratories of Milchpruefring Bayern e.V. (Wolnzach, Germany). Measurements of protein, fat and lactose were done by infrared-spectrophotometry (MilkoScan-FT-6000, VOSS GmbH, Rellingen, Germany). Analysis of SCC was conducted by fluorescence-optical counting (Fossomatic-FC, FOSS GmbH, Rellingen, Germany).

Blood

Blood samples were collected 14 and 7 d before expected calving and at d 1, 8, 15, 22, 26 to 29, 32, 43, 57, 113, 141 to 144, 147 and 155 pp. Jugular venipuncture was done after milking and before feeding (0645 h). For collection 9-mL vacuum tubes (Vacuette®, Greiner Bio-One, Kremsmuenster, Austria) were used. After coagulation (maximum 1 h), serum was separated by centrifugation (2000 × *g*, 15 min at 4°C) and three 1.5 mL-aliquots 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). Glucose concentrations were measured 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). Determination of BHBA concentration was done by spectrophotometric enzymatic analysis (CV = 7.1%; Sigma-Aldrich Diagnostics, Munich, Germany). Energy balance (EB) was calculated using the formula EB = (DMI diet × NE_L diet) + (DMI concentrates × NE_L concentrates) - (0.293 × body weight$^{0.75}$) - (0.38 × fat%) - (0.21 × protein%) + 0.95) × milk yield) as described by Kamphues et al. (2004).

2.2.2 Liver and muscle tissue

Liver

Liver biopsies were obtained within 24 hours after calving and at d 15, 29, 57 and 144 pp by blind percutaneous needle biopsy (Bard®Magnum™, Covington, USA). Biopsies were done after milking and blood sampling and before feeding (0650 h). A field of 15 x 15 cm² was shaved, washed and degreased with 70% ethanol and disinfected with iodine solution. Local anaesthetic (7 mL Procasel® (Procainhydrochlorid, 2%), Selectavet, Weyarn, Germany) was used to desensitize skin and underlying body wall and muscle. A small incision was made through the skin at the intersection of a line running from the tuber coxae to the shoulder joint with the 11th and 12th intercostal space and was just large enough to admit the trocar. Liver tissue (nearly 100 mg) was directly given into RNA stabilization solution (RNA*later*®, Applied Biosystems, Darmstadt, Germany) and stored at -80°C until mRNA extraction.

Muscle

Muscle tissue samples of musculus semitendinosus (approximately 600 mg) were obtained from the animals by an open muscle biopsy procedure at three times of lactation: within 24 hours after parturition, at d 43 and 113 pp. Cows received a local subcutaneous anesthesia (7 mL Procasel®, Selectavet, Weyarn, Germany) and caudal epidural anesthesia (5 mL Procasel®, Selectavet, Weyarn, Germany). Samples were cut free of visible connecting tissue and divided into two aliquots, frozen in liquid nitrogen, and stored at -80° until analysis of mRNA levels.

Slaughtering and tissue collection

All cows were slaughtered approximately at d 155 pp on their 12th day of estrus cycle. Slaughtering was conducted in slaughter house, Grub (Germany). Cows were stunned with a captive bold and exsanguinated until death. Within 30 min after slaughter tissue samples from derma, lung, liver, spleen, kidney, adrenal gland,

tongue, skeletal muscle, adipose tissue, heart, mammary gland, mesenterial lymph node, small intestina, colon, abomasum, cecum, rumen, cerebrum and pituitary gland were taken and divided into four aliquots. One aliquot was directly given into RNA stabilization solution (RNA*later*®, Applied Biosystems, Darmstadt, Germany) and three were frozen in liquid nitrogen, and all samples were stored at -80° until further analysis.

2.2.3 pBMEC from milk

During experimental timeframe, 10 morning milk samples were obtained from each cow at d 8, 15, 26, 29, 43, 57, 113, 141, 144 and 155 pp. Whole morning milk was separated during milking into a sterile bucket. Milk yield was determined with a spring scale and one aliquot (40 mL) was stored at 4°C for a maximum of 7 d with a preservative (acidiol) until analysis of milk composition. 3.6 L milk were filled into autoclaved glass bottles and used for immunomagnetic cell isolation immediately. At d 15, 57, 113 and 155 pp additional 2 L milk were filled into autoclaved glass bottles and used for cell isolation for cell culture.

pBMEC immunomagnetic isolated from milk

Boutinaud et al. (2008) described an immunomagnetic method to purify pBMEC from somatic cells. In the present study, the method was refined and modified. Milk (1800 mL) was defatted by centrifugation at 1800 × *g* at 4°C for 30 min in four 450-mL corning tubes and skim milk was removed. Remaining total cell pellets were resuspended in 25 mL of phosphate buffered saline (PBS) and pooled in pairs. After a second centrifugation step (1850 × *g*, 15 min at 4°C) the two total cell pellets were resuspended and pooled in 1 mL of PBS containing 1% bovine serum albumin (BSA). Purification of pBMEC was performed applying an immunomagnetic-bead based separation technique (Figure 3). Cell suspension was first incubated for 10 min on a rotary mixer at 4°C with a primary mouse monoclonal antibody against cytokeratin 8 antibody (clone C-43, EXBIO, Praha, Czech Republic), which is specific to bovine epithelial cells. Unbound antibodies were removed from the cell-antibody complex by 8 min of centrifugation at 300 × *g* at 4°C. After discarding the supernatant, cell-antibody complex was resuspended in 1 mL of 1% BSA-PBS. Dynabeads (25 µL; PanMouse IgG, Invitrogen, Dynal AS, Oslo, Norway) were added and the suspension was incubated for 20 min on a rotary mixer at 4°C. Antibody-bound cells were collected by placing the sample vials into the Dynal MPC™-L

(Dynal AS, Oslo, Norway) for 2 min and withdrawing of the supernatant. A second washing including a magnetic separation step was performed with 1 mL of 1% BSA-PBS followed by are suspension of pBMEC in 1 mL of 1% BSA-PBS. A 7-µL aliquot was removed to perform a hematocytometer cell count and a 10-µL aliquot was collected to stain pBMEC immunohistochemically. Purified pBMEC were obtained by centrifugation of tubes at 1800 × *g* at 4°C for 5 min, resuspended in 700 µL Qiazol (Qiagen GmbH, Hilden, Germany) and stored at -80°C until RNA extraction.

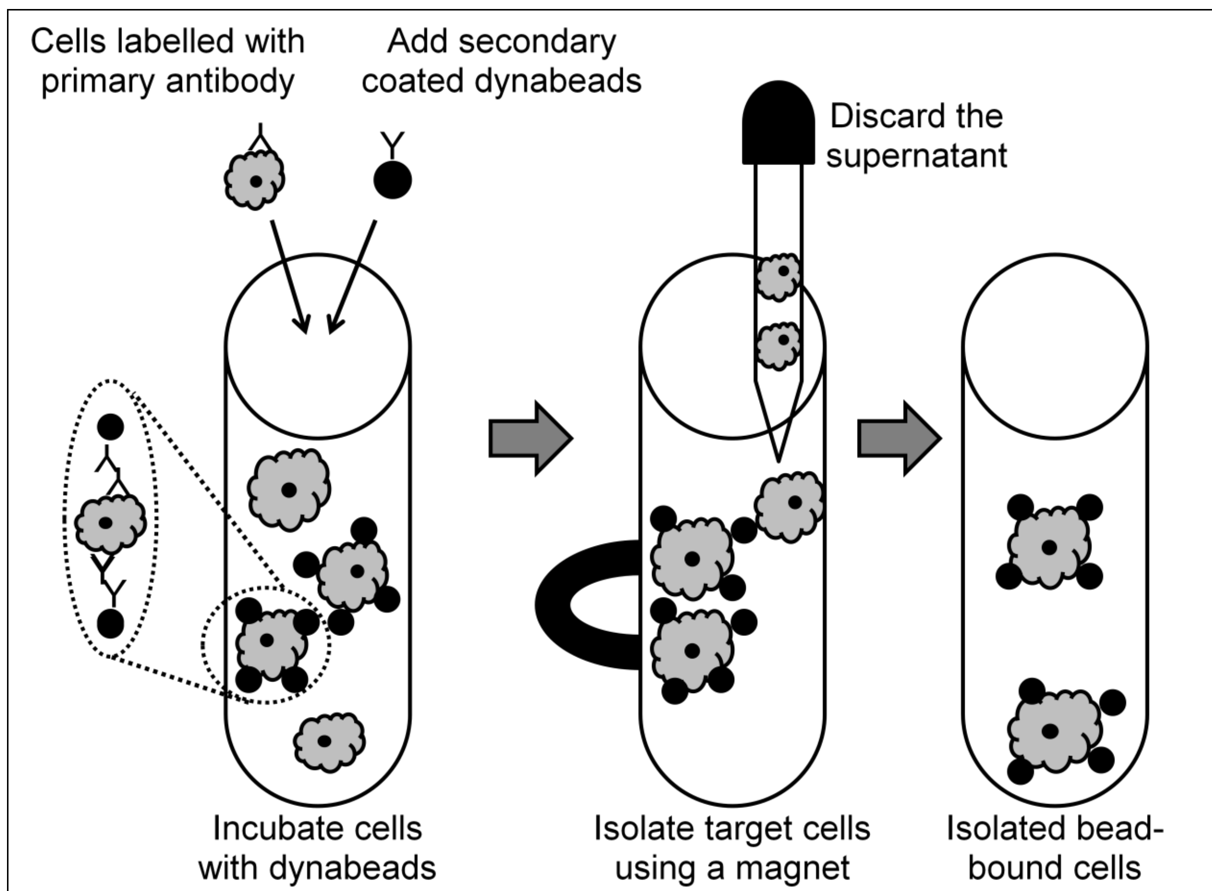

Figure 3. Mammary epithelial cell isolation using indirect technique (adapted from manufacturing instructions for Dynabeads® Pan Mouse IgG)

pBMEC from cell culture

The method presented in this thesis was developed referring to Buehring (1990) who described a method to recover pBMEC from milk and to grow in cell culture. Milk (1000 mL) was defatted by centrifugation at 1800 × *g* at 22°C for 15 min in two 500-mL corning tubes and skim milk was removed. Remaining total cell pellets were resuspended in 50 mL washing solution (135 mL autoclaved H_2O, 15 mL Hanks' Balanced Salt Solution, 0.12 mL Amphotericin B, 0.3 mL Gentamycin (all three from Sigma-Aldrich, Hamburg, Germany), 3 mL Penicillin/Streptomycin (PenStrep; Carl

Roth GmbH, Karlsruhe, Germany). After a second centrifugation step (1500 × *g*, 5 min at room temperature) the two total cell pellets were resuspended, filtered through a 200 µm nylon membrane and pooled in 50 mL washing solution. After a final centrifugation step (1500 × *g*, 5 min at room temperature) cell pellet was resuspended in 7 mL Dulbecco's modified Eagle's Medium F-12 Ham (Gibco®, Invitrogen, Darmstadt, Germany) supplemented with antimicrobials (0.1% Gentamycin, 0.5% ITS (Sigma Aldrich, Hamburg, Germany), 1% PenStrep, 0.02% Amphotericin B) and 5% fetal bovine serum (Gibco®, Invitrogen, Darmstadt, Germany) and placed in a sterile cell culture flask. Culture of cell culture flasks were maintained at 37°C in a humidified atmosphere of 5% CO_2 and the medium was changed twice per week.

When cell layer was confluent or after four weeks in culture, pBMEC were detached from culture flask bottom with 2 mL StemPro® Accutase® (Invitrogen, Darmstadt, Germany), harvested and counted in a hematocytometer.

2.3 Purification and quantification of ribonucleic acids (RNA)

Liver tissue

mRNA was extracted from 100 mg liver tissue by using peqGOLD TriFast™ (Peqlab, Erlangen, Germany) according to the manufacturing instructions. RNA was dissolved in 50 µL sterile RNase-free water and quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg).

pBMEC from milk

Total RNA was extracted from the purified pBMEC applying the miRNeasy MiniKit (Qiagen GmbH, Hilden, Germany) according to the manufacturing instructions. RNA was dissolved in 30 µL sterile RNase-free water and quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg).

pBMEC from cell culture

mRNA was purified applying the NucleoSpin® RNA II kit (Macherey-Nagel, Dueren, Germany) according to the manufacturing instrucutions. RNA was dissolved in 30 µL sterile RNase-free water and quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg).

2.4 Complementary deoxyribonucleic acid synthesis

Constant amounts of 1 µg RNA of liver samples, 250 ng RNA of pBMEC from milk and 500 ng RNA of pBMEC from cell culture were reverse transcribed to complementary DNA (cDNA) using 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 200 U of MMLV-H^- reverse transcriptase (Promega, Regensburg, Germany). According to the manufacturing instructions, reaction of reverse transcription was carried out in 60 µL volume, using a PCR thermocycler (Biometra, Goettingen, Germany) and was achieved by successive incubations at 21°C for 10 min and 48°C for 50 min, finishing with enzyme inactivation at 90°C for 2 min. Reverse transcript products of liver tissue and pBMEC samples were stored at -20°C.

2.5 Target gene selection and primer design

Gene sequences for primer design were obtained from the gene bank of the National Center for Biotechnology Information. Exon-spanning primers were designed using National Center for Biotechnology Information primer tool and synthesized at Eurofins MWG (Ebersberg, Germany) except hepatocyte nuclear factor-4A (HNF4A) which was according to Loor et al. (2005), peroxisome proliferator activated receptor (PPAR) α and PPARG which were according to Sigl et al. (2010), and sterol regulatory element binding transcription factor 1 (SREBF1) which was according to Van Dorland et al. (2009). Primers, accession numbers and product lengths for each gene measured in liver tissue are listed in Table 5.

Table 5. Primer sequences, accession numbers and product lengths for genes measured in liver tissue

Gene[1]	Sequence (5’ → 3’)	Product size (bp)	GeneBank accession no.
Reference genes			
ACTB for	AACTCCATCATGAAGTGTGAC	202	NM_173979.3
ACTB rev	GATCCACATCTGCTGGAAGG		
GAPD for	GTCTTCACTACCATGGAGAAGG	197	NM_001034034.1
GAPD rev	TCATGGATGACCTTGGCCAG		

H3F3A for	ACTCGCTACAAAAGCCGCTCG	232	NM_001014389.2
H3F3A rev	ACTTGCCTCCTGCAAAGCAC		
Lipid metabolism			
ACACA for	CTCTTCCGACAGGTTCAAGC	248	NM_174224.2
ACACA rev	ACCATCCTGGCAAGTTTCAC		
ACADVL for	CGTACATGGTGAGTGCCAAC	209	NM_174494.2
ACADVL rev	GTCATTTGTCCCCTCGAAGA		
CPT1A for	CCATACTCACATAATTGGTAGCC	144	XM_001789518.1
CPT1A rev	GCAACTAGTGAAGCCTCTTATGAA		
ECHS1 for	GCTGCTGTCAATGGCTATGC	192	NM_001025206.2
ECHS1 rev	ACCAGTGAGGACCATCTCCA		
GPAM for	TCTGACTGAAGATGGGGATG	148	NM_001012282.1
GPAM rev	ATGGGGAATTTGCCGCTTAT		
Protein metabolism			
CTSL for	CACTGGTGCTCTTGAAGGACA	177	NM_174032.2
CTSL rev	TAAGATTCCTCTGAGTCCAGGC		
TAT for	ACCCTTGTGGGTCAGTGTTC	165	NM_001034590.1
TAT rev	ACAGGATGGGGACTTTGCTG		
Carbohydrate metabolism			
CS for	TGGACATGATGTATGGTGG	217	NM_001044721.1
CS rev	AGCCAAGATACCTGTTCCTC		
PC for	ATCTCCTACACGGGTGACGT	214	NM_177946.3
PC rev	TGTCGTGGGTGTGGATGTGCA		
PCK1 for	TTTGGCGTCGCTCCGGGAAC	244	NM_174737.2
PCK1 rev	GGCACTGGCTGGCTGGAGTG		
PCK2 for	TACGAGGCCTTCAACTGGCGT	365	NM_001205594.1
PCK2 rev	AGATCCAAGGCGCCTTCCTTA		
Glucose transport			
SLC2A2 for	GGACCTTGGTTTTGGCTGTC	275	NM_001103222.1
SLC2A2 rev	CACAGACAGGGACCAGAACA		
Hormone receptor			
INSR for	CCAACTGCTCAGTCATCGAA	164	XM_590552.5
INSR rev	GTTGGGGAACAAGTCCTTCA		
Immune response			
TNFA for	TCTGCCATCAAGAGCCCTTGCC	185	NM_173966.2

TNFA rev	GCGATGATCCCAAAGTAGACCTGCC		
Ketogenesis			
HMGCS2 for	CGCCCGGCGTCCCGTTTAAA	294	NM_001045883.1
HMGCS2 rev	GGACCCGCCACACTTTCGGTC		
Translation			
EIF4B for	CCACGCCGGGACATGGATCG	164	NM_001035028.1
EIF4B rev	TCATAGCGGTCCCCGCCTCC		
Transcription regulation			
HNF4A for	GCATGGCCAAGATCGACAA	73	NM_001015557.1
HNF4A rev	TGGGCATGAGGTGCTTCAC		
PPARA for	GGATGTCCCATAACGCGATTCG	235	NM_001034036.1
PPARA rev	TCGTGGATGACGAAAGGCGG		
PPARG for	CTCCAAGAGTACCAAAGTGCAATC	198	NM_181024.2
PPARG rev	CCGGAAGAAACCCTTGCATC		
SREBF1 for	CCAGCTGACAGCTCCATTGA	67	NM_001113302.1
SREBF1 rev	TGCGCGCCACAAGGA		
Anabolism			
IGF1 for	CATCCTCCTCGCATCTCTTC	239	NM_001077828.1
IGF1 rev	CTCCAGCCTCCTCAGATCAC		

[1]ACTB = actin beta; GAPD = glyceraldehyde-3-phosphate dehydrogenase; H3F3A = H3 histone family 3A; 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; CS = citrate synthase; PC = pyruvate carboxylase; PCK1 = phosphoenolpyruvate carboxykinase, cytosolic; PCK2 = phosphoenolpyruvate carboxykinase, mitochondrial; SLC2A2 = facilitated glucose transporter, member 2; INSR = insulin receptor; TNFA = tumor necrosis factor α; HMGCS2 = 3-hydroxy-3-methylglutary-coenzyme A synthase 2; EIF4B = eukaryotic translation initation factor 4B; HNF4A = hepatocyte nuclear factor-4A (Loor et al., 2005); PPARA = peroxisome proliferator activated receptor-α (Sigl et al., 2010); PPARG = peroxisome proliferator activated receptor-γ (Sigl et al., 2010); SREBF1 = sterol regulatory element binding transcription factor 1 (Van Dorland et al., 2009); IGF1 = insulin-like growth factor 1

Primers, accession numbers and product lengths for each gene measured in pBMEC are listed in Table 6.

Table 6. Primer sequences, accession numbers and product lengths for genes measured in pBMEC

Gene[1]	Sequence (5’ → 3’)	Product size (bp)	GeneBank accession no.
Major milk protein genes			
CSN1S1 for	ATGAAACTTCTCATCCTTACCTGTCTT	179	NM_181029.2

CSN1S1 rev	CCAATATCCTTGCTCAGTTCATT		
CSN1S2 for	AGCTCTCCACCAGTGAGGAA	150	NM_174528.2
CSN1S2 rev	GCAAGGCGAATTTCTGGTAA		
CSN2 for	GTGAGGAACAGCAGCAAACA	233	NM_181008.2
CSN2 rev	AGGGAAGGGCATTTCTTTGT		
CSN3 for	TGCAATGATGAAGAGTTTTTTCCTAG	150	NM_174294.1
CSN3 rev	GATTGGGATATATTTGGCTATTTTGT		
LALBA for	CTCTCTGCTCCTGGTAGGCAT	247	NM_174378.2
LALBA rev	GTGAGGGTTCTGGTCGTCTT		
PAEP for	AGAAGGTGGCGGGGACTTGG	375	NM_173929.3
PAEP rev	TGTCGAATTTCTCCAGGGCCT		
Marker of epithelial cells			
KRT8 for	GCTACATTAACAACCTCCGTC	237	NM_001033610.1
KRT8 rev	TCTCATCAGTCAGCCCTTCC		
Receptors			
IGF1R for	CCCAAAACCGAAGCTGAGAAG	200	XM_606794.3
IGF1R	TCCGGGTCTGTGATGTTGTAG		
INSR for	CCAACTGCTCAGTCATCGAA	164	XM_590552.5
INSR rev	GTTGGGGAACAAGTCCTTCA		
NR3C1 for	ACCAATTCCTGTCGGTTCAG	166	NM_001206634.1
NR3C1 rev	TGAGGAACTGGATGGAGGAG		
PRLR for	CATGGTGACCTGCATCCTC	172	NM_001039726.1
PRLR rev	ACCCTCATGCCTCTCACATC		
Transcription factors			
CEBPB for	GCACAGCGACGAGTACAAGA	152	NM_176788.1
CEBPB rev	GTTGCTCCACCTTCTTCTGG		
ELF5 for	ATACTGGACGAAGCGCCACGTC	134	NM_001024569.1
ELF5 rev	ACTCCTCCTGTGTCATGCCGCA		
JAK2 for	TCTGGTATCCACCCAACCATGTCT	201	XM_865133.2
JAK2 rev	AATCATGCCGCCACTGAGCAA		
RUNX2 for	ACCATGGTGGAGATCATCG	207	XM_002684501.1
RUNX2 rev	CCGGAGCTCAGCAGAATAA		
STAT5A for	GTGAAGCCACAGATCAAGCA	176	NM_001012673.1
STAT5A rev	TCGAATTCTCCATCCTGGTC		
SOCS2 for	CCGGAACGGCACTGTTCACCTT	109	NM_177523.2
SOCS2 rev	CCAGACGGTGCTGGTACACTTGTT		

YY1 for	GCTTGCCCTCATAAAGGCTGCACA	192	NM_001098081.1
YY1 rev	GCAGCCTTCGAACGTGCACTGA		
Glucose transporters			
SLC2A1 for	GTGCTCCTGGTTCTGTTCTTCA	84	NM_174602.2
SLC2A1 rev	GCCAGAAGCAATCTCATCGAA		
SLC5A1 for	TACGAGCGCATCCGCAATGCA	129	NM_174606.2
SLC5A1 rev	ACCTGCCAGGAAGAAGCCTCCA		
Translation factors			
AKT1 for	GATCACCGACTTCGGACTGT	202	NM_173986.2
AKT1 rev	CTTCTCGTGGTCCTGGTTGT		
EIF4EBP1 for	GAA CTC ACC TGT GAC CAA GA	157	NM_001077893.1
EIF4EBP1 rev	CTCAAACTGTGACTCTTCACC		
mTOR for	CGGGACTACAGGGAGAAAAA	340	XM_001788228.1
mTOR rev	CCTCAAAGCAGTCCCCAAAG		
OAT for	ATACAGGAGTGGAGGCTGGA	150	NM_001034240.1
OAT rev	CAGTGGAGCTGGAGATAGCA		
RPS6KB1 for	GGCAGCCCACGAACACCTGT	96	NM_205816.1
RPS6KB1 rev	AGGCGTCTGCGGATTTGCCG		
References genes			
GAPD for	GTCTTCACTACCATGGAGAAGG	197	NM_001034034.1
GAPD rev	TCATGGATGACCTTGGCCAG		
H3F3A for	ACTCGCTACAAAAGCCGCTCG	232	NM_001014389.2
H3F3A rev	ACTTGCCTCCTGCAAAGCAC		
RPS9 for	CCTCGACCAAGAGCTGAAG	64	NM_001101152.1
RPS9 rev	CCTCCAGACCTCACGTTTGTTC		
UBB for	GTC TTC ACT ACC ATG GAG AAG G	197	NM_174133.2
UBB rev	TCA TGG ATG ACC TTG GCC AG		

[1] CSN1S1 = α_{S1}-casein; CSN1S2 = α_{S2}-casein; CSN2 = β-casein; CSN3 = κ-casein; LALBA = α-lactalbumin; PAEP = progestagen-associated endometrial protein, known as β-lactoglobulin; KRT8 = cytokertin 8; IGF1R = insulin-like growth factor 1 receptor; INSR = insulin receptor; NR3C1 = nuclear receptor subfamily 3, group C, member 1, known as glucocorticoid receptor; PRLR = prolactin receptor; CEBPB = CCAAT/enhancer binding protein β; ELF5 = E74-like factor 5; JAK2 = janus kinase 2; RUNX2 = runt-related transcription factor 2; STAT5A = signal transducer and activator of transcription 5A; SOCS2 = suppressor of cytokine signaling 2; YY1 = yin yang 1; SLC2A1 = solute carrier family 2, member 1; SLC5A1 = solute carrier family 5, member 1; AKT1 = v-akt murine thymoma viral oncogene homolog 1; EIF4EBP1 = eukaryotic translation initiation factor 4E binding protein 1; mTOR = mammalian target of rapamycin; OAT = ornithine δ-aminotransferase; RPS6KB1 = ribosomal protein S6 kinase; GAPD = glyceraldehydes-3-phosphate dehydrogenase; H3F3A = H3 histone family 3A; RPS9 = ribosomal protein 9; UBB = polyubiquitin

2.6 Quantitative polymerase chain reaction

Liver and pBMEC from milk

Quantitative PCR was performed using MESA Green qPCR MasterMix plus for SYBR® Assay w/fluorescein (Eurogentec, Cologne, Germany) with a standard protocol recommended by the manufacturing instructions. All components necessary for real-time RT-qPCR were mixed in the reaction wells of semi-skirted twin.tec PCR plate 96 (Eppendorf, Hamburg, Germany). The mastermix was prepared as follows: 7.5 µL 2 × MESA Green qPCR MasterMix, 1.5 µL forward primer (10 pmol/µL), 1.5 µL reverse primer (10 pmol/µL), 3.0 µL RNase free water. Per well, 13.5 µL mastermix plus 1.5 µL cDNA were added. The plate was sealed, placed in the iQ™5 Cycler (Bio-Rad, Munich, Germany), and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program (95°C, 3 s; primer specific annealing, 60 s) and melting curve analysis.

pBMEC from cell culture

Quantitative PCR was performed using LightCycler® DNA Master SYBR Green (Roche, Mannheim, Germany) applying a standard protocol recommended by the manufacturer. All components necessary for real-time qPCR were mixed in the 20 µL LightCycler® capillaries. The mastermix was prepared as follows: 6.4 µL RNase free water, 1.2 µL $MgCl_2$, 0.2 µL forward primer (20 pmol/µL), 0.2 µL reverse primer (20 pmol/µL), and 1 µL LightCycler® FastStart DNA Master SYBR Green I. Per well, 9 µL mastermix plus 1 µL cDNA was added. After centrifugation, capillaries were placed in the LightCycler 2.0 (Roche, Mannheim, Germany), and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program (95°C, 3 s; primer specific annealing temperature, 60 s) and melting curve analysis.

2.7 Immunohistochemical staining methods

pBMEC from milk

For immunohistochemical detection of pBMEC from milk 10 µL of the cell suspension was spread on an object slide, treated with 7 µL of Poly-L-Lysine solution (Science Services, Munich, Germany), and cells were fixed for 10 min with 100% ethanol. Addition of methanol (99.8%) for 5 min permeabilized cell surfaces. Thereafter, samples were washed twice with PBS for 5 min. Endogenous peroxidase activity was blocked with 1% H_2O_2 for 20 min. Following blocking with 10% goat serum, samples

were incubated with cytokeratin 8 antibody at 37°C for 45 min. After washing with PBS (twice for 10 min), samples were incubated with a secondary anti-mouse IgG peroxidase-conjugated antibody (2.5 mg/mL, Sigma-Aldrich, Munich, Germany). Therefore, samples were washed twice with PBS. Binding of antibody was detected by incubation with PBS containing 0.01% diaminobenzene and 0.01% H_2O_2 for 15 min. Cells were counterstained using Mayer`s Haemalaun (Carl Roth GmbH, Karlsruhe, Germany). pBMEC coated with magnetic beads are shown in Figure 4.

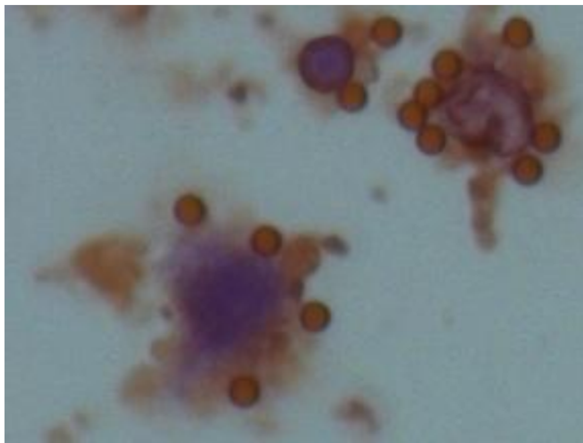

Figure 4. Mammary epithelial cells attached to Dynabeads coated with anti-cytokeratin 8. Cells are counterstained in blue, cytokeratin filaments stained in brown and magnetic beads appeared as brown circles. Source: Own illustration

pBMEC from cell culture

For immunohistochemical detection of pBMEC from cell culture, cells were harvested and quantified. Therefore, 1000 cells per well were disseminated on a coated 16-well-object slide and covered with 300 µL culture medium per well. Object slides were maintained at 37°C in a humidified atmosphere of 5% CO_2 and medium was changed 24 hours later. When cell layer was confluent, approximately 3 days later, culture medium was removed and cells were fixed for 10 min with 100% ethanol. Subsequent operations were done like described for pBMEC from milk and staining is shown in Figure 5.

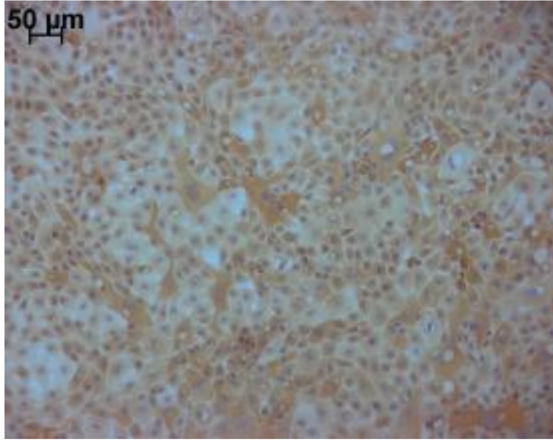

Figure 5. Mammary epithelial cells attached to microscope slide after cultivation for three days at 37°C. Cells are counterstained in blue and cytokeratin filaments stained in brown; Source: Own illustration

3 STATISTICAL ANALYSIS

3.1 Statistical analysis of functional parameters in blood and performance data

For any metabolic key parameters in blood, for milk yield and milk constituents as well, treatment effects and differences among groups were determined using the Restricted Maximum Likelihood in the Mixed Model procedure in Statistical Analysis System (SAS; SAS Institute, 2002). The model contained fixed effects of group and days in milk (DIM) and random effects of cow within group.
The following model was used by defining covariance structure as described above:

$$\mathbf{Y_{ijk} = \mu + group_{ijkl} + cow_{j}(group)_{ijkl} + DIM_{l} + (group\ DIM)_{ikl} + \varepsilon_{ijkl}}$$

Y =	dependent variable
μ =	the overall mean
group =	fixed effect of group ijkl (i = MP, j = mp, k = Mp, l = mP)
DIM =	fixed effect of DIM l postpartum (l = 1, 2, 3, …, 155)
cow (treatment)=	random effect of cow within group
interaction	group × week

Measures on different animals are independent, so covariance concern is only with measures on the same animal. The covariance structure refers to variances at individual times and to correlation between measures at different times on the same animal. For each variable analyzed, three covariance structures were evaluated: compound symmetry, autoregressive order 1, and unstructured. The covariance structure of repeated measurements that resulted in the Akaike's information criterion or Schwarz Bayesian criterion closest to zero was used (Littell et al., 1998). Differences between treatments were determined using the PDIFF option.
Results are reported as least square means (LSM) ± standard error of means (SEM). Means were considered to differ significantly in case $P<0.05$.

3.2 Statistical analysis of mRNA abundance in liver

Data of gene expression in liver were also analyzed using Restricted Maximum Likelihood in the MIXED procedure in SAS but including also parity as fixed effects. In each model, animal was used as repeated subject. Genes were selected as reference genes using GenEx Pro Software Version 5.2.7.44 (MultiD Analyses, Gothenburg, Sweden). The mean of the three housekeeping genes, actin beta

(ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPD) and H3 histone family 3A (H3F3A) was calculated for the reference index and used for normalization. Quantitative cycle (Cq)-values were calculated by Bio-Rad iQ^{TM}5 Optical System Software Version 2.1 with the analysis mode ‘PCR base line substracted curve fit’. The ΔCq-values were calculated as $\Delta Cq = Cq_{target\ gene} - meanCq_{refence\ genes}$ (Pfaffl, 2001). In order to avoid negative digits while allowing an estimation of a relative comparison between two time points, data are subtracted from the arbitrary value 10 (10-ΔCq).

3.3 Statistical analysis of mRNA abundance in pBMEC

The ΔCq-values were normalized individually in relation to the housekeeping gene index of GAPD, H3F3A and ribosomal protein 9 (RPS9) before using the MIXED procedure of SAS with repeated measurements. The analysis of variance models used contained the fixed effect DIM and a random cow effect. Genes were selected as reference genes using GenEx Pro Software Version 5.2.7.44 (MultiD Analyses, Gothenburg, Sweden). The mean of the three housekeeping genes, GAPD, H3F3A, and ribosomal protein S9 (RPS9) was calculated for the reference index and used for normalization. Cq-values 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 $\Delta Cq = Cq_{target\ gene} - meanCq_{refence\ genes}$ (Pfaffl 2001). In order to avoid negative digits while allowing an estimation of a relative comparison between two time points, data are presented as LSM ± SEM subtracted from the arbitrary value 2 (2-ΔCq).

4 RESULTS AND DISCUSSION

4.1 100-d performance

100-d milk yield, milk protein and fat percentage were calculated to select cows for the experiment. After the experiment individual performances of the previous and current lactation were calculated (Table 7). 100-d performance of the previous lactation was obtained by the common calculation of Arbeitsgemeinschaft Deutscher Rinderzuechter e.V. whereas 100-d performance of current lactation was calculated in the following way: daily milk yield was pooled to weekly means and for calculation of milk fat and milk protein yield weekly means of milk fat and protein yield were multiplied by 7 and summed. All data obtained during restricted feeding (d 26 to 28 pp) were excluded for this calculation. Data of Arbeitsgemeinschaft Deutscher Rinderzuechter e.V. are based on the first three measurements of milk performance test whereas results of current lactation were obtained by 97 measurements for milk yield and by 32 measurements for milk protein and fat yield, respectively. These different calculation methods and the different housing, feeding, milking and management systems (Table 7) make the comparison of the 100-d performance irreducibly complex.

Table 7. 100-d performance for milk yield, milk protein and fat of cows during current lactation

	Previous lactation			Current lactation					
Cow-ID	Group[1]	Milk yield, kg	Milk protein, %	Parity	Milk yield, kg	Milk protein, %	Milk protein, kg	Milk fat, %	Milk fat, kg
15263	MP	3721	3.57	2	3565	3.70	132	4.99	178
25564	MP	4478	3.34	3	4084	3.28	134	4.77	195
34439	MP	4082	3.32	3	4536	3.50	159	5.11	232
34460	MP	4153	3.48	3	3089	3.66	113	4.56	141
63660	MP	4766	3.41	3	3388	3.04	103	5.79	196
15265	mp	3111	2.84	2	4583	3.03	139	4.63	212
15366	mp	3904	2.90	2	3625	3.01	109	3.89	141
15625	mp	2880	2.84	2	5180	2.93	152	3.80	197
15662	mp	3182	2.71	2	3474	3.25	113	4.32	150
20073	mp	3250	2.77	2	3599	2.95	106	4.33	156

20330	mp	2910	2.98	2	4142	3.21	133	4.47	185
34303	mp	3642	2.96	3	3227	2.80	92	3.97	128
03827	Mp	4021	2.84	2	4495	3.09	139	3.94	177
03863	Mp	3740	2.86	2	4190	3.25	136	4.25	178
25242	Mp	5009	2.92	4	4854	3.07	149	4.29	208
34456	Mp	4959	2.88	3	-	-	-	-	-
63689	Mp	4562	2.61	2	4373	3.00	131	4.28	187
03463	mP	2904	3.65	3	3771	3.58	135	5.38	203
03642	mP	2650	3.33	2	4181	3.09	129	5.14	215
03870	mP	3094	3.35	2	3967	3.40	135	4.59	182
15582	mP	2618	3.38	2	4100	3.27	134	5.49	225
24232	mP	3594	3.35	5	-	-	-	-	-
34230	mP	2977	3.60	4	3067	3.72	114	5.51	169
34311	mP	3356	3.74	3	3504	3.71	130	4.79	168
34346	mP	3966	3.30	3	4029	3.23	130	5.06	204
57758	mP	4449	3.28	4	4168	3.10	129	5.76	240

[1]M represents cows with high milk yield, m represents cows with low milk yield, P represents cows with high milk protein concentration, and p represents cows with low milk protein concentration.

Previous to the experimental period, all cows housed in a large farm (800 dairy cows) in Saxony (Germany). About two month before expected calving, dry-off cows were transferred to the Versuchsstation Veitshof in Freising (Bavaria, Germany), where the experimental trial occurred. Both dairy farms were different in husbandry, management, feeding and individual care of cows (Table 8).

Table 8. Housing, milking management and feeding system at the dairy farm in Saxony and Versuchsstation Veitshof Freising

	Dairy farm in Saxony	Versuchsstation Veitshof
Housing		
Stall	cubicle house, slatted floor	cubicle house, slatted floor
Floor covering	brushed concrete	rubber coated
Cubicle	high-lying with chalk	low-lying with straw powder
Milking management		
Parlour	Side-by-side with fast all-exit release	2 x 2 tandem parlour

Milking per day	3	2
Milking cluster and removing	DeLaval, automatic	GEA Westfalia, manual
Feeding system		
Feeding rack	no	yes
Feed	Total mixed ration according to milk yield and days of lactation	Partial mixed ration for 22 kg milk
Concentrates	no additional concentrates	automatic feeder dependent on day of lactation

Some earlier studies showed effects of these different management systems on milk yield and milk protein concentration. Compared with the concrete floor surface, rubber coated floor led to a increased walking behavior (step length and steps per day) and had positive effects on comfort (licking) and estrus (mounting) behavior in dairy cows (Platz et al., 2008). Regarding the milking management, earlier studies demonstrated that more frequent milking led to more milk yield (Hillerton and Winter, 1992; Erdman and Varner, 1995). Additionally, Kruip et al. (2002) reported a substantially decreased milk yield in cows changing from thrice to twice day milking a day. Nevertheless, feeding the dairy cow is indisputably the major challenge to influence protein synthesis in the mammary gland and the output in milk. Variabilities in milk protein content were associated with differences in nutritional factors that influence protein metabolism in mammary gland (DePeters and Cant, 1992; Burgos et al., 2010). Metabolizable protein intake stimulates milk protein yield but is supposed to increase the supply of EAA that limit protein synthesis (Toerien et al., 2010). Moreover, the extraction rate of AA from the blood by the mammary gland is very high and the overall efficiency of mammary utilization of AA for milk protein synthesis exceeds 80% in the dairy cow (Mackle et al., 2000). Therefore, AA composition and amount of EAA in the diet influence milk protein yield (Shingoethe, 1996).

In present study, half of the cows had scarcely finished their first lactation. It is well established that the mammary gland of primiparous cows is not completely developed and without a doubt, the milk yield during first lactation is not representative for the individual live-time achievement of milk yield capacity (Fleischer et al., 2001). In our study, all cows with parity 1 or 2 in previous lactation showed higher milk yields during their subsequent 100-d lactation period. Earlier

studies reported that advancing parity is associated with increase in milk production (Dematawewa et al., 1998 and Lee and Kim, 2006). Nevertheless, 7 cows had less or similar milk yields during following lactation. Milk yield depression of three cows (15263, 15366, 34303) remain inexplicable, whereas one cow suffered lameness (63689), one cow (25564) showed retained placenta and suffered lameness, one cow (34460) had an abortion, showed retained placenta and suffered lameness and another cow (63660) had mastitis, absorbed a foreign body and was euthanized at d 108 pp. Prevalence data for the different claw disorders were panaritium and interdigital dermatitis. Heuer et al. (1999) reported an increased incidence of lameness as milk yield increase. Occurrence of diseases in our study is summarized in Table 9.

Table 9. Occurrence of diseases in the cows during study

Diseases	Timeframe		
	Prepartum (wk 2 ap until calving)	Calving and early lactation (until wk 8 pp)	Mid lactation (from wk 9 until end of the study)
Retained placenta		7 (03642, 03827, 25564, 34346, 34460, 57758, 63660)	
Endometritis		4 (03827, 25564, 34346, 34439)	
Lameness	4 (03827, 03863, 34346, 57758)	8 (03827, 15662, 25564, 34230, 34346, 34460, 57758, 63689)	7 (03827, 15263, 15582, 25242, 25564, 34230, 63689)
Mastitis		2 (34311, 63660)	5 (15263, 15625, 15662, 34311, 63660)
Ketosis		1 (57758)	

Five cows had a 100-d milk yield lower than 3000 kg in previous lactation. During the succeeding lactation, milk yield increased by 1000 kg in four cows, that were in their second or 3rd lactation during experiment, whereas in one cow which get in her 4th lactation, milk yield remained below 3000 kg. In another two 4th parity cows, milk yield decreased in current lactation. Additionally, during the periparturient period the three cows with parity 4 lost more body condition, determined by loss of body weight and decrease in BCS and backfat thickness, than cows with lower parity (Figure 6).

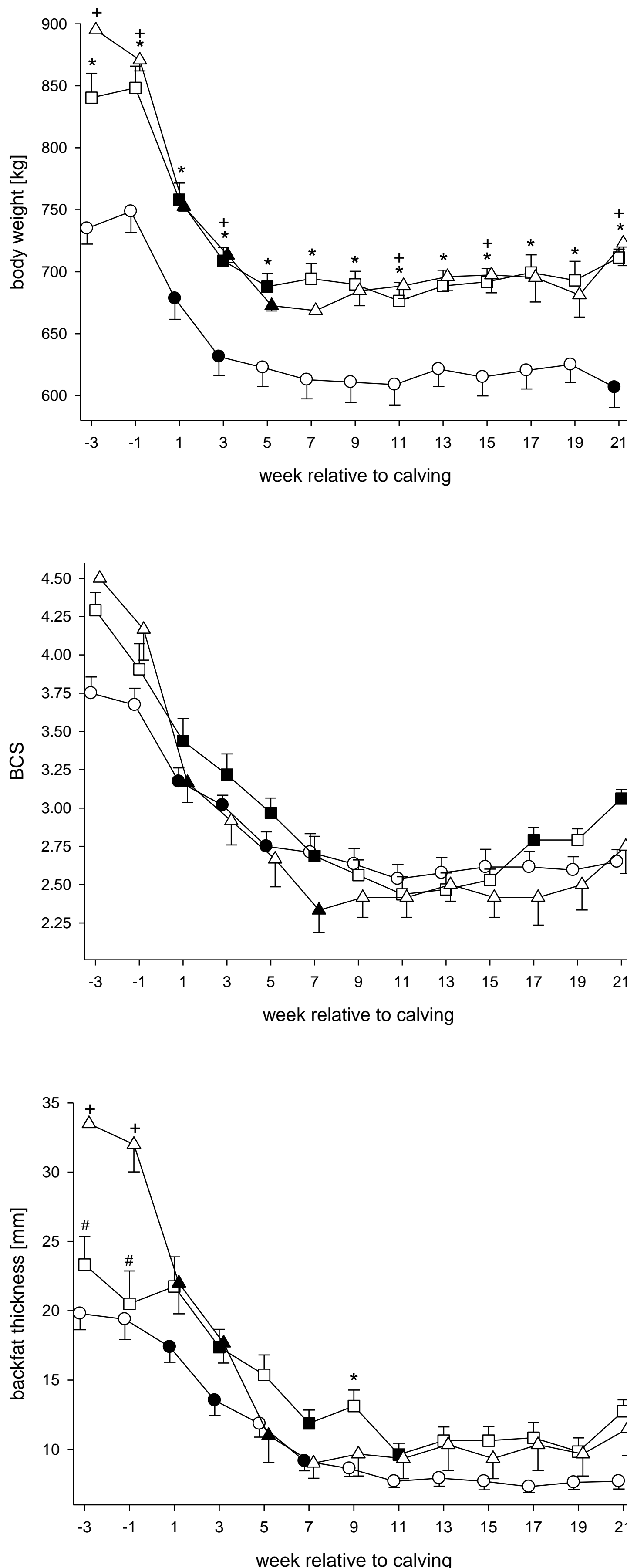

Figure 6. Body weight (kg), body condition score and backfat thickness (mm) from week -3 before expected parturition up to week 21 of lactation for 24 Holstein-Friesian dairy cows assigned for parity 2 (○), 3 (□) or 4 (△). Values that differ significantly from the prior value are filled. Asterisks indicate differences between parity 2 and 3, plus signs indicate differences between parity 2 and 4, and hash signs indicate differences between parity 3 and 4 ($P<0.05$). Values are LSM ± SEM.

Simultaneously, one cow (57758) with parity 4 suffered from retained placenta and ketosis and the other two cows with parity 4 suffered from lameness. Kim and Suh (2003) determined a greater occurrence of metabolic and reproductive diseases in cows with marked condition loss during early lactation and even body condition loss during the dry period until calving had harmful effects on the occurrence of postpartum reproductive diseases (Markusfeld et al., 2008). The days of first ovulation are shown in Table 10.

Table 10. Occurrence of first ovulation measured by progesterone concentration in skim milk samples

	First ovulation < DIM 30	First ovulation > DIM 30	No estrus cycle
Number of cows	13	6 (03642, 03827, 15625, 34303, 34346, 57758)	3 (15265, 25242, 25564)

In conclusion, expected variations in milk yield and milk composition of the experimental cows were induced by multiple factors, e.g. different management and feeding systems, but especially by the influence of parity.

4.2 Feed restriction during early lactation

23 Holstein-Friesian cows were blocked into four groups according to mean milk yield and mean milk protein content at d 23 to 25 pp (Table 11). From d 23 until 31 pp cows were moved to a tie-stall with separated feed troughs and free access to water. During the first three days (d 23 to 25 pp), cows were fed ad lib with LD and additional concentrates (6 kg). From d 26 to 28 pp, cows received RD (70% of ad lib; Table 4) and received no additional concentrates.The objective of this experiment was to study the effect of a short-term FR on metabolic adaptation and milk productivity, and on hepatic key performance indicators during early lactation in cows classified according to milk yield and milk protein concentration.

Table 11. LSM ± SEM of milk yield and composition and blood serum parameters during feed restriction

	Group[1]			
Item	MP	mp	Mp	mP
Milk, kg/d				
d 23 to 25 pp	$42.1 \pm 1.13^{a*}$	$36.8 \pm 0.51^{b*}$	$45.2 \pm 1.33^{a*}$	33.4 ± 0.69^{b}

d 26 to 28 pp	$39.1 \pm 0.97^{a+}$	33.7 ± 1.45^{b}	42.3 ± 1.39^{a}	31.4 ± 1.23^{b}
d 29 to 31 pp	40.1 ± 1.41^{a}	32.9 ± 1.37^{b}	40.7 ± 1.43^{a}	31.7 ± 1.18^{b}
Milk protein, %				
d 23 to 25 pp	3.28 ± 0.07^{a}	2.84 ± 0.06^{b}	2.90 ± 0.06^{b}	3.40 ± 0.05^{a}
d 26 to 28 pp	3.19 ± 0.07	2.89 ± 0.08^{a}	2.89 ± 0.04^{a}	3.28 ± 0.05^{b}
d 29 to 31 pp	3.02 ± 0.06	2.77 ± 0.11	2.91 ± 0.06	3.11 ± 0.08
Milk protein, g/d				
d 23 to 25 pp	$1381 \pm 55^{ac*}$	1045 ± 28^{b}	1313 ± 49^{c}	1134 ± 37^{bc}
d 26 to 28 pp	1250 ± 56^{a}	974 ± 57^{b}	1221 ± 40^{a}	1031 ± 4^{b}
d 29 to 31 pp	1215 ± 63^{a}	914 ± 66^{b}	1181 ± 32^{a}	987 ± 56^{b}

[1]M represents cows with high milk yield, m represents cows with low milk yield, P represents cows with high milk protein concentration, and p represents cows with low milk protein concentration.
[abc]Means with alphabetic superscripts indicate differences between groups ($P < 0.05$).
[*+]Means with figurative superscripts indicate differences between timepoints ($P < 0.05$).

It is widely recognized that NEB leads to a marked decrease in milk protein concentration in the immediate postpartum period of dairy cows and therefore to an undesirable loss in average 305-d milk protein yield (DePeters and Cant, 1992, Murphy and O'Mara, 1993 and 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 our experimental trial to evaluate those physiological adaptive responses in cows with significantly different milk protein concentrations during early lactation and concomitant significantly varying milk yield under same housing and feeding conditions.

As expected, classification of the 23 Holstein-Friesian cows in four groups according to milk yield and milk protein concentration also affected yields of milk fat and milk lactose as well as milk fat concentration. Nevertheless, serum metabolites were comparable among groups during the first 8 weeks of lactation. However, we found higher blood serum glucose levels in MP- compared to mp-cows at the day of calving. Because average time of sampling tended to be earlier in MP- (6 h after parturition) than in mp-cows (12.5 h after parturition), the results of blood sampling within 24 h after parturition could be influenced by the physiological high blood glucose level during calving. Furthermore, two weeks before expected calving MP-cows showed higher blood serum concentrations of NEFA compared to Mp-cows

which might reflect a higher energy deficit in those cows. In the present study, ovarian cycle activity was not influenced by milk volume and milk protein concentration during early lactation. Nevertheless, three cows were excluded from statistical analysis of cyclicity because of ovarial cysts. Of those three animals, one mp-cow suffered inflammation of uterus (retained placenta), whereas the other two cows had the highest milk yield during early lactation (49.3 ± 2.0 kg/d). Infectious diseases of genital tract and risk of metabolic imbalances over the course of periparturient period due to high milk yield are the two main reasons leading to decreased fertility (Walsh et al., 2011). Crowe (2008) reported that dairy cows in good nutritional state ovulate around 15 d pp. In the present study, cows ovulated at d 23 ± 2 but within the physiological timeframe approximately up to 30 days pp. Incidence of lameness and retained placenta was highest in dairy cows with high milk yields during early lactation whereas mp-cows had lowest incidence of clinical diseases. Previous studies confirmed that high-yielding dairy cows are more susceptible to diseases (Mallard et al., 1998 and LeBlanc, 2010).

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 behavior such as excessive mooing or restlessness were detected during tied-stall housing. The average DMI of all animals was 16.3 ± 0.75 kg from d 23 until 25 pp which was slightly lower (approx. 17 kg; Figure 7) compared with previously reported DMI for multiparous cows on d 24 pp (Ingvartsen and Andersen, 2000). As expected, mp-cows showed lowest ad lib 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, which was roughly met only by mP-cows (66.9%). DMI of MP-, mp-, and Mp-cows during restriction was still lower (63.6%; 62.0%; 59.5% for Mp-, mp- and Mp-Cows, respectively) due to a marked decrease during the first day of restriction associated with slower adaption to straw-supplemented RD. Moreover, hyperketonemia could have decreased feed intake, but this metabolic challenge had to be faced by all animals during FR.

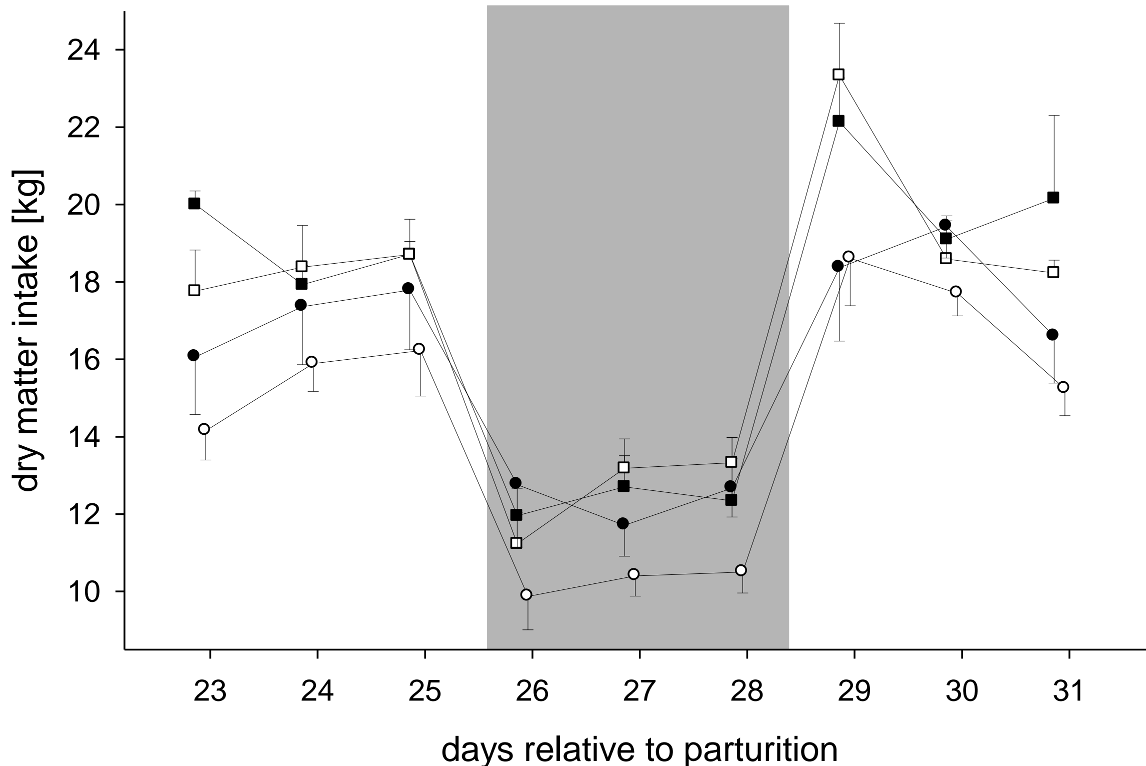

Figure 7. Dry matter intake (kg) during the first eight weeks of lactation and during three days of restricted feeding. M = high milk yield, m = low milk yield, P = high milk protein concentration, p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey bars show the three days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $P<0.001$, group: $P = 0.06$, time × group: $P = 0.80$

Our results are supported by the well-known fact, that during early lactation nutrient energy intake regularly lags behind milk-production related energy demands leading to a NEB (Bell, 1995 and Drackley, 1999). All cows, regardless of group membership, experienced a NEB in the third week of lactation (average -64.9 ± 5.7 MJ $NE_{L,}$ Figure 8) but NEB was even lower compared to Kessel et al. (2008; approx. -35 MJ NE_L). On the first day of FR, a severe decline of EB to values below -110 MJ NE_L in MP- and Mp-cows was found. Subsequently, in those animals EB increased slightly during the following two restriction days. These findings reflect the fast metabolic adaptation of high-yielding animals to increased NEB. In low-yielding cows, the decline in EB was more moderate and remained on the level of the first day of FR during remaining FR period. In agreement with Nielsen et al. (2003) and Agenäs et al. (2003) milk protein concentration of all cows was unaffected by FR.

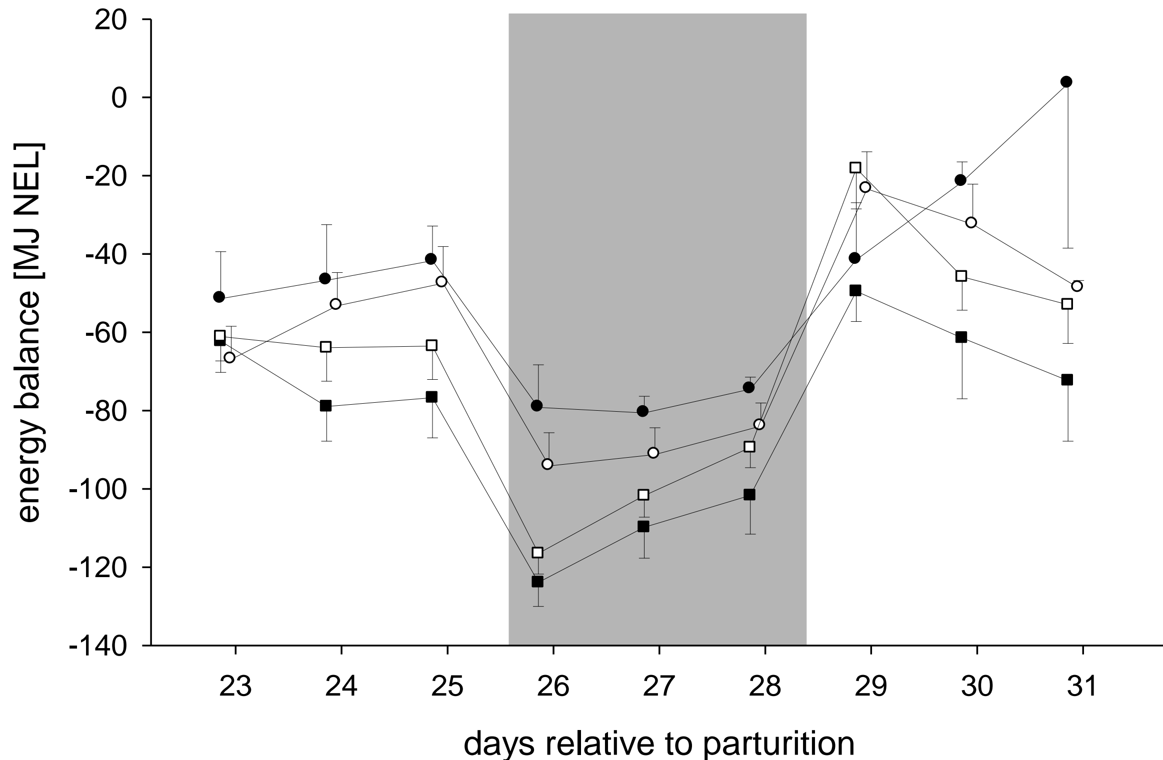

Figure 8. Energy balance (MJ NEL) during the first eight weeks of lactation and during three days of restricted feeding. M = high milk yield, m = low milk yield, P = high milk protein concentration, p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey bars show the three days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $P<0.001$, group: $P< 0.01$, time × group: $P = 0.54$

Agenäs et al. (2003) illustrated a distinct decline in milk protein concentration during subsequent first two days of realimentation. In our study, milk protein concentration also declined to a nadir one day after FR, particularly in mP-cows. As expected due to the decreased milk yield and milk protein concentration (Figure 9 and 10), milk protein yield also declined over the course of FR. Moreover, in all groups milk fat concentration and yield did not vary during FR. 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 a decrease in milk fat yield (Velez and Donkin, 2005). Throughout our experiment, milk lactose concentration was constant in all cows which can be explained by the osmotic role of lactose and the fact that milk volume is mainly depending on lactose synthesis (Peaker, 1978). As shown before, lowest level of protein content was reached on the first day of refeeding, while milk fat and lactose content were not responsive to the reduced feeding level. The subsequent minimum of protein content in FR is not unusual, as fat and lactose

synthesis have top priority due to breeding preferences over the last decades. Also, fat mobilization 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 fat-protein ratio (FPR) more than 1.5 during early lactation are at risk for ketosis or are already affected with it (Heuer et al., 1999). Almost all cows in our experiment, regardless of classification, showed higher FPR values before and during FR.

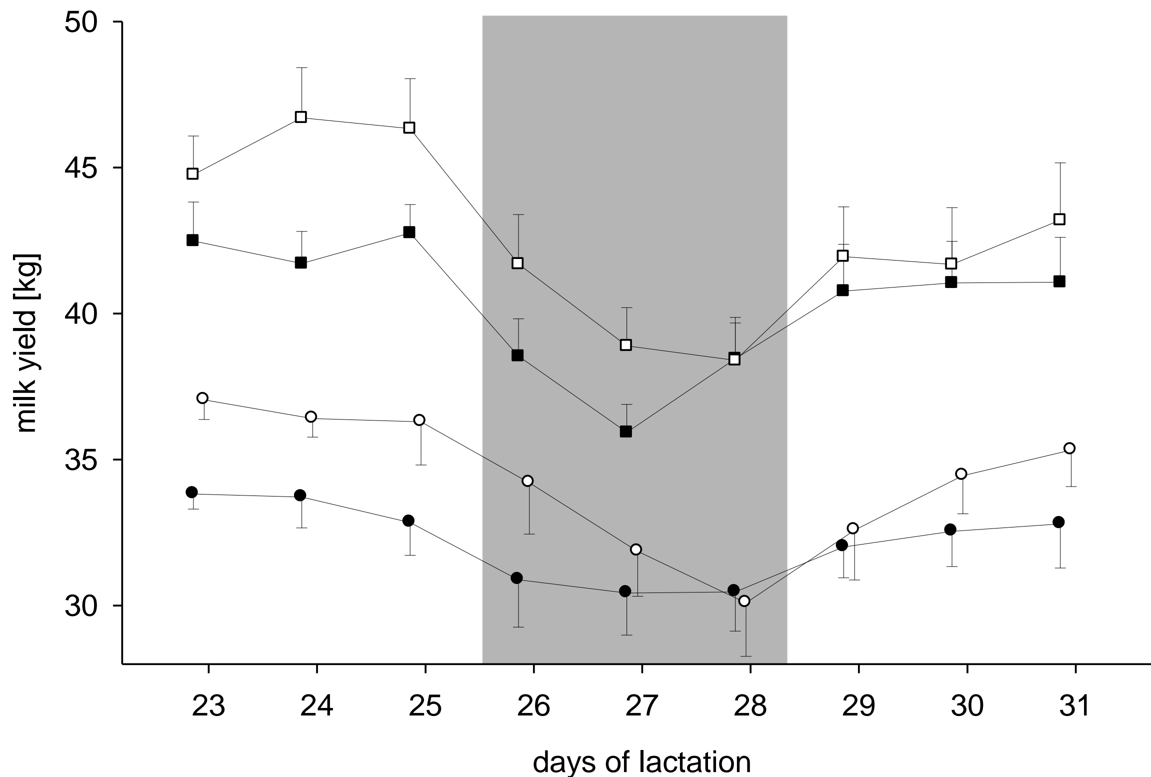

Figure 9. Milk yield (kg) during three days of restricted feeding. M = high milk yield, m = low milk yield, P = high milk protein concentration, p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey bars show the three days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: *P*<0.001, group: *P*<0.001, time × group: *P* = 0.22

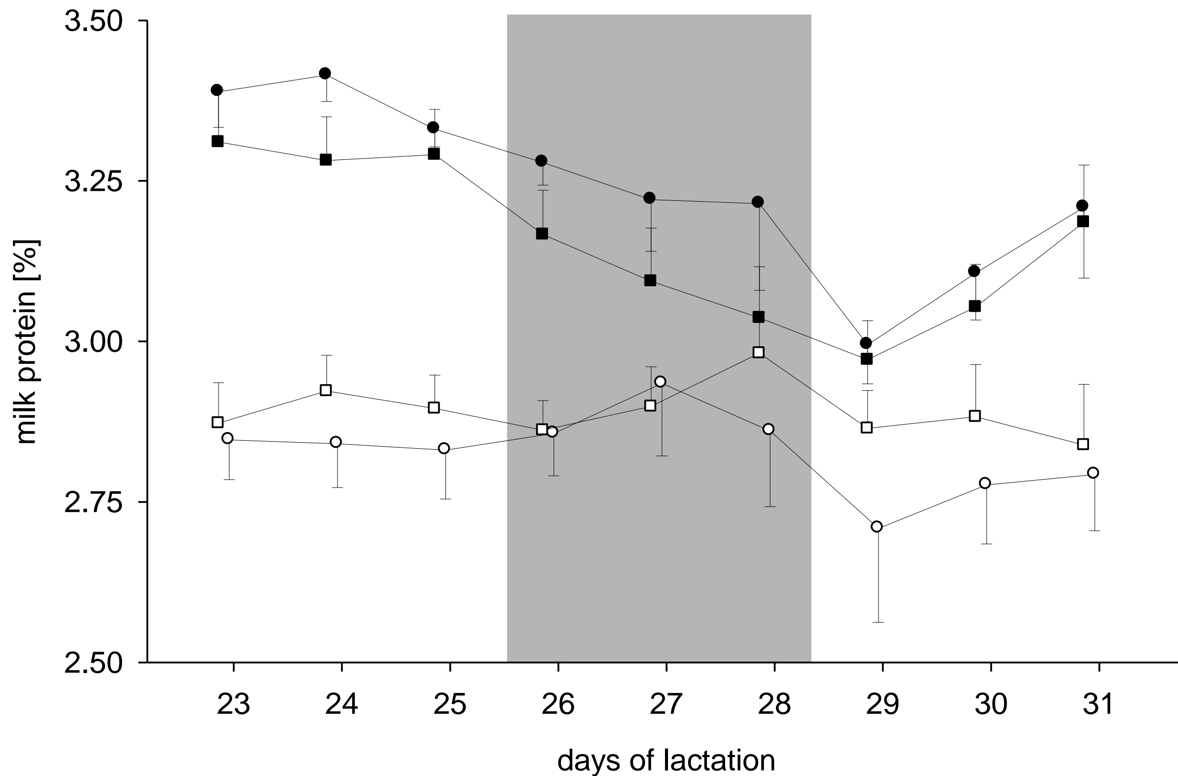

Figure 10. Milk protein (%) during three days of restricted feeding. M = high milk yield, m = low milk yield, P = high milk protein concentration, p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey bars show the three days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $P = 0.26$, group: $P<0.001$, time × group: $P = 0.23$

As previously shown, blood serum glucose levels decreased (Figure 11) and blood serum NEFA concentrations (Figure 12) increased in all groups and reached the initial level during subsequent ad lib feeding (Nielsen et al., 2003; Loor et al., 2007). The steep decrease of glucose far below the basal level of 3.0 mmol/L (Rosenberger, 1990) in high-yielding dairy cows could be explained by the largely distribution of blood glucose to milk synthesis. Insufficient energy supply results in lipolysis of adipose tissue (Mashek and Grummer, 2003). This is indicated by the higher concentration of circulating NEFAs in blood, which are supplied to gluconeogenesis and β-oxidation in hepatocytes. In the present study, average blood serum NEFA levels rose in all groups above threshold levels of 1,000 µmol/L during restricted feeding, except in mp-cows. Lower serum NEFA levels in early lactation for mp-cows suggested a more stable metabolic status together with a sustained physiological serum glucose concentration. In addition, due to deficiency of glucose, product of β-oxidation acetyl-CoA is not metabolized in citrate-cycle and induces ketogenesis

during NEB (Zammit, 1983). Blood serum BHBA levels increased drastically up to the third day of FR (Figure 13) above threshold values for subclinical ketosis of 1.2 -1.4 mmol/L in all groups (LeBlanc, 2010). However, our 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. Therefore, individual regulation of these metabolic adaptations requires further investigations.

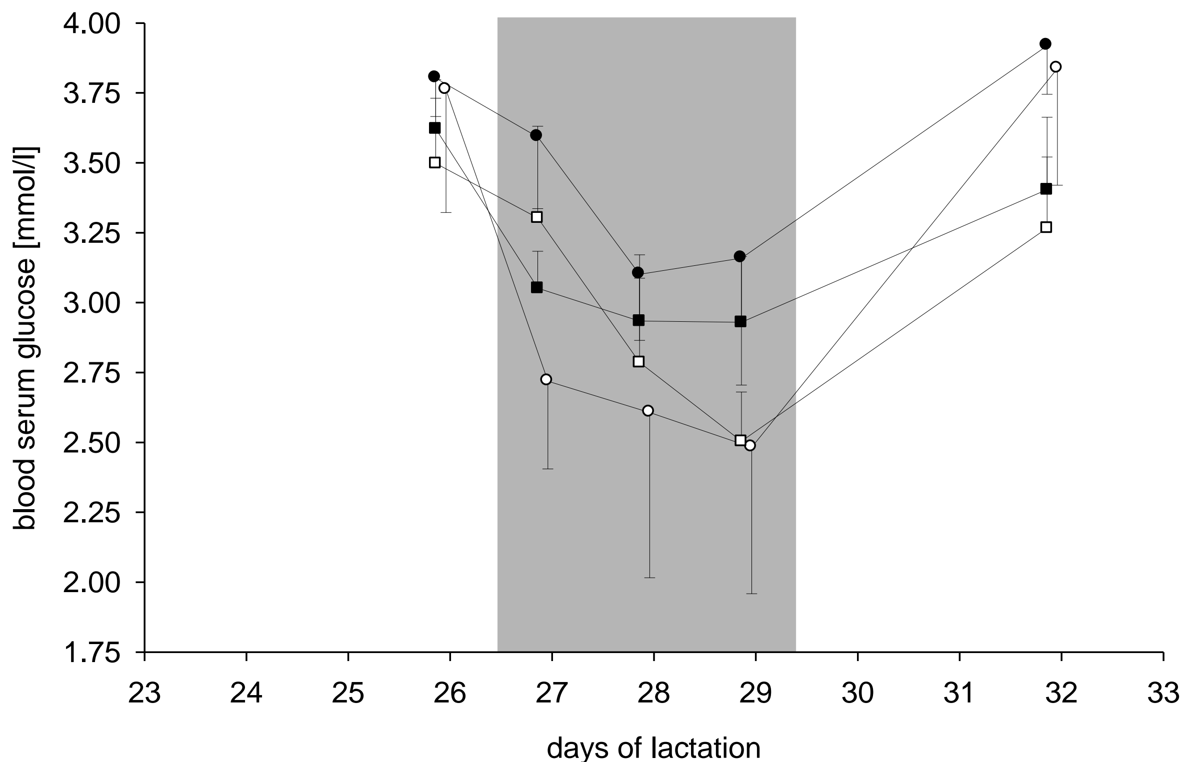

Figure 11. Blood serum glucose levels (mmol/L) during three days of restricted feeding. M = high milk yield, m = low milk yield, P = high milk protein concentration, p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey bars show the three days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $P<0.001$, group: $P = 0.40$, time × group: $P = 0.61$

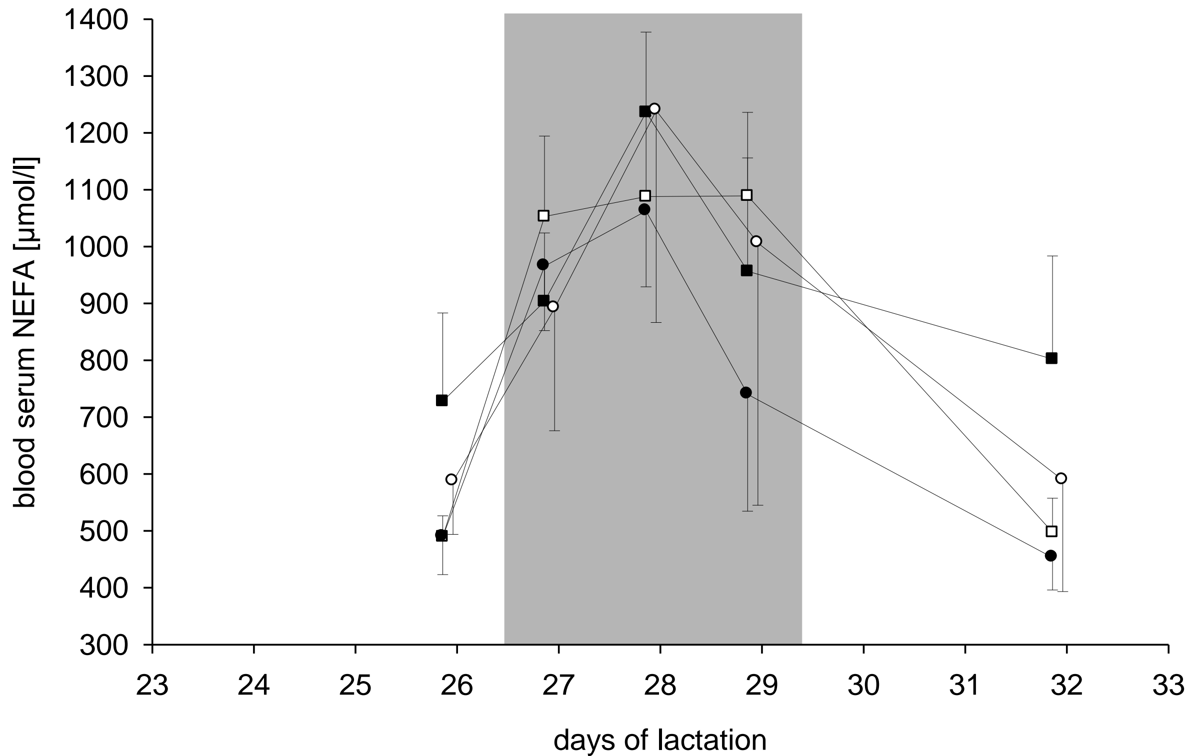

Figure 12. Blood serum non-esterified fatty acid levels (µmol/L) during three days of restricted feeding. M = high milk yield, m = low milk yield, P = high milk protein concentration, p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey bars show the three days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $P<0.001$, group: $P = 0.71$, time × group: $P = 0.79$

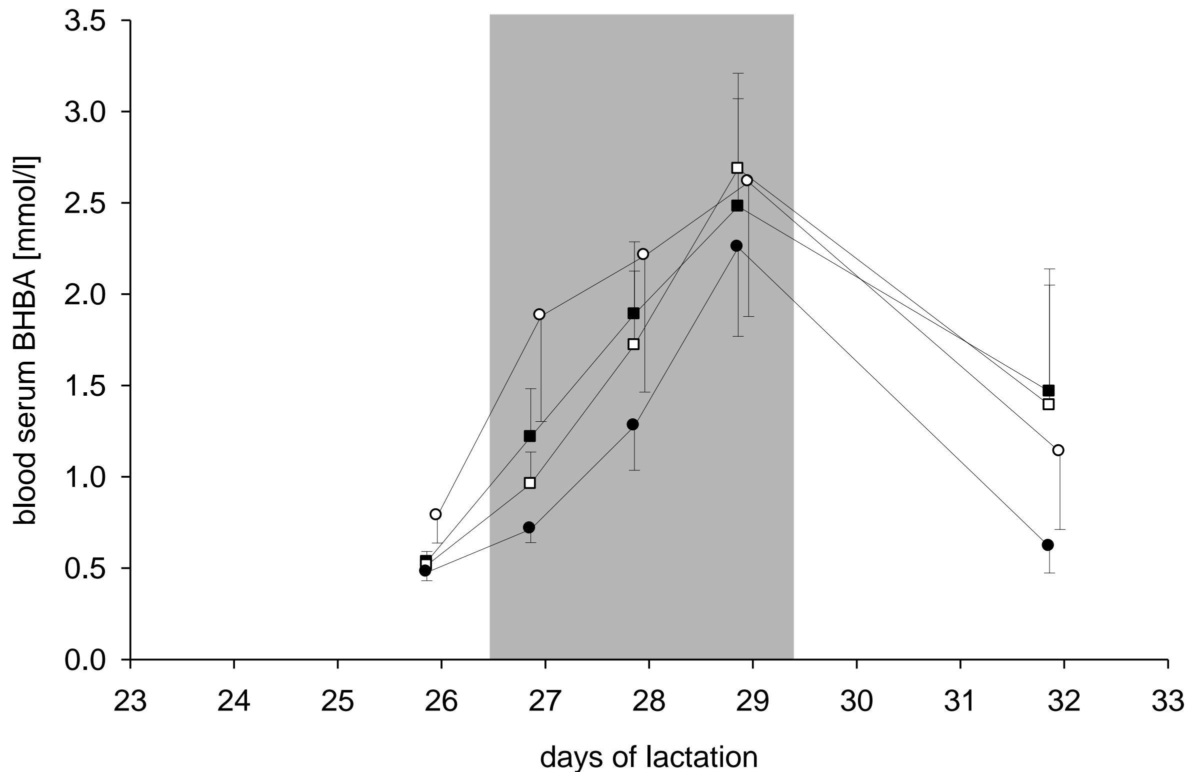

Figure 13. Blood serum β-hydroxybutyric acid levels (mmol/L) during three days of restricted feeding. M = high milk yield, m = low milk yield, P = high milk protein concentration, p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles ○, Mp-cows as empty squares □ and mP-cows as filled circles ●. Grey bars show the three days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: P<0.001, group: P = 0.80, time × group: P = 0.14

One principal aim was to analyze gene networks related to hepatic metabolism in early lactation and particularly after an induced FR (d 29 pp; Figure 14). Due to the enormous increase of blood serum concentrations of NEFA and BHBA during FR we suggested that our FR model was appropriate to enhance hepatic fatty acid oxidation, gluconeogenesis and ketogenesis, diminished fatty acid synthesis and modified amino acid catabolism during early lactation. The nuclear protein PPAR-α is a mediator of NEFA to mitochondrial fatty acid oxidation and upregulates genes involved in ketogenesis (Mandard et al., 2004). However, mRNA levels of PPARA did not correlate to levels of transcripts encoding for ACADVL and CPT1A in contrast to previous findings (Drackley 1999). At d 15 and 29 pp mRNA levels of genes related to β-oxidation (ACADVL and CPT1A; enoyl CoA hydratase, ECHS1; and glycerol-3-phosphate acyltransferase, GPAM; respectively) was lowest in mp-cows. Based on higher milk fat yields in Mp-cows, those cows were expected to experience increased

lipolysis of body fat tissue. Concerning the de novo fatty acid synthesis, mRNA levels of acetyl-CoA carboxylase α (ACACA) were lower after calving, at d 15 pp and after FR in all animals, regardless the classification. These results go in line with Loor et al. (2007) who found down regulation of ACACA associated with FR and ketosis in high-yielding dairy cows.

NEFA blood levels and its uptake in liver tissue influence expression of transcription factors PPARA and HNF4A as well as expression of liver triacylglycerol content-related genes SREBF1 and GPAM (Loor et al., 2005). It is well established, that transcript abundances for SREBF1 and GPAM increase gradually, yet markedly throughout early lactation (Loor et al., 2005). Kim et al. (2004) and Romics et al. (2004) reported that upregulation of SREBF1 in mice was followed by upregulation 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. However, only in Mp-cows a further increase was measured during FR in both genes, which might suggest a higher risk of liver-related disorders. HNF4A plays an important role for PPARA in fatty acid oxidation and gluconeogenesis (Odom et al., 2004), through binding to the promoter region of ACADVL and PCK1 (Loor et al., 2005). Our results indicated higher mRNA levels of HNF4A, which was associated with increased fatty acid oxidation. As lactation advanced, mRNA levels of PCK1 increased from d 15 pp through d 57 pp. Greenfield et al. (2000) reported comparable results. The upregulation of PCK1 during early lactation in our study is associated with the large demand of glucose for milk synthesis and describes increased gluconeogenesis initiated by FR. During calving, mRNA levels of PCK2 were lower in Mp-cows compared to mp- and mP-cows. Therefore, the absence of increased mRNA levels of PCK2 around calving (Loor, 2010) was associated with the previously reported higher risk for liver-related disorders in Mp-cows. With onset of lactation, increasing milk yield and demand for lactose is associated with upregulation of mRNA levels of PC (Greenfield et al., 2000). At d 15 pp, transcript abundances of PC were lowest in mp-cows and highest in mP-cows. Due to the high milk production in high-yield dairy cows, higher transcript abundances of PC were presumed. During periparturient period, hepatic ketogenesis occurs frequently in dairy cows with HMGCS2 acting as a controlling enzyme (Loor et al., 2005; Voet and Voet, 2004). Present results showed higher mRNA levels of HMGCS2 after FR which are supported by Hegardt et al. (1999) who

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. Liver tyrosine aminotransferase (TAT) and CTSL are proteinases associated with AA catabolism, which is diminished during periparturient period (Loor et al., 2005). Our data showed downregulation of CTSL in MP-cows at d 1 and 15 pp, whereas CTSL was upregulated in mp-cows. Additionally, we measured highest mRNA levels of TAT and CTSL after FR. Enhanced activities of TAT and CTSL are associated with increased amino acid fragments, partly used for gluconeogenesis or ketogenesis.

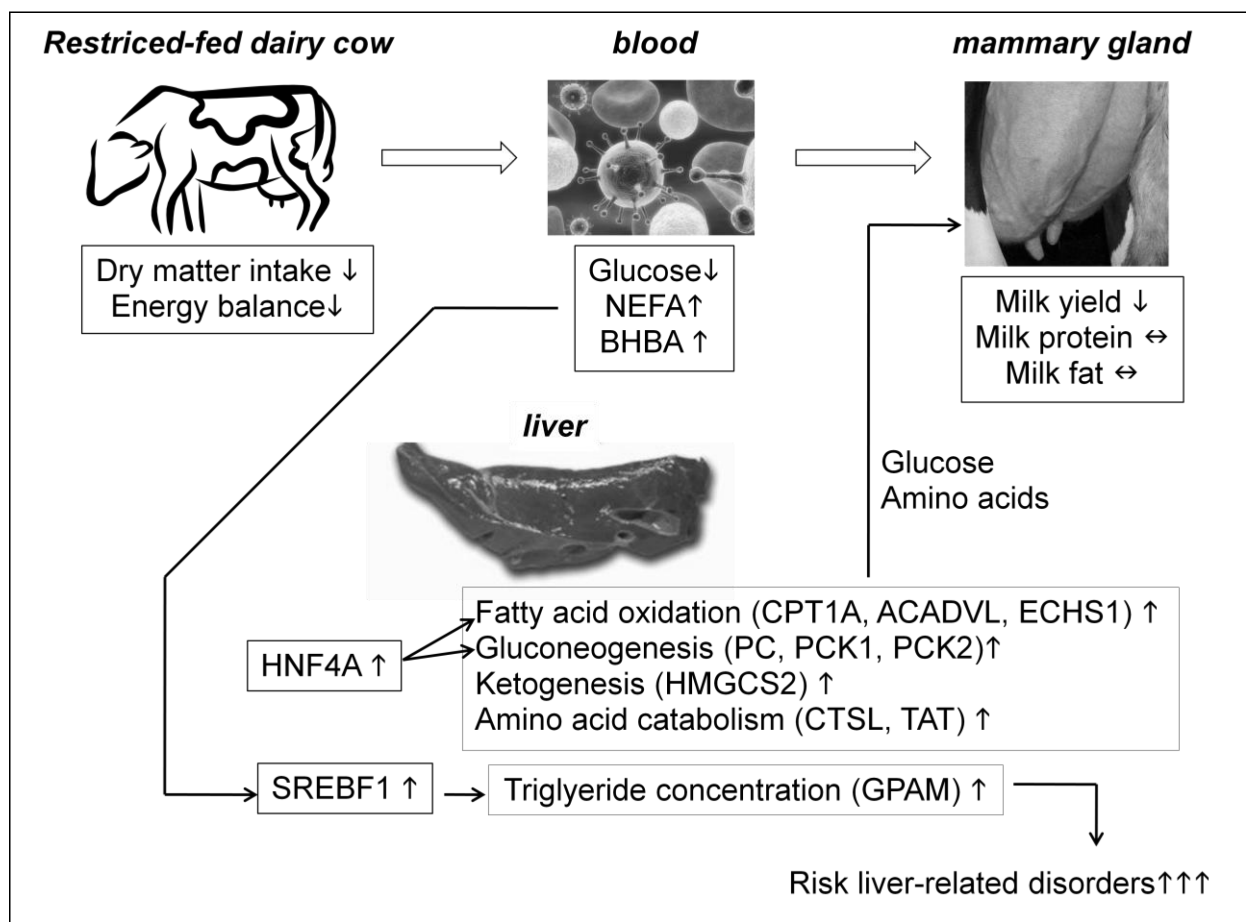

Figure 14. Metabolic interactions among blood serum parameters, mammary milk synthesis and gene expression in liver (adapted from Loor et al., 2005) in dairy cows after 3 days of restricted feeding during early lactation. Restricted feed intake results in calculated negative energy balance and decreased blood glucose concentrations, increased blood NEFA and BHBA levels and causes milk yield depression. Cytokines from liver increase blood serum NEFA and BHBA levels. Circulating NEFA are ligands for HNF4A resulting in its upregulation and downstream activation of genes involved in fatty acid oxidation (CPT1A, ACADVL, ECHS1), gluconeogenesis (PC, PCK1, PCK2), ketogenesis (HMGCS2) and amino acid catabolism (CTSL, TAT). The end result initated by HNF4A upregulation is net hepatic glucose synthesis and sparing of amino acids for milk synthesis. Upregulation of SREBF1, via cytokines or

fatty acids, and GPAM is associated with greater concentrations of liver triacylglycerol and therefore increased risk for hepatic health disorders.

In conclusion, high yielding dairy cows with low or high milk protein concentrations during early lactation resulted in comparable milk protein yields during the first nine weeks of lactation. However, physiological adaptation to a metabolic challenge seemed to be in part diminished in Mp-cows. Therefore, efforts to increase milk protein concentration in high yielding dairy cows during early lactation could be a lucrative, sustainable and animal-appropriate management tool to increase dairy economic outcome and to reduce metabolic imbalances.

4.3 Comparison of expression of several genes in pBMEC purified from milk and harvested from cell culture

A total number of 27 morning milk samples was obtained from 14 cows at d 15, 57 and 113 pp to compare mRNA expression of milk protein genes, STAT5A, glucocorticoid receptor (GR), runt-related transcription factor 2 (RUNX2) and SLC2A1 in magnetic bead-isolated pBMEC (BE) and pBMEC harvested from cell culture (CE).

Compared to mammary gland biopsies and mammary tissue from slaughtered cows, raw milk contains a relatively small number of pBMEC. But the ability of pBMEC to attach to the substrate in cell culture enable to increase number of pBMEC and demonstrated that shed cells are not necessarily dead (Buehring, 1990). Expectedly, number of harvested pBMEC was higher in samples from cell culture compared to number of pBMEC purified with magnetic beads. Consequently, amount of extracted mRNA was higher in CE. Nevertheless, RNA quantity of BE was sufficient for qPCR analysis (Table 12).

Table 12. Comparison of two pBMEC isolation methods.

Item[1]	Isolation method					
	pBMEC purified with beads (BE)			pBMEC from cell culture (CE)		
DIM	15	57	113	15	57	113
Number of samples	12	10	5	12	10	5
Number of MEC for RNA extraction,	1.2 ± 0.02[a]	1.4 ± 0.03[a]	1.3 ± 0.03[a]	521 ± 60[b]	391 ± 93[b]	483 ± 68[b]

(×10^3)						
RNA, µg	3.6 ± 0.4[a]	3.0 ± 0.8[a]	4.4 ± 1.5[a]	8.0 ± 1.0[b]	6.8 ± 1.3[b]	9.3 ± 1.1[b]
A 260/280	1.64 ± 0.07[a]	1.53 ± 0.08[a]	1.78 ± 0.09[a]	2.14 ± 0.00[b]	2.18 ± 0.01[b]	2.15 ± 0.00[b]
A 260/230	0.73 ± 0.09[a]	0.52 ± 0.10[b]	0.68 ± 0.20[ab]	1.15 ± 0.21[c]	1.00 ± 0.22[c]	1.12 ± 0.26[c]

[1]Values are presented as LSM ± SEM.

The epithelial keratins have been found to be useful markers for epithelial cells (Taylor-Papadimitriou et al., 1989). In this context, keratin 8 was obtained as a marker. The higher amount of pBMEC in cells from cell culture caused a 19-fold higher mRNA abundance of keratin 8 (KRT8) in CE compared to BE (Figure 15).

Regarding the milk protein gene expression in CE, levels of transcripts were extremely slight for CSN1S1 and CSN1S2, low for CSN2, CSN3 and LALBA and did not exist for LGB, whereas in BE high transcript abundances for the six major milk protein genes were measured. Due to the omitted lactogenic hormones, prolactin, insulin and hydrocortisone in the culture medium, CE had no ability for milk protein synthesis. Therefore, milk protein gene expression was minimized or absent (Figure 15).

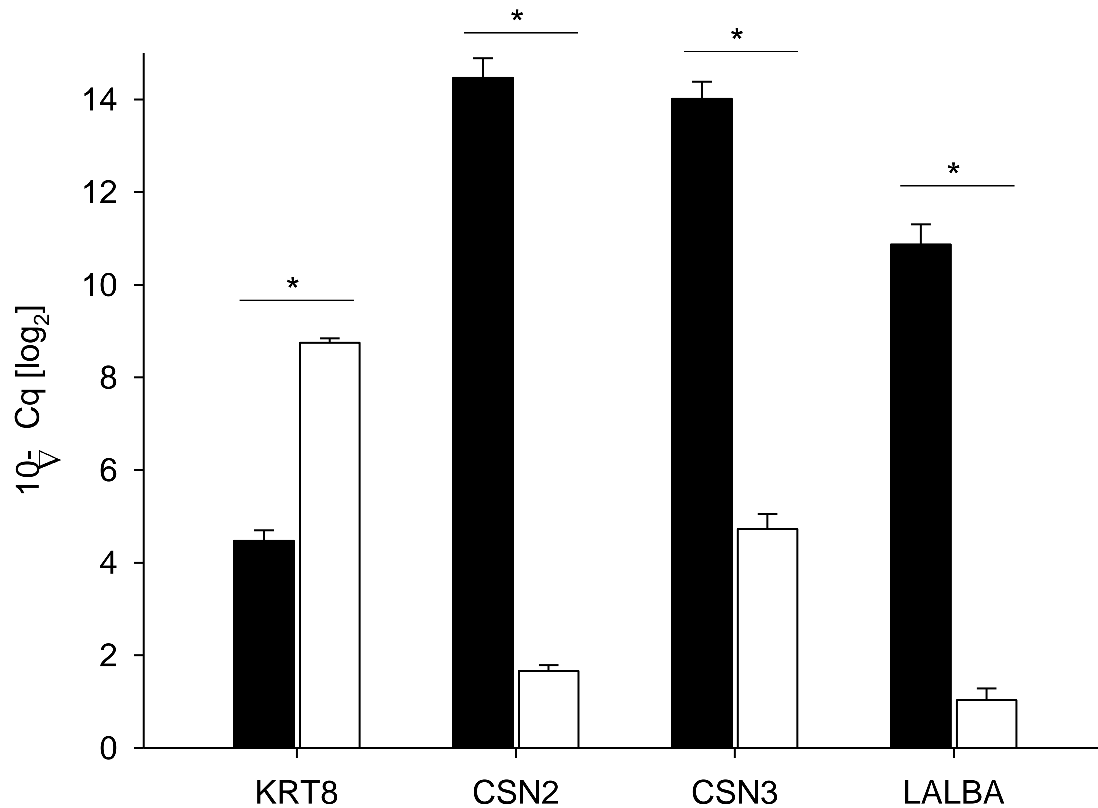

Figure 15. Transcript abundance of Cytokeratin 8 (KRT8), β-casein (CSN2), κ-casein (CSN3) and α-lactalbumin (LALBA) during lactation in mammary epithelial cells immunomagnetic purified from bovine milk (BE; black bars, ■) and harvested from cell culture (CE; empty bars, □). Values are LSM ± SEM. Asterisks indicate significant differences between BE and CE

The primary transcription factor STAT5A is responsible for the signal transduction of prolactin in the mammary gland and all milk protein genes contain at least one STAT5 binding site (Rosen et al., 1999). In addition, varies of hormones, growth factors and cytokines can activate STAT5 (Wood et al., 1995; Gouilleux et al., 2005). Furthermore, STAT5A is expressed in numerous tissues (Kazansky et al., 1995) and is in the mammary gland not limited to lactation (Rosen et al., 1999). Level of transcripts encoding for STAT5A were 3-fold higher in BE compared to CE. Although the transcription factor STAT5 is permanent expressed in pBMEC and therefore measurable in cell culture, missing concentration of prolactin in the culture medium might have been resulted in lower mRNA levels of STAT5A in CE (Figure 16).

Although culture medium was without lactogenic hormones and receptors met in a deficit in stimuli, mRNA of receptors for glucocorticoids was still measurable after

cultivation of pBMEC for four weeks. Level of transcripts for GR were even higher in CE compared to BE ($P = 0.03$; Figure 16).

Furthermore, RUNX2 is a master regulator of bone development and is also expressed in MEC (Barnes et al., 2003; Inman and Shore, 2003). Three essential regulatory elements have been identified in the promoter of CSN2 (Inman et al., 2005): the renowned transcriptional activation via STAT5 and GR and contrary the little known molecular mechanism by the RUNX2/OCT1 complex. Although omitted lactogenic hormones in culture medium mRNA levels of RUNX2 were higher in CE compared to BE. In addition, levels of transcripts encoding for the three CSN2 promoters were measurable in CE but there was no activation of CSN2 transcription without lactogenic hormones (Figure 16).

SLC2A1 is known as the predominant facilitative glucose transporter in the lactating bovine mammary gland (Zhao et al., 1999) and plays a key role in maintaining glucose homeostasis during lactation (Bell and Bauman, 1997). Komatsu et al. (2005) detected no change in the mRNA expression of SLC2A1 between peak and late lactation, but mRNA was barely detectable in dry cows. Furthermore, transcript abundances of SLC2A1 were higher in CE compared to BE ($P < 0.001$). Nevertheless, the applied cell culture medium contained glucose. Therefore, the glucose transporter, member 1, which is concentrated in the cells of blood-tissue barriers, was responsible for basal glucose uptake. Higher transcript abundances of SLC2A1 in CE are explained by the constant and potentially higher amount of utilizable glucose in the culture medium compared to glucose supply in the mammary gland (Figure 16).

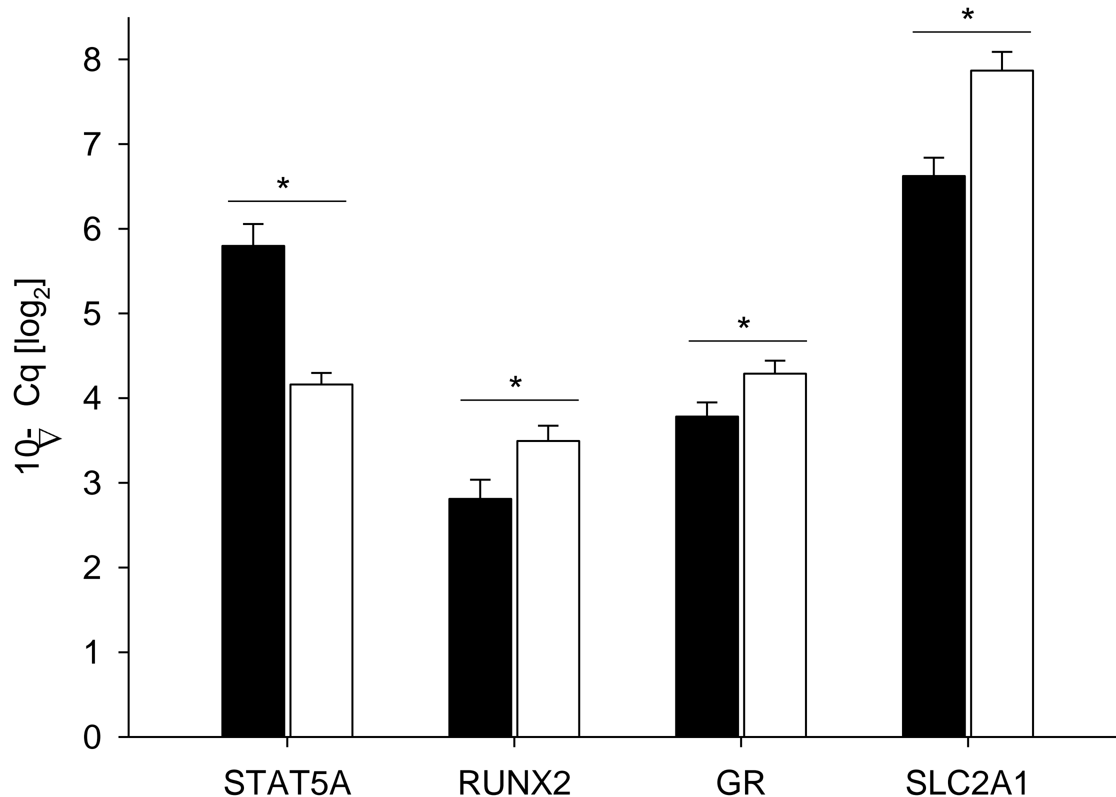

Figure 16. Transcript abundances of signal transducer and activator of transcription 5A (STAT5A), runt-related transcription factor 2 (RUNX2), glucocorticoid receptor (GR) and solute carrier family 2, member 1 (SLC2A1) during lactation in mammary epithelial cells immunomagnetic purified from bovine milk (BE; black bars, ■) and harvested from cell culture (CE; empty bars, □). Values are LSM ± SEM. Asterisks indicate significant differences between BE and CE

MEC culture models and especially lactating MEC culture models are useful for studies of milk synthesis and milk protein synthesis. Nevertheless, cell cultures demand a high range of morphological and functional adjustment strategies from the MEC to the varied physiological and metabolic ambiance. While BE were used immediately after releasing alveolar cell cluster in the mammary gland, CE were culture for four weeks to increase number of pBMEC. After attaching on cell culture flask surface, pBMEC population doubled within 76.6 h (Buehring 1990) and experienced a pivotal alteration of their metabolism. Therefore, differences in gene expression of BE and CE could be explained by the specific treatments.

In conclusion, the comparison between pBMEC purified from milk and pBMEC harvested from cell culture showed different gene expression pattern: in CE milk protein genes could not be measured because of no hormone administration in

culture medium and mRNA levels of STAT5a were lower whereas transcript abundances of GR, RUNX2 and SLC2A1 were higher in CE. pBMEC immunomagnetic purified from milk were evaluated as facsimile representative samples and obtained for further investigations.

4.4 Milk protein gene expression during the first 20 weeks of lactation in pBMEC

A total number of 152 morning milk samples from 23 cows was obtained to study milk protein gene expression during the first 20 weeks of lactation (d 8, 15, 26, 43, 57, 113 and 141 after calving).

The number of total milk cells tended to be lowest on d 43 pp and highest on d 113 pp. Numbers of pBMEC were comparable among all time points, whereas the percentage of pBMEC in relation to total milk cells differed during lactation. Percentage of pBMEC increased from d 8 pp to d 43 and 57 pp, respectively. Afterwards, fractions of pBMEC decreased to d 141 pp. Extracted quantity of pBMEC mRNA did not vary during experimental timeframe. Expression levels of KRT8 were used as a marker for epithelial cells. Transcript abundance of this marker was constant during all time points (Table 13).

Table 13. Number of samples and LSM ± SEM of number of total milk cells, number of separated mammary epithelial cells, RNA quantity and cytokeratin mRNA levels.

	Day of sampling postpartum						
	8	15	26	43	57	113	141
Number of analyzed milk samples, n	21	22	22	23	22	21	21
Number of total milk cells, $\times 10^3$/mL of milk	83 ± 18[ac]	89 ± 34[ac]	77 ± 42[ac]	38 ± 8[b]	48 ± 17[b]	123 ± 80[c]	108 ± 41[a]
Number of mammary epithelial cells, $\times 10^3$/mL of milk	1.2 ± 0.04	1.3 ± 0.04	1.1 ± 0.06	1.2 ± 0.04	1.2 ± 0.03	1.4 ± 0.03	1.1 ± 0.06
MEC (%) of total milk cells	2.0 ± 0.2[a]	3.4 ± 0.4[b]	3.6 ± 0.3[b]	5.6 ± 0.8[c]	6.7 ± 1.0[c]	4.9 ± 0.9[c]	2.2 ± 0.3[a]
RNA quantity, µg	4.1 ±	3.3 ±	2.5 ±	3.6 ±	2.6 ±	3.6 ±	4.5 ±

	0.7	0.3	0.5	0.9	0.5	0.6	0.7
KRT 8 mRNA level, arbitrary value	6.8 ± 0.3	6.7 ± 0.4	6.9 ± 0.4	7.0 ± 0.5	6.6 ± 0.4	7.7 ± 0.5	7.6 ± 0.5

[a]Means with different superscripts within the same row are significantly different.

Percentage of pBMEC and milk yield were correlated during the first 20 weeks of lactation (R = 0.79; *P*<0.05; Figure 17).

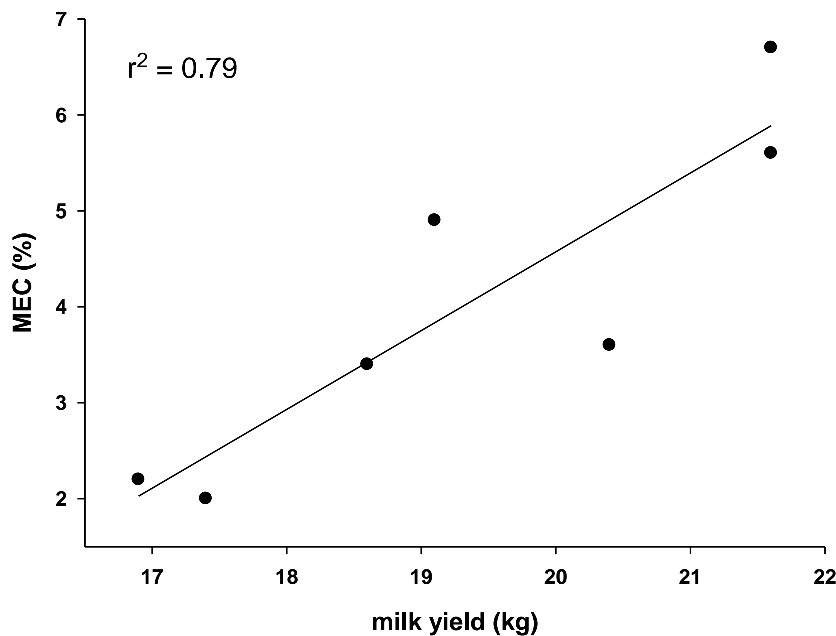

Figure 17. Relationship between milk yield and percentage of milk epithelial cells immunomagnetic isolated of total milk cells (R = 0.79; *P*<0.05). Results are LSM ± SEM of milk samples on day 8, 15, 26, 43, 57, 113, 141 pp

In numerous previous studies on mRNA expression, mammary gland tissue was obtained at one or at few time points via intricate biopsies (Farr et al., 1996; Finucane et al., 2008) or once after slaughtering of precious experimental animals (Capuco et al., 2001; Colitti and Pulina, 2010). In those samples, mRNA was extracted directly from all cells or after a preceding step of pBMEC cultivation (Talhouk et al., 1990; Griesbeck-Zilch et al., 2008). In addition, techniques to culture pBMEC from milk were described (Buehring 1990). Next to the disadvantage of a potential influence of cell culture condition on pBMEC mRNA expression, cell cultivation from mammary gland tissue partly resulted in samples that included a large fraction of non-pMEC, like fibroblasts and adipocytes. To circumvent that drawback, Gomm et al. (1995) described the isolation of pure pMEC from human mammary tissue applying an

immunomagnetic separation technique. Boutinaud et al. (2008) refined that method further to extract pBMEC directly from milk. During milking, the pBMEC detach from the alveolar epithelium and discard the mammary gland within the milk. Moreover, milk is a noninvasive source of viable pBMEC (Boutinaud and Jammes, 2002). The number of pBMEC (2.1×10^{6}) purified from a similar volume of milk (1800 mL) did not vary among sampled time points in our study reflecting a constant renewal during lactation. Boutinaud et al. (2008) isolated approximately 162 d pp comparable 2.7×10^{6} pBMEC from 1750 mL milk of Holstein Friesian cows which comprised 2% of total milk cells. Despite the constant discharge of pBMEC, it is well established that fraction of pBMEC of total milk cells is low (Miller et al., 1991; Boutinaud and Jammes 2002). In our study, pBMEC represented about 2% to 6.7% of total milk cells and that proportion was highest during peak lactation. Capuco et al. (2001) who found a peak of pBMEC number during early lactation with a subsequent decrease during following lactation and concluded that the proportion of pBMEC is influenced by stage of lactation supported these results. However, milk SCC depends mainly on immune status of the udder and only cows with a total somatic cell count below 2×10^{5} per mL were included in the study. Therefore, a varying proportion of pBMEC is expected in cases of clinical mastitis due to increased number of immune cells with or without increased shedding of pBMEC. Contrary to the SCC, number of pBMEC depends predominantly on structure of the mammary epithelium, stage of lactation and milking methods (Boutinaud and Jammes, 2002).

Contrary to the direct cell isolation method described by Boutinaud et al. (2008), a method of indirect cell purification was established in the present work. Total cells were first coated with the monoclonal antibody directed against cytokeratin 8, and afterwards cells-antibody complexes were incubated with the immunomagnetic particles resulting in higher purification results. Previously, it was postulated, that milk yield depended primarily on the size of the mammary gland (Linzell, 1966; Sorensen et al., 1998). However, it was demonstrated more recently that milk yield is regulated by the quantity of mammary secretory cells and their secretory activity (Capuco et al., 2001). According to this, in our study the ratio of pBMEC of total milk cells and milk yield were found to be correlated during the experimental timeframe ($R = 0.79$), whereas correlation of milk yield and total somatic milk cells was lower ($R = 0.62$). Earlier studies revealed that the number of pBMEC found in milk is correlated with milk yield. Annen et al. (2007) supported the hypothesis that increased milk yield

during early lactation is associated with an increased accumulation of new pBMEC during late gestation and increased pBMEC shedding during early lactation. In addition, increased expression of genes related to cell proliferation occurred during increased milk production (Connor et al., 2008).

The relative expression of the six major milk protein genes CSN1S1, CSN1S2, CSN2, CSN3, LALBA and PAEP showed similar patterns during the first 20 weeks of lactation. Maxima of mRNA abundances were reached during the first two weeks of lactation followed by respective declines towards the end of the experimental period (Figure 18).

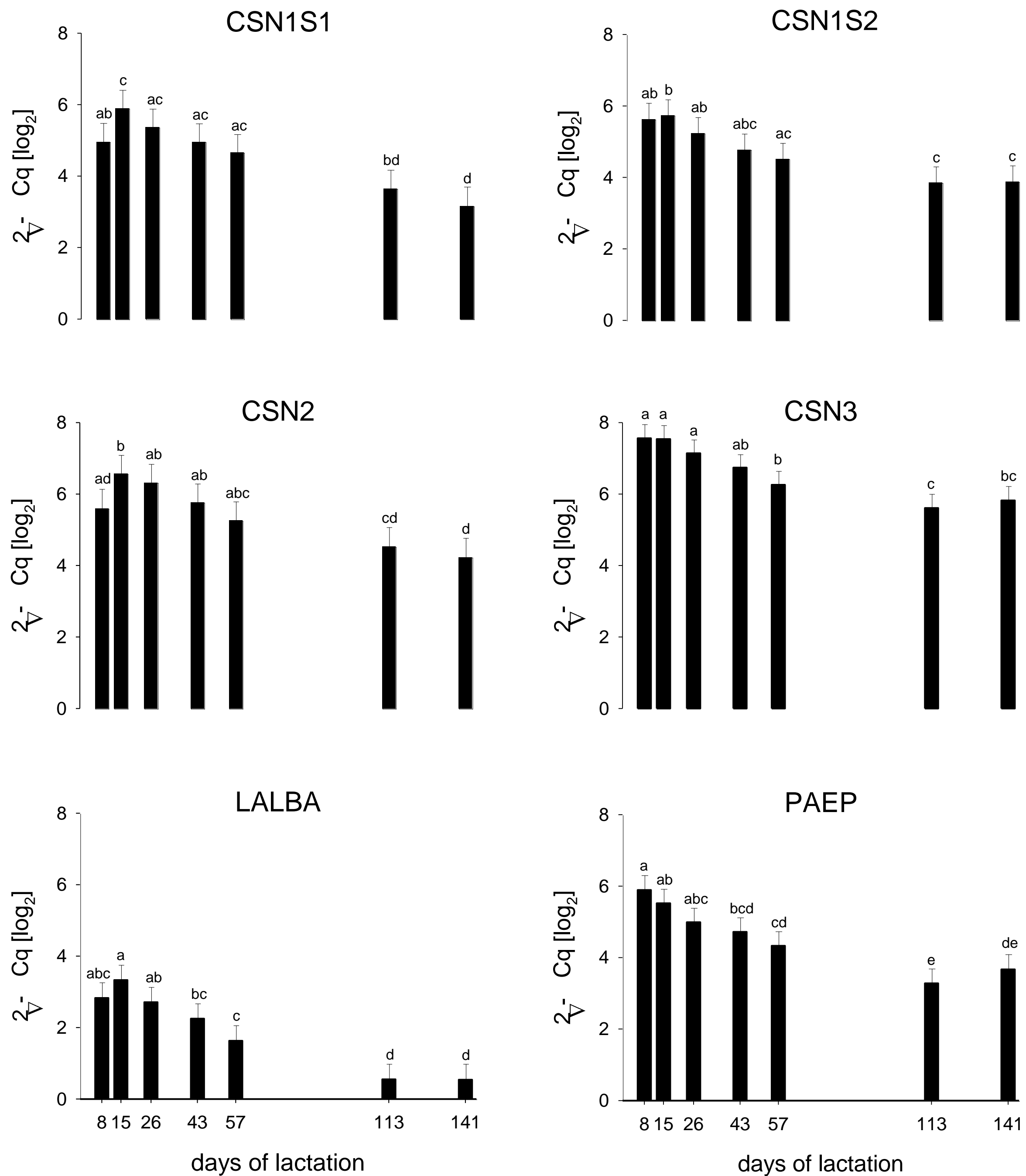

Figure 18. Transcript abundance of α_{S1}-, α_{S2}-, β-, κ-casein, α-lactalbumin and β-lactoglobulin (during the first 20 weeks of lactation in bovine mammary epithelial cells purified from milk. ΔCq was calculated as $Cq_{target\ gene}$ - $meanCq_{refence\ genes}$. Results are shown as $2^{-\Delta Cq}$ ± SEM. Letters indicate significant differences (*P*<0.05)

Although mRNA levels of CSN3 tended to be higher in our studies, general casein mRNA expression was comparable to levels reported by Bevilacqua et al. (2006). In their work similar mRNA abundance of CSN1S1, CSN1S2, CSN2 and CSN3 (all

approximately 25%) was demonstrated in mammary tissue obtained from three lactating cows. Due to applying the immunomagnetic isolation method of pBMEC from fresh milk, it was possible for the first time to determine milk protein gene expression profiles in the same animal over the course of lactation. Nonetheless, major milk protein gene expression patterns in mammary tissue of other species like common brushtail possum or mouse during pregnancy, lactation and dry period were elaborated (Demmer et al., 1998; Stein et al., 2004; Anderson et al., 2007). Colitti and Farinacci (2009) examined gene expression of LALBA in mammary tissue in dairy ewes, collected after slaughtering, during peak (d 30 pp), mid (d 60 pp) and end of lactation (d 150 pp). In contrast to our findings in lactating cows, the relative expression level of LALBA in ewes reached the highest value only at the end of the lactation. Furthermore, Colitti and Pulina (2010) analyzed transcripts of the four caseins CSN1S1, CSN1S2, CSN2 and CSN3 in mammary tissue after slaughter in dairy ewes. Respective to the study of Colitti and Pulina (2010) gene expression of the four caseins was up-regulated during peak, mid and late lactation but down-regulated during pregnancy and involution. Those findings correspond to ovine milk protein composition during lactation. Concentrations of caseins, total albumins and β-lactoglobulin in whole milk increased significantly over the course of lactation (Poulton and Ashton, 1970). No milk protein fractions were analyzed in the present study, but previous studies in dairy cows show different composition during lactation by contrast with ewes. Early reports stated peak concentrations of total caseins and serum proteins approximately five days after calving followed by a decline during the remaining 310 d-lactation period, except for a slight increase during time of peak yield (Larson and Kendall, 1957). In contrast, Ng-Kwai-Hang et al. (1987) determined a decline in concentrations of major milk proteins only between d 30 and 90 pp during peak milk yield followed by a marked increase until d 365 pp concomitant to lowering volumes of milk. Nevertheless, highest total protein production was found, as described previously, during the first months of lactation (Friggens et al., 2007). The ratio of total caseins to whey proteins does not vary depending on stage of lactation, reflecting no changes in the rates of synthesis for both main fractions (Coulon et al. 1998). Yet, relations between specific caseins differed depending on stage of lactation (Kroeker et al., 1985; Çardak, 2009). During the first two months, a marked decrease of α-casein and a reciprocal systematical increase of β-CN as a proportion of the casein fraction were demonstrated. The relative amount of κ-casein

remained constant during the whole lactation cycle. Concentrations of β-LG were on their minimum level during the second month of lactation, whereas proportions of α-LA, which is involved in milk lactose synthesis, decreased with progress in lactation as a result of lowering milk yields. In present study, transcripts of all six milk protein genes were found to peak during the first two weeks of lactation and to decline continuously towards mid lactation. We hypothesized that milk protein gene expression has a pivotal effect on milk protein composition whereas milk protein concentration was not influenced. Bionaz and Loor (2007) confirmed this assumption. In this context, the translational efficiency of milk protein transcripts also has to be taken into account. Bevilacqua et al. (2006) measured equal proportions of casein gene transcripts, which is roughly comparable to our findings. However, the four casein mRNAs were not translated with the same efficiency. They showed that CSN1S1 and CSN2 were translated 3- to 4-fold more efficiently in comparison with CSN1S2 and CSN3 and explained their findings with differences in the mRNA leader region. Due to those differences in translational efficiency, the differences in quantities of milk proteins could be explained. Milk proteins α_{S1}- and β-CN account for the major part of milk proteins (15 g/L and 11 g/L, respectively), whereas α_{S2}- and κ-casein represent only a minor part (both 4 g/L) in skim milk (Farrell et al. 2004). In addition, whey proteins only amount to 5.5 g/L (1.5 g/L α-LA and 4 g/L for β-LG). However, no data is available on translational efficiency throughout lactation, which could be influenced by different factors such as genetics, epigenetic, nutrition, milking frequency, hormonal status or diseases.

Furthermore, milk protein synthesis may be regulated at multiple levels within the mammary epithelial cells including transcription, post-transcription, translation and amino acid supply (Menzies et al., 2009). The genes encoding these proteins are regulated by the complex interplay of peptide and steroid hormones, predominantly the lactogenic hormones prolactin, insulin and hydrocortisone, and cell-cell and cell-substratum interactions. Moreover, the uptake of amino acids from feed and their metabolic conversion are important preludes to milk protein synthesis. Therefore, Shennan and Peaker (2000) reported that the transport rate of amino acids seems to be the limited factor for milk protein synthesis.

The indirect immunomagnetic bead based method was appropriate to isolate pBMEC directly from fresh milk for further qPCR analysis. The percentage of shed pBMEC in relation to somatic milk cells was highly correlated to milk yield. Expression patterns

of the six major milk protein genes in 24 Holstein-Friesian cows were comparable during the first 20 weeks of lactation and respective proportions were comparable to previous findings on casein and whey protein concentrations in milk.

4.5 Gene expression of key enzymes in pBMEC

All morning milk samples for pBMEC isolation, taken from 24 Holstein Friesian cows at d 8, 15, 26, 29, 43, 57, 113, 141, 144 and 155 pp, were used to analyze gene expression of key enzymes during the first 21 weeks of lactation and to study the influence of restricted feeding on their expression profiles. pBMEC were purified from milk samples and mRNA abundances of central key enzymes were quantified by qPCR.

Gene expression profiles of all major milk proteins during the first 21 weeks of lactation were shown in 5.4. Decreased levels of transcripts with increased day of lactation for LALBA and CSN3 were also reported by Bionaz and Loor (2007 and 2011, respectively). Regarding the FR, results showed increased mRNA levels for all milk protein genes after restricted feeding in early lactation (Figure 19), whereas milk yield, milk protein content and yield remained stable at this time.

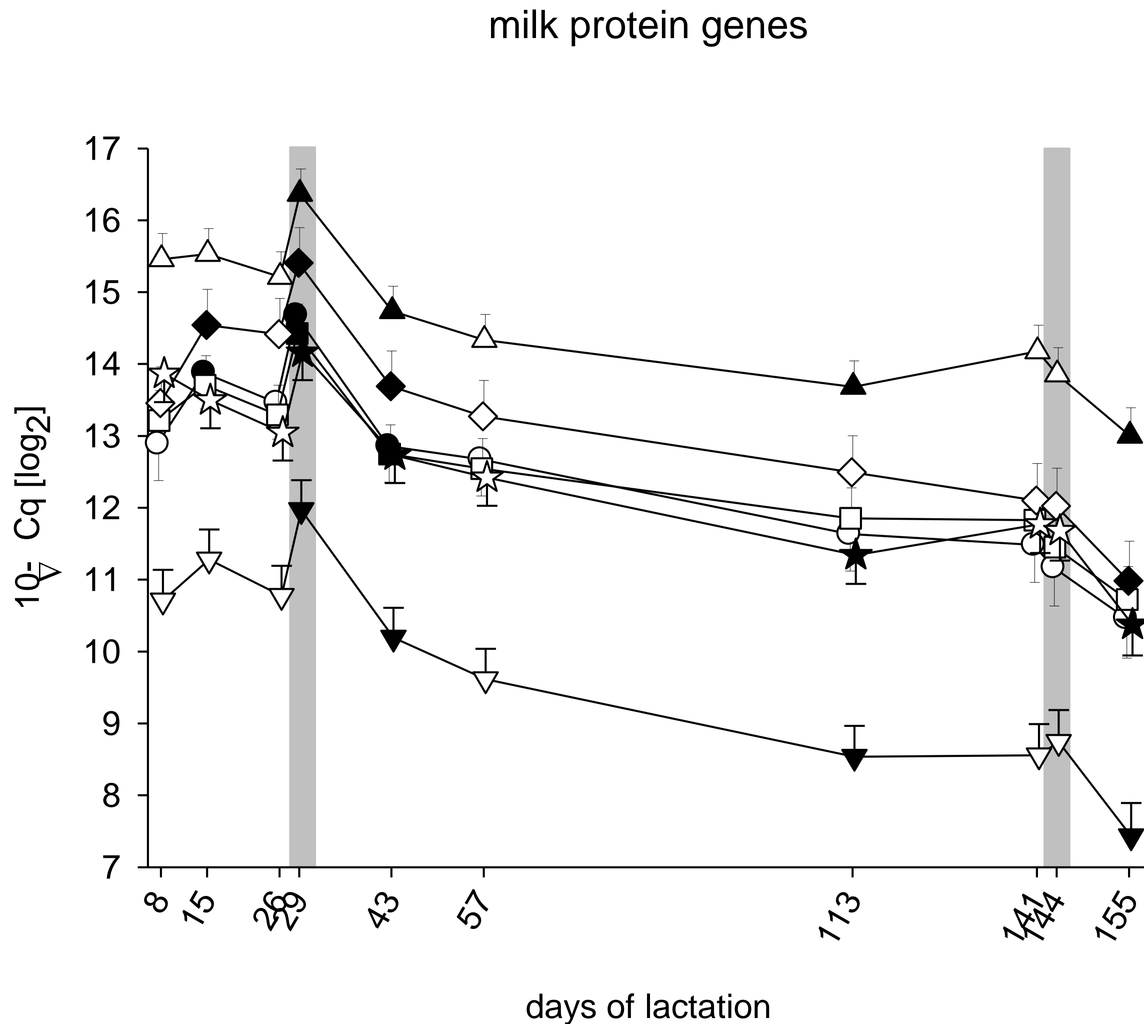

Figure 19. Transcript abundance of CSN1S1 (○), CSN1S2 (□), CSN2 (◇), CSN3(△), LALBA (▽) and PAEP (☆) during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Values that differ significantly from the prior value are filled. Expression of all six genes was significant affected by time (*P*<0.001)

The epithelial keratins have been found to be useful markers for epithelial cells (Taylor-Papadimitriou et al., 1989). In this context, keratin 8 was obtained as a marker for pBMEC. Previous results indicated constant amounts of pBMEC during lactation cycle whereas the percentage of pBMEC of total milk cells increased during early lactation and peaked with peak lactation at d 57 pp (results presented in Figure 16). Due to the unchanged amount of pBMEC we expected similar levels of KRT8-mRNA during experimental timeframe. Surprisingly, increased levels of transcripts encoding for KRT8 were determined until d 26 pp. Thereafter, levels remained on a constant level until end of the study and were unaffected by restricted feeding.

Milk protein synthesis may be regulated at multiple levels within the mammary epithelial cells including transcription, post-transcription, translation and amino acid supply (Menzies et al., 2009). The genes encoding the milk proteins are regulated by the complex interplay of peptide and steroid hormones, predominantly the lactogenic hormones prolactin, insulin and hydrocortisone, and cell-cell and cell-substratum interactions.

JAK2/STAT5 pathway. Prolactin is involved in MEC proliferation and differentiation during pregnancy and is essential for the secretion of milk into alveolar lumen (Riley et al., 2009). Furthermore, it is important for milk protein gene expression and binds to the extracellular portion of prolactin receptor (PRLR) and initiates events in the JAK/STAT signal transduction cascade (Darnell Jr. 1997). mRNA levels of PRLR were constant during the study (Figure 20) and results are confirmed by the work of Bionaz and Loor (2011) who indicated constant PRLR-mRNA expression during the first 240 d of lactation. Auchtung et al. (2003) found an inverse relationship between circulation prolactin and mRNA expression of PRLR in mammary parenchymal tissue of steers exposed to different photoperiods. In general, prolactin causes a down-regulation of its receptor (Gratton et al., 2001). Results from Accorsi et al. (2005) and Bionaz and Loor (2011) who reported constant plasma prolactin levels during early lactation and increased levels until d 150 pp in dairy cows suggest these data. Therefore, PRL induces STAT5A binding activity in mammary gland (Jahn et al., 1997). The primary transcription factor STAT5A is responsible for the signal transduction of prolactin in the mammary gland and all milk protein genes contain at least one STAT5 binding site (Rosen et al., 1999). In addition, varies of hormones, growth factors and cytokines can activate STAT5 (Wood et al., 1995; Gouilleux et al., 1995). Furthermore, STAT5A is expressed in numerous tissues (Kazansky et al., 1995) and in the mammary gland, and it is not limited to lactation (Rosen et al., 1999). Moreover, STAT5A is the key for controlling suppressor of cytokine signaling 2 (SOCS2) transactivation in the mammary gland (Davey et al., 1999). In the present study, levels of transcripts encoding for STAT5A remained unaffected during experimental timeframe, whereas mRNA levels of SOCS2 increased until d 43 pp and declined afterwards (Figure 20). We hypothesized that constant mRNA levels of STAT5A resulted from the continuously influx of lactogenic hormones during lactation cycle and STAT5A is essential for milk protein synthesis but the decreased expression of the milk protein genes during the first half of lactation seems not to be

under control by the STAT5A transcription factor. Bionaz and Loor (2011) also found this argumentation.

Besides prolactin, insulin has a direct effect on the bovine mammary gland and plays an important role in the coordinated induction of milk protein gene expression. Among others, insulin receptor (INSR) and insulin-like growth factor 1 receptor (IGF1R) were reported to be possible stimulators for JAK2 (Gual et al., 1998) and STAT5 (Okajima et a., 1998). Regarding JAK2, transcript abundances increased from d 57 pp until end of the study. Transcript abundance of INSR arose during early lactation, peaked at d 57 pp, and subsequently decreased until end of the study. IGF1R mRNA levels arose during the first 21 weeks of lactation with peak at d 113 and 141 pp (Figure 20). Sharma et al. (1994) compared mRNA level of insulin-like growth factor 1 (IGF1) in mammary gland biopsies among early and late lactation and detected no changes during lactation cycle. High transcript abundance of JAK2 was associated with the high mRNA levels of INSR and IGF1R during the experimental timeframe. In turn, JAK2 triggers a cascade of signaling events that involve the insulin receptor substrate (IRS), followed by the phosphoinositide 3-kinase (PI 3-kinase) and protein kinase B (also known as v-akt murine thymoma viral oncogene homolog 1, AKT1; Harrington et al., 2005). In present study, levels of transcripts encoding for AKT1 were unaffected by time (Figure 20).

The requirement of insulin may primarily be facilitated by the major milk protein transcription factor E74-like factor 5 (ELF5; Menzies et al., 2009). With increased day of lactation, mRNA levels of ELF5 declined continuously. These results were associated with the decreased mRNA levels of all six major milk protein genes during the first 21 weeks of lactation. Bionaz and Loor (2011) also reported the pivotal role of ELF5 in the bovine mammary protein synthesis. They explained that the decrease in CSN3 expression at d 240 pp is a consequence of the decreased transcript levels of STAT5B and ELF5. The combination of prolactin and insulin promoted the phosphorylation of eIF4E-binding protein 1 (4E-BP1), an initiation factor-binding protein, in cow mammary tissue (Barash, 1999). While plasma insulin levels remain on a low level during lactation, Accorsi et al. (2005) found increased plasma prolactin levels until d 150 after parturition in dairy cows. Other studies showed that the expression of ELF5 was induced by insulin in bovine and mouse mammary gland. Those observations confirmed the pivotal role of insulin in the expression and translation of milk-related genes supported by the results of Bionaz and Loor (2011).

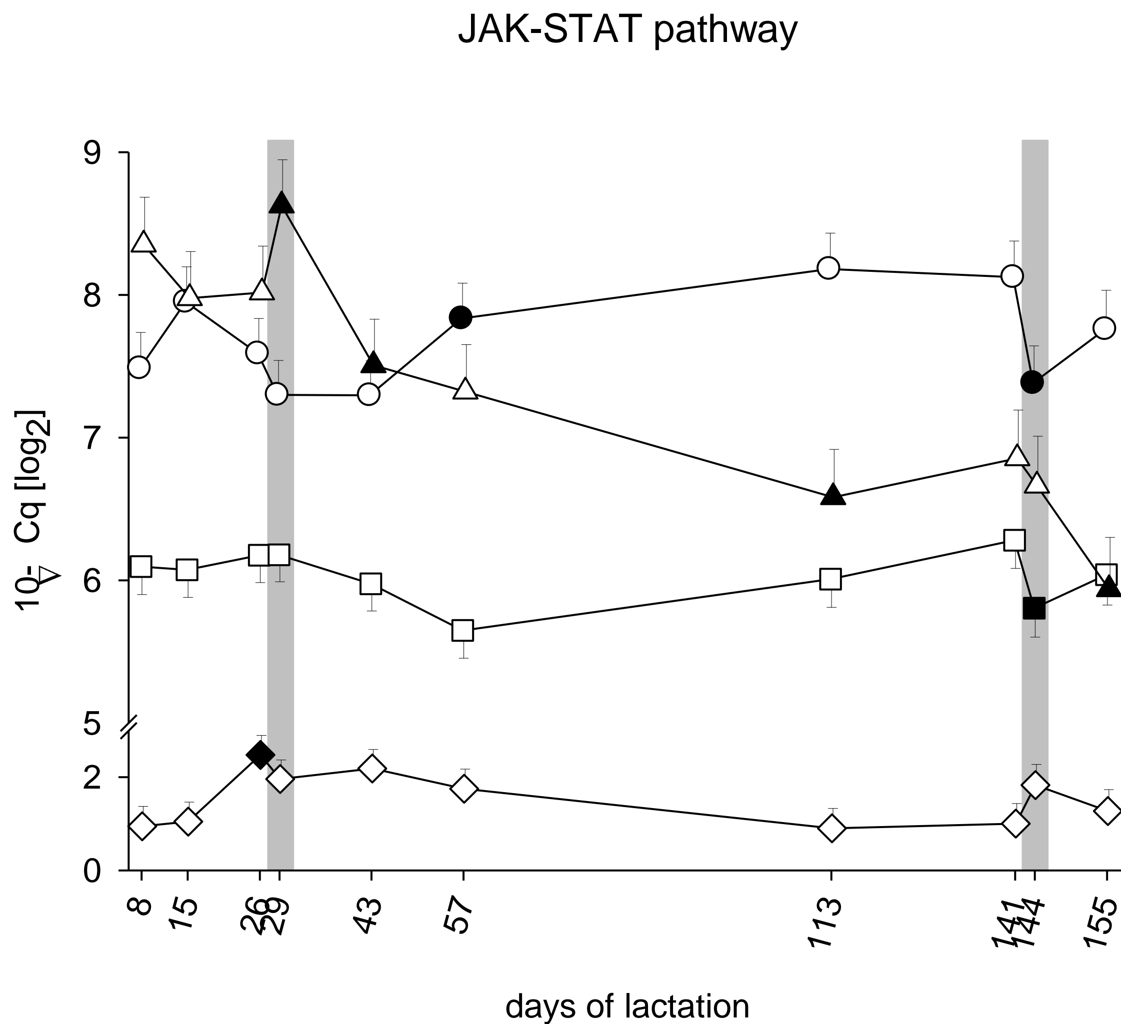

Figure 20. Transcript abundance of JAK2 (○), STAT5A (□), PRLR (◇) and ELF5 (△) during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Values that differ significantly from the prior value are filled. Expression of JAK2 ($P = 0.02$) and ELF5 ($P<0.001$) was significant affected by time

mTOR pathway. Besides the JAK/STAT pathway, recent studies in ruminants had highlighted a crucial role of the mTOR pathway in the regulation of milk protein synthesis (Torien et al., 2010 and Bionaz and Loor, 2011). Similar to growth hormone (GH), IGF-1 also acts through an IRS cascade (LeRoith et al., 1995) and it is likely to account for some of the GH effects in mammary mTOR signaling (Cui et al., 2003). The effects of nutrients and hormones on protein translation are mediated by mammalian target of rapamycin (mTOR) signaling (Yang et al., 2008). The mTOR signaling cascade integrates AA availability, cellular energy status, and endocrine signals to regulate protein synthesis through changes in the phosphorylation status of eukaryotic initiation factor 4E (eIF4E)-binding protein-1 (4E-BP1), a translational

repressor, and p70 ribosomal protein S6 kinase-1 (RPS6KB1; Burgos et al., 2010). In the present study, mRNA abundances for mTOR were not affected by day of lactation. Levels of transcripts encoding for RPS6KB1 decreased during FR in mid lactation. Furthermore, transcripts encoding for 4E-BP1 decreased with increased day of lactation (Figure 21). Due to the restricted availability of nutrients, we expected changes in mRNA levels of mTOR and RPS6KB1 during FR.

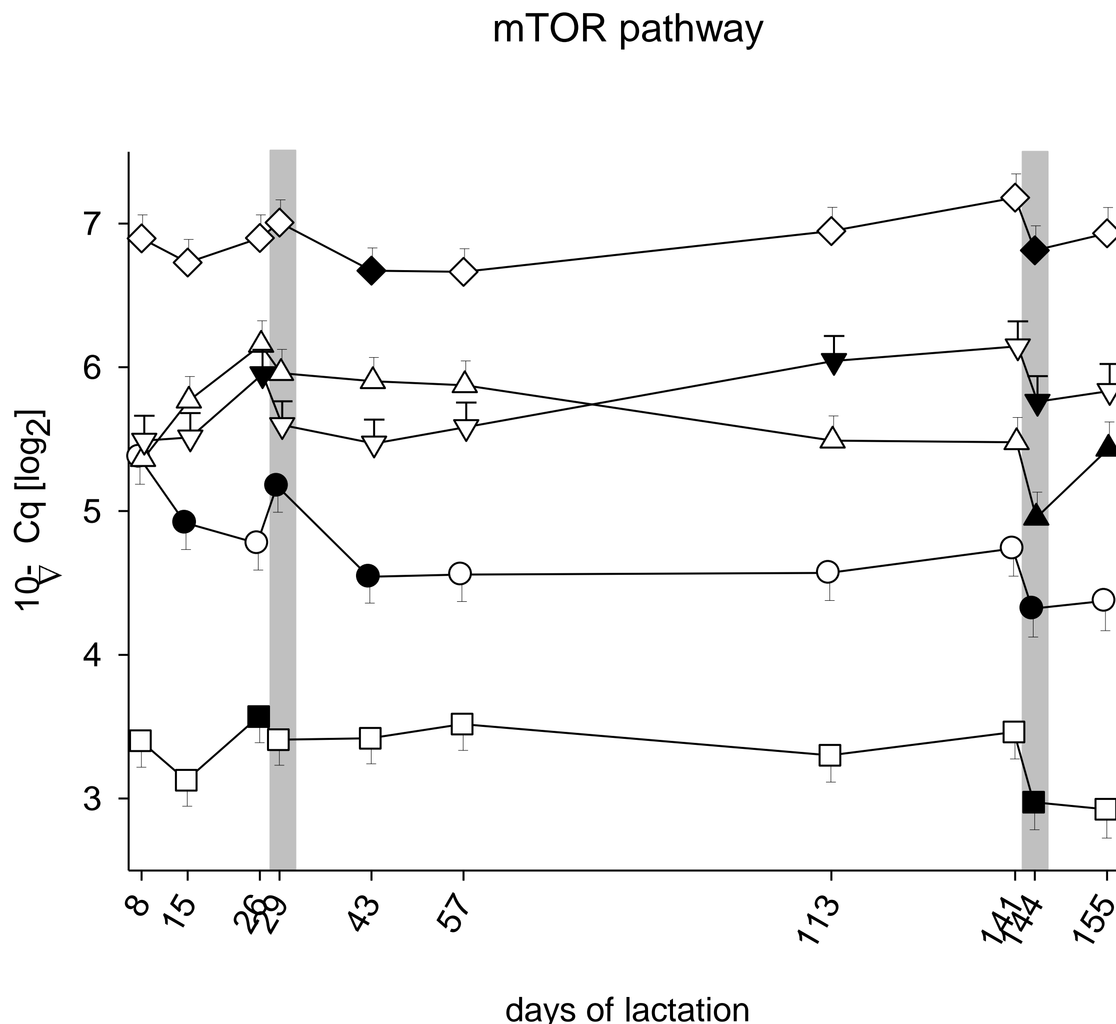

Figure 21. Transcript abundance of EIF4EBP1 (○), RPS6KB1 (□), AKT1 (◇), INSR (△) and IGF-1R (▽) during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Values that differ significantly from the prior value are filled. Expression of EIF4EBP1 (*P*<0.001), RPS6KB1 (*P*<0.05), INSR (*P*<0.001) and IGF-1R (*P* = 0.02) was significant affected by time

β-casein transcription. The CCAAT/enhancer binding proteins (CEBP) play important functional roles in mammary development and lactation and are expressed during pregnancy and involution (Rosen et al., 1999). Furthermore, CEBP beta (CEBPB) is essential for milk protein gene expression and CSN2 contains four CEBPB binding

sites. Therefore, the absence of CEBPB reduces β-casein gene expression and effects whey acidic protein expression undetectable in lactating mice (Robinson et al., 1998 and Seagroves et al., 1998). Transcript abundances of CEBPB increased during the first three weeks after parturition and remained on a high level during the following 20 weeks of lactation (Figure 22). Increasing mRNA expression levels of CEBPB were associated with the onset of lactation and the enormous increase of milk yield during the first weeks of lactation. Although milk protein gene expression decreased until d 155 pp, the continuously high levels of CEBPB were linked with the important relevance for the maintained lactation.

RUNX2 has a functional role in the regulation of gene expression in mammary epithelial cells (Inman and Shore, 2003). Besides STAT5A and GR, RUNX2 is an essential regulatory element and is required for the β-casein transcription via forming a complex with OCT1 (Inman et al., 2005). Moreover, Yin Yang 1 (YY1) is a multifunctional protein that can either activate or repress transcription but it predominantly acts as a repressor of β-casein gene expression (Rosen et al., 1999). Transcript abundances of YY1 did not change during study (Figure 22). Earlier studies reported that lactogenic hormones (Meier and Groner, 1994; Raught et al., 1994) did not change the level of YY1. Although decreased transcript abundances occurred for CSN2, mRNA expression of RUNX2 and YY1 were constant during experimental timeframe.

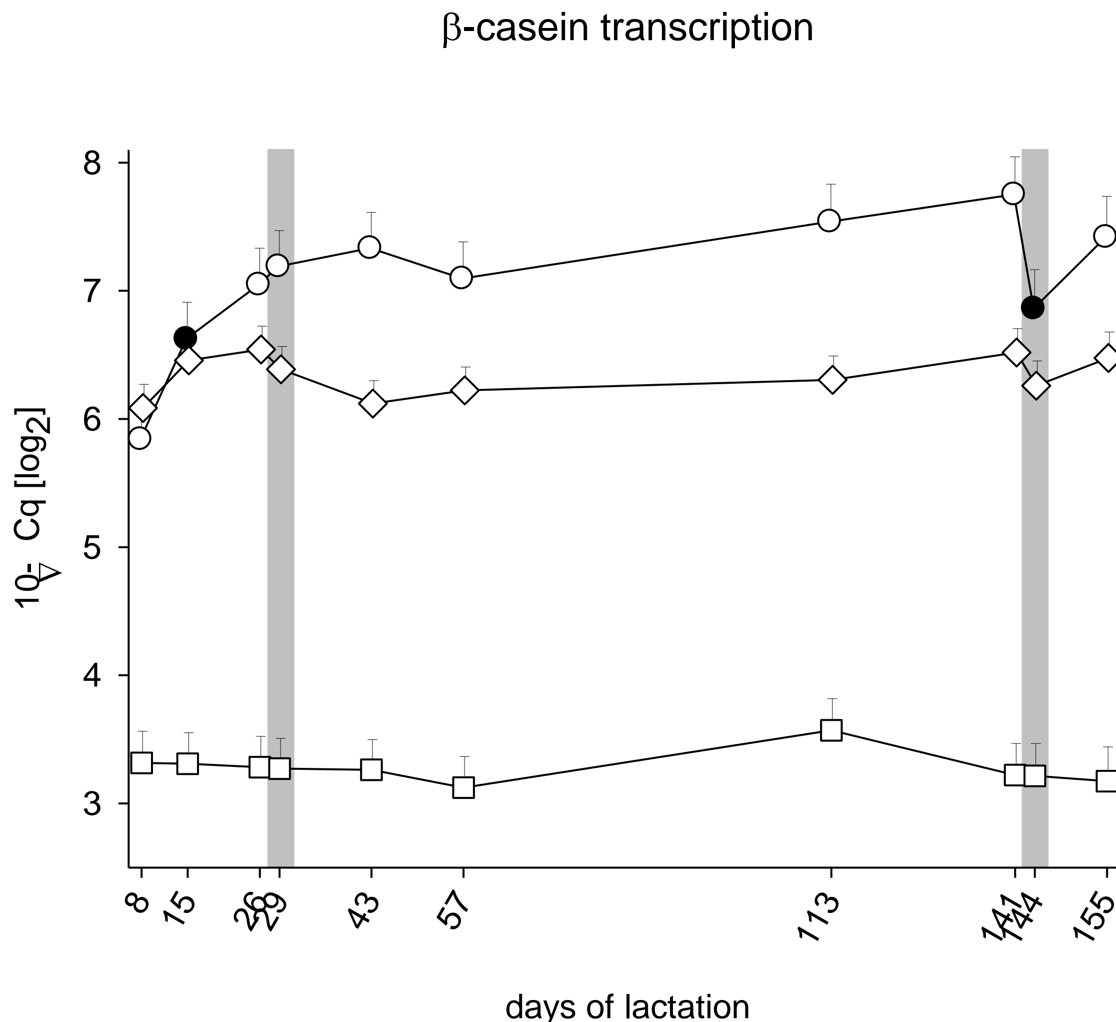

Figure 22. Transcript abundance of CEBPB (○), RUNX2 (□) and YY1 (◇) during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Values that differ significantly from the prior value are filled. Expression of CEBPB ($P<0.01$) was significant affected by time

Amino acid transfer and glucose transporter. The uptake of AA from feed and their metabolic conversion are important preludes for the milk protein synthesis. Therefore, deficits in nonessential AA can be overcome by synthesis of other precursors. Proline, a not adequately taken up amino acid, is required in rather high amounts for casein synthesis and ornithine δ-aminotransferase (OAT) is a key enzyme in this process (Basch et al., 1995). The unaffected mRNA levels of OAT during the experimental timeframe (Figure 23) were associated with the same feeding components during the study and therefore the identical AA composition of the feed. Blood plasma concentrations of the AA were not determined.

SLC2A1 is responsible for the transport of glucose into mammary cells through a passive mechanism (Zhao et al., 1996) and type 1 sodium glucose transporter

(SLC5A1) through an active mechanism (Zhao et al., 2005). SLC2A1 is known as the predominant facilitative glucose transporter in the lactating bovine mammary gland (Zhao et al., 1999) and plays a key role in maintaining glucose homeostasis during lactation (Bell and Bauman, 1997). Komatsu et al. (2010) detected no change in the mRNA expression of SLC2A1 between peak and late lactation, but mRNA was barely detectable in dry cows. mRNA levels encoding for SLC2A1 peaked at d 26 pp and remained subsequent on a constant level (Figure 23).

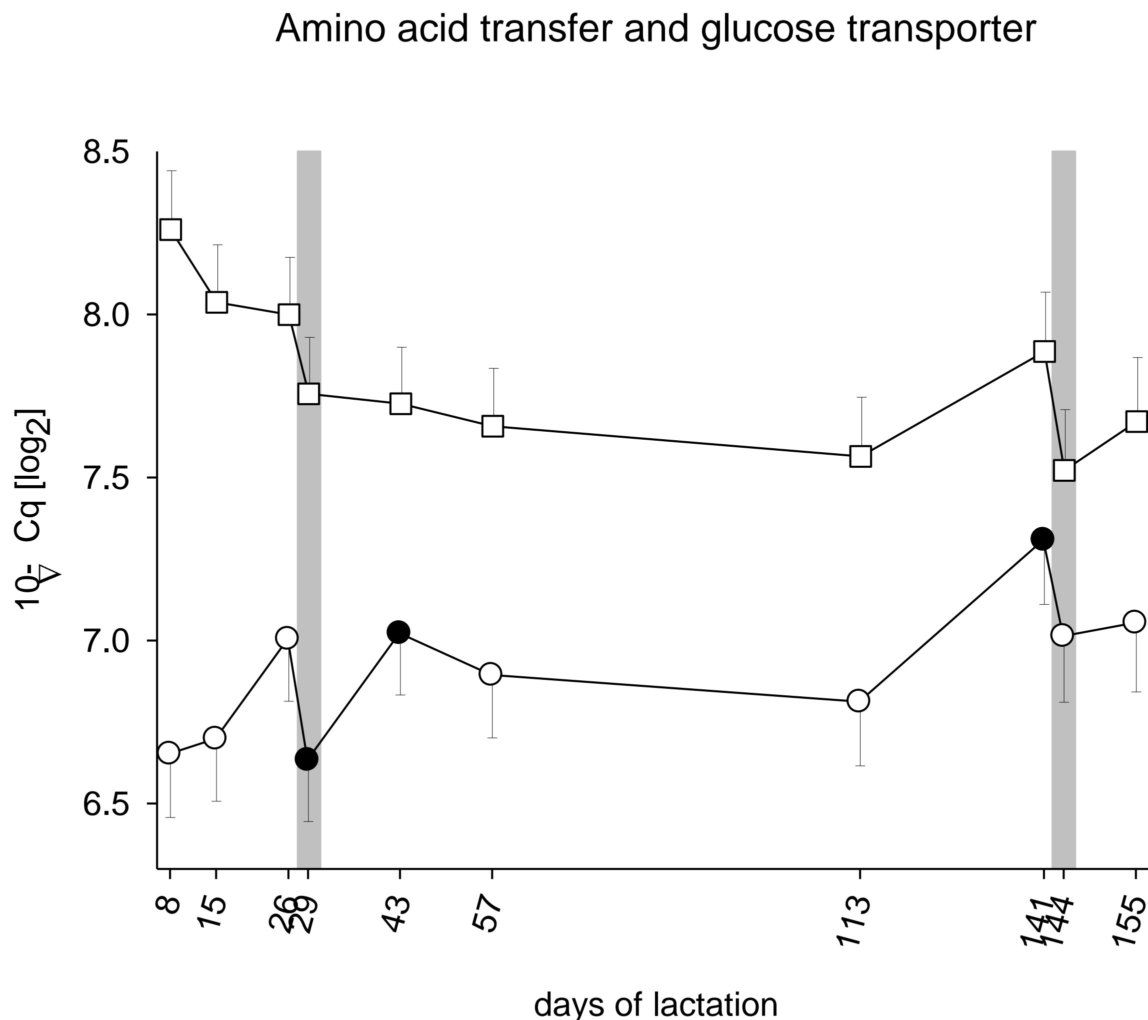

Figure 23. Transcript abundance of SLC2A1 (○) and OAT (□) during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Values that differ significantly from the prior value are filled. Expression of SLC2A1 (P = 0.03) was significant affected by time

In conclusion, pathway visualization indicated that during lactation the expression of investigated genes was quite stable but with few changes after restricted feeding. Transcript abundances encoding for ELF5 decreased with increased day of lactation.

This might explain the decrease of all major milk protein gene expression observed during the first half of lactation. Especially, the increase of mRNA levels for ELF5 after FR in early lactation and the simultaneous increase of mRNA levels of all milk protein genes clarified the pivotal role of the transcription factor ELF5 for milk protein gene expression. Bionaz and Loor (2011) referred this result. Considering all other investigated pathways, we can infer an overall increase in transcript abundances during mid lactation. This might explain the maintenance of relative proportions of the different caseins and whey proteins in milk during lactation despite the decrease in their expression (Bionaz and Loor 2011) and perhaps the increasing milk protein content during mid and end of lactation. In addition to this, the enormous demand of glucose and AA for milk protein synthesis, especially during mid and end of lactation when milk protein content increase, is reflected by the increased levels of transcripts for the glucose transporter and AA transfer during mid lactation. Bionaz and Loor (2011) indicated similar observations.

Dividing the experimental cows in two groups concerning their average milk protein content during the first half of lactation, homogenous groups of 12 cows could be built. Milk protein content was 3.08 ± 0.04 % and 3.50 ± 0.04 % for low (LP) and high milk protein cows (HP), respectively (Figure 24). Regarding milk yield and other milk constituents, the two groups had similar performances during the experimental timeframe (Figure 25).

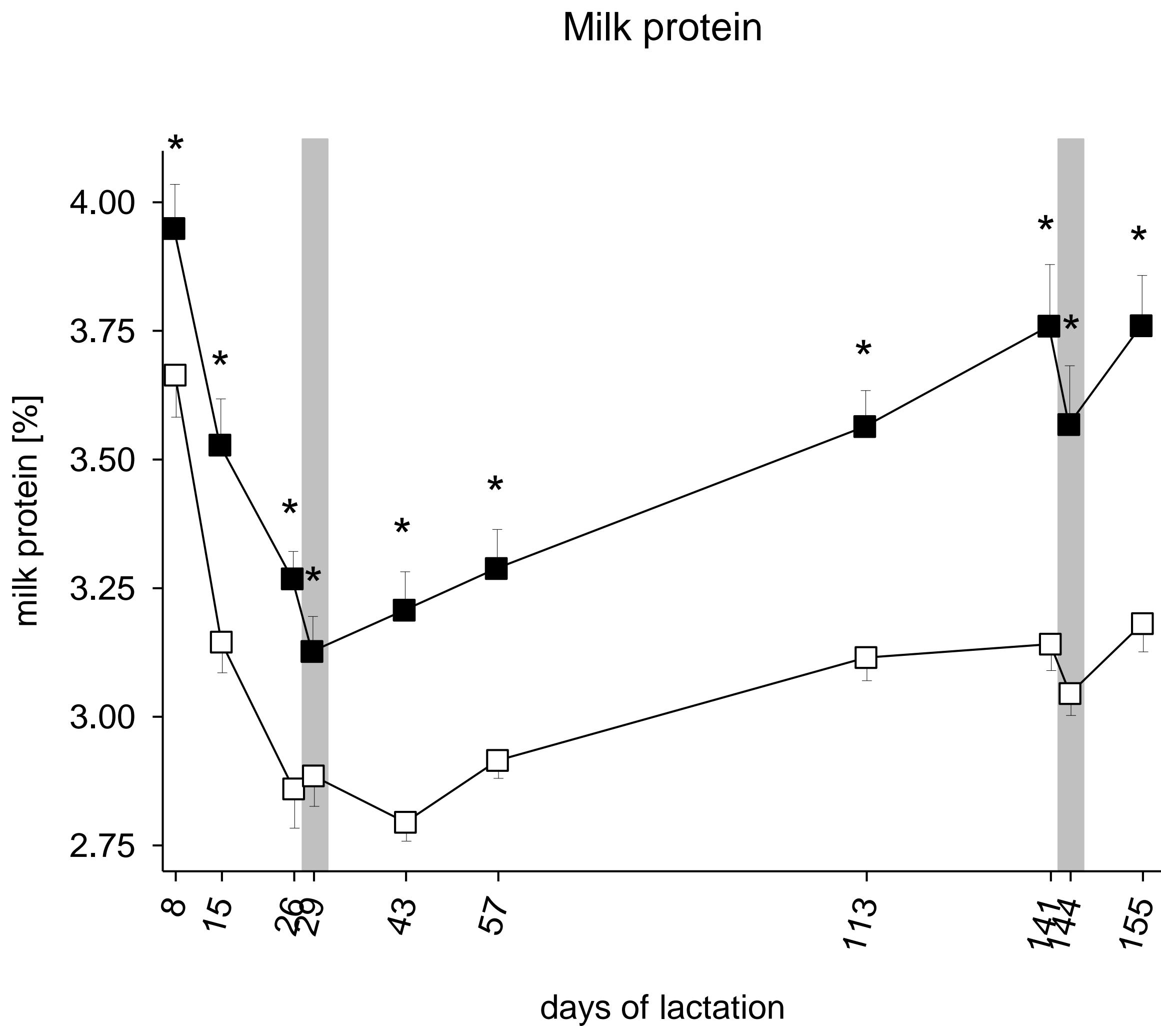

Figure 24. Milk protein content of HP (■) and LP (□) cows during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Asterisks indicate significant differences between HP and LP

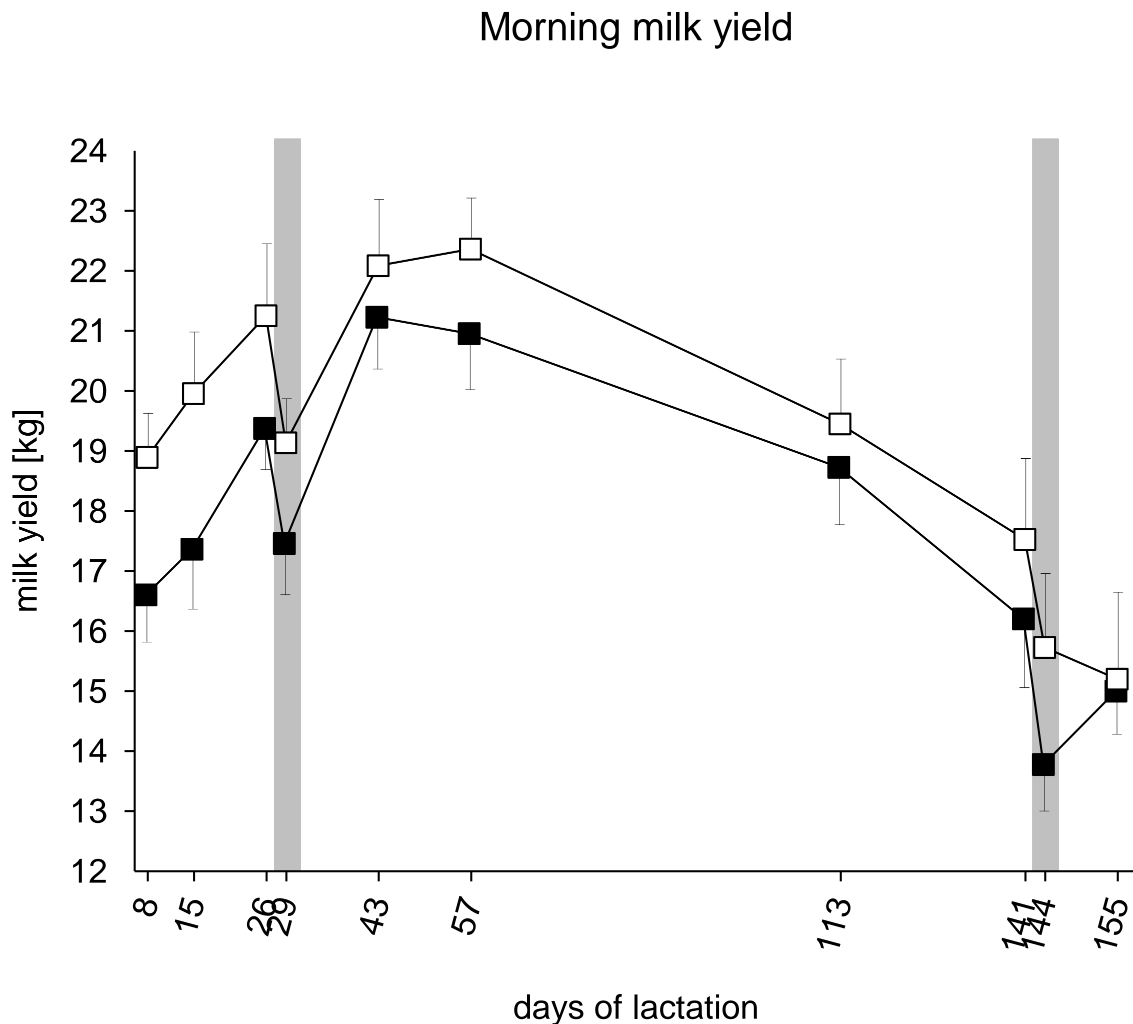

Figure 25. Morning milk yield of HP (■) and LP (□) cows during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Asterisks indicate significant differences between HP and LP

Transcript abundances of all investigated genes were found to be comparable, except SLC2A1. Starting at d 57 pp until end of the study, levels of transcript for SLC2A1 were significant higher in HP compared to LP cows (Figure 26). Transcript abundances of SLC2A1 increased during lactation with higher mRNA levels in HP cows. SLC2A1 is the predominant glucose transporter in the lactating mammary gland and is involved in milk synthesis. It is unknown if SLC2A1 is also involved in milk protein synthesis which could be inferred by the higher mRNA levels of the HP cows.

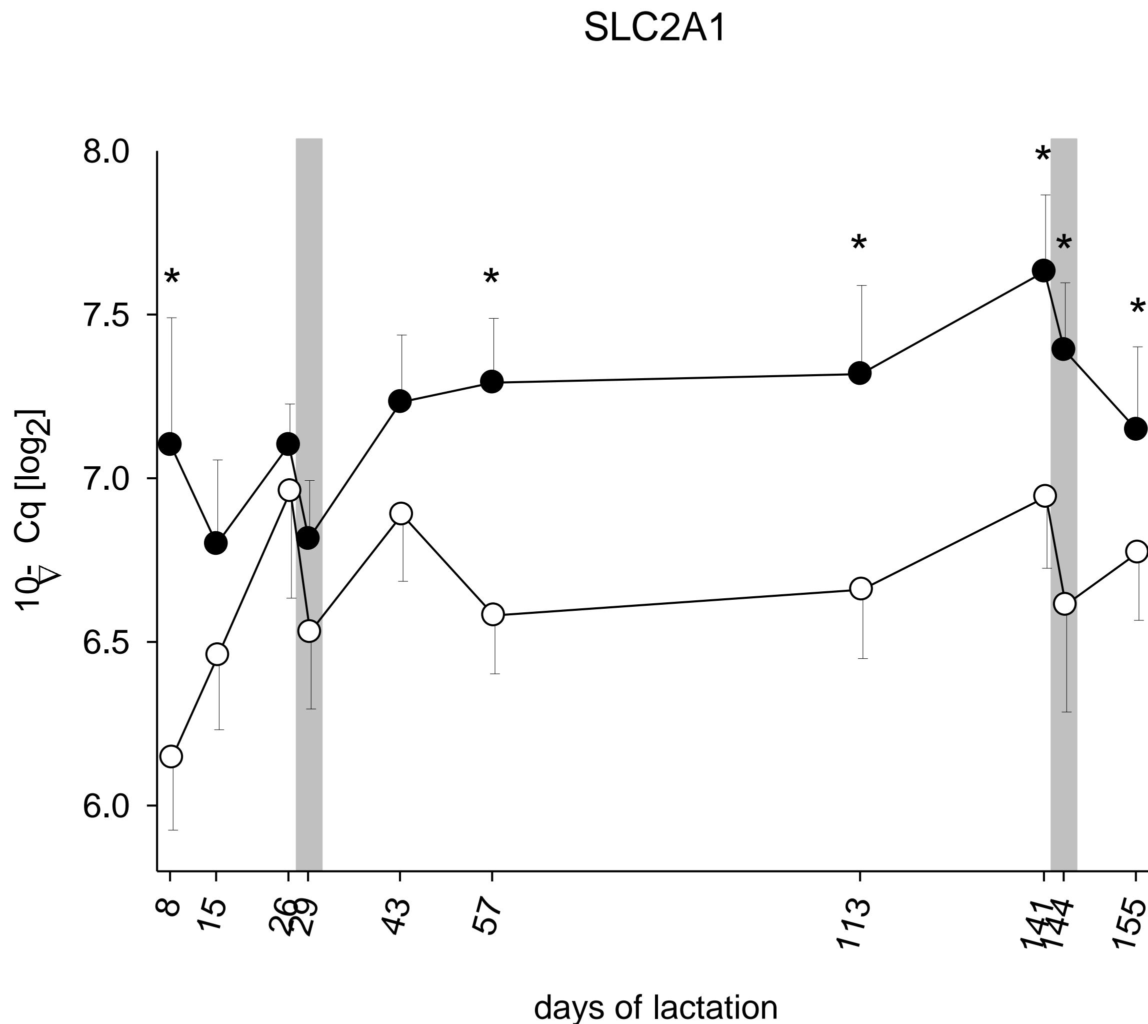

Figure 26. Transcript abundance of SLC2A1 in HP (●) and LP (○) cows during the first 155 days of lactation and after three days of restricted feeding during early (d 29 pp) and mid lactation (d 144 pp). Grey bars show sampling timepoint after FR. Asterisks indicate significant differences between HP and LP

In conclusion, during FR in early lactation transcript abundances of milk protein genes and ELF5 increased whereas levels of transcripts of JAK2, STAT5A, mTOR pathway and β-CN transcription remained unaffected. Our data showed that milk protein content and yield remained stable during this metabolic challenge. These results suggested that the constant transcript abundances of the lactogenic hormone receptors and the enzymes involving in pathways concerning milk protein biosynthesis were predominantly responsible for the maintenance of the milk protein content and milk protein yield during restricted feeding.

Further investigations are necessary to determine the differences in gene expression of all pathways concerning and involving in milk protein biosynthesis over a whole

305-d lactation period and to reveal differences in the regulation of milk protein biosynthesis in cows with low or high milk protein content.

5 CONCLUSIONS AND OUTLOOK

The present thesis revealed that pBMEC extracted from raw milk were suitable for qPCR analysis of milk protein biosynthesis. Lastly, milk protein gene expression and central pathways involved in milk protein biosynthesis were quite stable and with few changes among dairy cows with low or high milk protein content. Overall, our data suggested that these cows showed different metabolic adaptation to restricted feeding.

Influences of changed environment on 100-d performance of dairy cows

It is well accepted that changing environmental conditions affects the physiological state of dairy cows. The management system, under which cows were kept before and during the experiment, differed in housing, feeding and milking. Nevertheless, half of the cows got in their second lactation and parity influenced amount of milk production. Generally, all cows with parity 1 or 2 in their previous lactation showed higher milk yields during their subsequent 100-d lactation period. Cows with parity >3 suffered from an increased risk for metabolic diseases, shown by a decrease in milk yield and markedly higher loss of body weight and backfat thickness during early lactation. Variations in milk production and milk composition were found to be caused to a low part by the different management systems, but mostly by increased parity.

Metabolic challenge during early lactation

In this study, experimental cows were categorized in four groups (MP, mp, Mp and mP) according to averaged values for milk yield and milk protein percentage at days 23 to 25 pp. Dry matter intake was reduced to 68% for three subsequent days during early lactation. Restricted feed intake resulted in decreased blood glucose concentrations, increased blood NEFA and BHBA levels, calculated negative energy balance and was accompanied by milk yield depression. However, in Mp-cows, physiological adaptation to restricted feeding seemed to be in part diminished by the large effort for milk synthesis. Circulating NEFA during FR were ligands for HNF4A resulting in upregulation and downstream upregulation of genes with key function in fatty acid oxidation, gluconeogenesis, ketogenesis and amino acid catabolism. Restricted feeding triggered upregulation of SREBF1 via cytokines or fatty acids and triggered upregulation of GPAM, which was associated with greater concentrations of liver triacylglcerol and therefore facilitates the risk for liver-related disorders. Therefore, since MP- and Mp-cows had comparable amounts of milk protein yield,

efforts to increase milk protein concentration in high yielding dairy cows during early lactation could be a lucrative, sustainable and animal-appropriate management tool to increase dairy economic outcome and to reduce metabolic imbalances.

Development of a method to isolate pBMEC from milk and comparison with pBMEC harvested from cell culture

Primary MEC represent a good model to study lactogenesis, milk constituents biosynthesis, virus or immunity transmission and cancer research in ruminants and monogastric species. Infection studies can only be performed in vitro, e.g. using cells from raw milk in culture. Nevertheless, cell cultures demand a high range of morphological and functional adjustment strategies from the pMEC to the varied physiological and metabolic ambiance and lead to different expression profiles in pBMEC harvested from cell culture compared to pBMEC from raw milk. Therefore, studies concerning metabolic situations should be performed in pMEC from mammary gland biopsies or in desquamated pMEC extracted from fresh milk. Finally, the presented method for isolating pBMEC from milk is frequently repeatable and circumvents the drawback of mammary gland biopsies. Additionally, pMEC from milk provide the basis for future research in lactating animals, not only in dairy cows.

Expression profiles of milk protein genes and key enzymes in pBMEC

Milk protein biosynthesis is regulated at many levels within the pBMEC and milk protein genes are regulated by the complex interactions of peptide and steroid hormones, especially the lactogenic hormones, and cell-cell and cell-substratum interactions. There are only a few studies available about the regulation of milk protein gene expression, mammary gland transport systems, hormonal regulation of milk protein biosynthesis and the different pathways involved in milk protein gene expression. Research on milk protein gene expression predominantly occurred in mice and rats, or in small ruminants but rarely in cows. Transcript abundances encoding for ELF5 decreased with increased day of lactation and might explain the decrease of all major milk protein gene expression observed during the first half of lactation. Especially, the increase of mRNA levels for ELF5 after FR in early lactation and the simultaneous increase of mRNA levels of all milk protein genes clarified the pivotal role of ELF5 for milk protein synthesis. In addition, ELF5 is predominantly responsible for the variation in the expression of the milk protein genes. Considering the JAK/STAT pathway, AA transfer and glucose transporter and the β-casein promoters, we could infer an overall increase in transcript abundances during mid

lactation. This might explain the maintenance of relative proportions of the different caseins and whey proteins in milk during lactation despite the decrease in their expression (Bionaz and Loor 2011) and perhaps the increasing milk protein content during mid and end of lactation. Dividing the experimental cows in two groups concerning their average milk protein content during the first half of lactation, transcript abundances of SLC2A1 were higher for cows with high milk protein content. Possibly, cows with high mRNA levels for SLC2A1, had an enhanced glucose transport and uptake and synthesized more milk protein. Further investigations are necessary to determine the differences in gene expression of further pathways concerning and involving in milk protein biosynthesis over a whole 305-d lactation period and to reveal differences in the regulation of milk protein biosynthesis in cows with low or high milk protein content.

Milk protein genes and milk protein composition

In the present study, milk protein fractions were not analyzed, but previous studies in dairy cows showed different composition during lactation. Early reports stated peak concentrations of total caseins and serum proteins approximately five days after calving followed by a decline during the remaining 310 d-lactation period. In contrast, other studies reported a decline in concentrations of major milk proteins only between d 30 and 90 pp followed by a marked increase until d 365 pp. Nevertheless, highest total protein production was found during the first months of lactation. Furthermore, previous studies investigated that the ratio of total caseins to whey proteins does not vary depending on stage of lactation, whereas relations between specific caseins differed depending on stage of lactation. In present study, the expression patterns of the six major milk protein genes were comparable to previous findings on casein and whey protein concentrations in milk during early and until mid lactation. This implies that changes in transcripts of the milk protein genes might have a large effect on milk protein composition but a rather small effect on total milk protein biosynthesis.

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SCIENTIFIC COMMUNICATIONS

Original Publications

Sigl T, Gellrich K, Meyer HHD, Kaske M, Wiedemann S. 2012. Multiparous cows categorized by milk protein concentration and energy-corrected milk yield during early lactation - metabolism, productivity and effect of a short-term feed restriction. Journal of Animal Physiology and Animal Nutrition. Jan 20. doi: 10.1111/j.1439-0396.2011.01268.x

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2008-2011	PhD thesis (Dr. rer. nat.), Technical University Munich, Research Center for Nutrition and Food Sciences, “Transcript regulation in primary bovine mammary epithelial cells purified from milk and effects of restricted feeding on mammary protein biosynthesis pathways, hepatic regulation, milk yield and composition in dairy cows selected for different milk protein content”
2003-2008	Study of Agricultural Science at Technical University Munich, Diploma thesis: „CLA-Supplementierung der Ration von Braunviehkuehen in den ersten vier Laktationswochen: Effekte auf Milchinhaltsstoffe, Blutmetaboliten und die Genexpression in Lebergewebe“ Graduation: Dipl.-Ing. agr.
1995-2003	Michaeli-Gymnasium Munich Graduation: Allgemeine Hochschulreife
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APPENDIX

Appendix I — Sigl T and Gellrich K, Meyer HHD, Kaske M, Wiedemann S. 2012. Multiparous cows categorized by milk protein concentration and energy-corrected milk yield during early lactation - metabolism, productivity and effect of a short-term feed restriction. Journal of Animal Physiology and Animal Nutrition. Jan 20. doi: 10.1111/j.1439-0396.2011.01268.x.

Appendix II — Sigl T, Meyer HHD, Wiedemann S. 2012. Gene expression of six major milk proteins in primary bovine mammary epithelial cells isolated from milk during the first 20 weeks of lactation. Czech Journal of Animal Science 57, 454-468.

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ORIGINAL ARTICLE

Multiparous cows categorized by milk protein concentration and energy-corrected milk yield during early lactation – metabolism, productivity and effect of a short-term feed restriction

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Summary

The objective of this experiment was to study milk productivity, metabolic adaptation and effect of a short-term feed restriction (FR) on key performance indicators during early lactation in cows classified according to energy-corrected milk (ECM) yield and milk protein concentration. Twenty-three multiparous Holstein–Friesian cows were categorized in four groups according to respective averaged values on Days 23–25 postpartum: high ECM yield and high protein concentration; low ECM yield and low protein concentration; high ECM yield and low protein concentration and low ECM yield and high protein concentration.

Dry matter intake was reduced to 68.3% for three subsequent days. Our results showed that short-time FR in early lactation succeeded in enhancing energy deficit of cows in all groups. Milk fat, milk protein and lactose concentrations as well as milk fat yield were not influenced by FR. Several hepatic genes encoding for enzymes involved in catabolism of amino acids, β-oxidation, gluconeogenesis and ketogenesis as well as mRNA encoding for insulin receptor showed increased transcript abundances after FR, primarily in cows with high milk yield and low milk protein concentration.

Introduction

Milk protein yield is presently considered to be the major economic outcome of the dairy industry. In Germany, approximately 40% of collected milk is devoted to protein-requiring production of cheese, yoghurt or yoghurt-based products (Bundesministerium fuer Ernaehrung, Landwirtschaft und Verbraucherschutz, 2011). Milk protein yield mainly depends on not only milk yield (genetic correlation 0.83), but also milk protein concentration (genetic correlation 0.06, Teepker and Swalve, 1988). Therefore, special emphasis of the overall genetic selection effort is devoted to increase milk protein concentration (Lipkin et al., 2008). Besides genetic improvement, milk protein concentration in individual cows could also be elevated by an optimized periparturient feeding regime, e.g. by elevating the amount of rumen-undegradable protein and/or by adequate roughage feeding (Jouany, 1994; Santos et al., 1998; Pop et al., 2001). Within physiological limits, dairy cows are also able to compensate insufficient supply of amino acids by mobilization of body reserves (Botts et al., 1979). Moreover, many factors such as stage of lactation (Ng-Kwai-Hang et al., 1984), parity (Waite et al., 1956), diseases of the mammary gland (Munro et al., 1984), length of dry period (Schlamberger et al., 2010), breed and environmental temperature (Feagan, 1979) affect the milk protein concentration and composition.

Earlier studies showed diverging results regarding influence of restricted feeding on milk yield and milk

composition (Gross et al., 2011). A 25% reduction of ingested dry matter for 3 weeks during mid lactation reduced milk yield by 12% and milk protein concentration from 3.36% to 3.09% (Guinard-Flament et al., 2007), whereas a more pronounced feed restriction (FR) of 51% over a 5-day period during mid lactation provoked a 22% reduction in milk yield, but had no effect on milk composition (Velez and Donkin, 2005). During early lactation most dairy cows suffer an individually varying period of negative energy balance (NEB) because of the lag in feed intake relative to increased energy demands for parturition and milk synthesis (Drackley, 1999). FR during the critical timeframe of early lactation enhances the effects on milk yield and milk constituents and causes an enormous metabolic stress. In addition, low feeding levels during early lactation which are further triggered by FR, cause dramatic metabolic regulations in key tissues such as liver (Loor, 2010). During early lactation, Greenfield et al. (2000) showed increased activity of enzymes involved in gluconeogenesis (pyruvate carboxylase, PC; phosphoenolpyruvate carboxykinase 1, PCK1; and PCK2) whereas in FR only PC was induced (Velez and Donkin, 2005). Moreover, less mRNA abundance of insulin receptor (INSR) was found in fatty compared to healthy liver tissue of cows (Liu et al., 2010) and increased activity of hepatic ketogenesis (2-hydroxy-3-methylglutary-coenzyme A synthase 2, HMGCS2) in fasting rats was reported by Hegardt (1999). During the transition period, Loor et al. (2005, 2006) showed changes of several hepatic genes of the lipid metabolism, such as β-oxidation (carnitine palmitoyltransferase 1A, CPT1A; acyl-CoA dehydrogenase very long chain, ACADVL; enoyl CoA hydratase 1, ECHS1) and triacylglycerol synthesis (acetyl-CoA carboxylase, ACACA; glycerol-3-phosphate acyltransferase, GPAM; sterol regulatory element binding transcription factor 1, SREBF1). In order to quantify modifications in protein metabolism, mRNA expression of tyrosine aminotransferase (TAT; Johnson et al., 1973) and cathepsin L (CTSL; Becker et al., 2010), both encoding for amino acid degrading enzymes, were measured. Loor et al. (2007) detected differential expression of hepatocyte nuclear factor 4A (HNF4A) and peroxisome proliferator activated receptor α (PPARA) during dry period through peak lactation, whereas Gingras et al. (2000) reported that nutrient availability is the primary signal that induces activation of eukaryotic translation initiation factor 4B (EIF4B). The glucose transporter, member 2 (SLC2A2) is apart from kidney and small intestine, located in liver tissue and is mainly involved in the release of hepatic glucose (Zhao and Keating, 2007).

Exposure to metabolic stress of dairy cows also depends largely on milk composition which requires individual energy expenses according to concentrations of energy carriers mainly milk fat, lactose and protein. Cows' energy demands for production of 1 g of milk fat are nearly twice as high (1.8-fold) as for 1 g of milk lactose and protein, respectively. However, selecting animals with low milk fat concentration to reduce energy output during the critical timeframe of early lactation could lead to an undesired concomitant reduction in milk protein due to the close correlation between milk fat and milk protein concentration. Also, milk lactose concentration cannot be reduced considerably due to its osmotic function and concomitant reduction in milk yield. However, protein yield cannot be increased only by selection effort to lactose and milk yield, which are extremely energy demanding. Milk protein yield should rather be increased by selecting cows with high milk protein concentration and concomitant constant milk yield.

Therefore, in this experimental trial dairy cows were classified in four groups according to ECM yield as well as milk protein concentration. The aim of the study was to characterize metabolism and milk productivity of those cows during early lactation and to analyze the effect of a short-term FR on those parameters under comparable management and feeding conditions.

Materials and methods

Classification of cows

The study was approved by the animal welfare committee of the government of Upper Bavaria, Germany and followed the federal guidelines on animal experimentation. The animal trial was conducted at the research farm Veitshof of the Technische Universitaet Muenchen (Freising, Germany) from August 2009 to January 2011. Twenty-four multiparous Holstein–Friesian cows were classified into four groups according to mean ECM yield and mean milk protein concentration on Days (d) 23–25 postpartum (pp): high ECM yield and high milk protein concentration (MP-cows), low ECM yield and low milk protein concentration (mp-cows), high ECM yield and low protein concentration (Mp-cows) and low ECM yield and high protein concentration (mP-cows). Differences between high and low ECM yields and high and low milk protein concentrations were proved to be significant.

Table 1 Components and nutritional values* of lactation (LD) and restriction diet (RD)

Variable	LD[†]	RD[‡]
Components, %		
Corn silage	60.0	56.4
Grass silage	23.0	21.6
Hay	4.0	3.8
Concentrates[§]	12.0	11.3
Mineral mix[¶]	1.0	0.9
Straw		6.0
Dry matter (DM)	45.2	47.6
Nutritional values (% of DM)		
Crude ash	6.3	6.3
Crude protein	16.7	15.9
Crude fiber	17.2	18.7
Crude fat	3.4	3.2
Non-fibre carbohydrates	56.4	55.7
Neutral detergent fiber	37.4	39.9
Acid detergent fiber	22.4	23.8
Available crude protein	15.7	15.2
Ruminal nitrogen balance	0.1	−0.2
ME (MJ/kg DM)	11.2	11.0
NE_L (MJ/kg DM)	6.8	6.6

*Nutritional values and composition of partial-mixed ration were determined by enhanced Weender-analysis, performed at the Bayerische Landesanstalt für Landwirtschaft (LfL), Zentrallabor Grub (Poing, Germany).

†Lactation diet was offered to all cows *ad libitum* from 2 weeks before parturition until end of experiment.

‡Restriction diet was offered to cows only during period of feed restriction from d 26 to 28 pp.

§Composition: 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, 0.40% plant oil (palm coconut) (Raiffeisen Kraftfutterwerke Sued GmbH, Wuerzburg, Germany).

¶Ingredients: 14% Ca, 10% Na, 5% P, 5% Mg (Josera, Kleinheubach, Germany).

Housing and feeding

Cows were housed in a freestall barn with rubber-coated slatted floors and cubicles bedded with straw powder. During calving, cows were kept in a single calving box bedded with barley straw. From 2 weeks before and continued after calving they were fed the lactation diet (LD; Table 1). The partly mixed ration, calculated for a basis milk yield of 22 kg/day, was delivered once daily at 0700 h and intended to offer *ad libitum* (*ad lib*) intake (residual feed >5%). To underline cows individual different milk yield and milk composition and to eliminate effects of performance-related feeding, additional concentrates were fed only depending on day of lactation. Additional concentrates (7 MJ NE_L/kg; Raiffeisen Kraft-futterwerke Sued, Wuerzburg, Germany) were offered in automated feeding stations. Amounts of concentrates increased from 2 kg after parturition by 0.3 kg daily up to 6 kg at d 14 pp which was fed until end of experiment at d 57 pp. Fresh water was available at all times. Cows were milked in a 2 × 2 tandem milking parlour (GEA WestfaliaSurge GmbH, Boenen, Germany) twice daily at 0420 and 1540 h.

Feed restriction

From d 23 until 31 pp cows were moved to a tie-stall with separated feed troughs and with free access to water. Cows had eye contact to the herd at all times. At d 23 pp, animals were weighed, using weighing elements underneath the claw stand (FX1, Texas Trading, Windach, Germany). The amount of feed offered and refused was weighed and recorded daily for calculation of DMI. During the first 3 days (d 23–25 pp), cows were fed *ad lib* with LD and additional concentrates (6 kg). From d 26 to 28 pp, cows received the restriction diet (RD; Table 1) and no additional concentrates. Offered amount of RD was 70% of previously measured feed intake of LD. Fresh feed was mixed daily and cows were fed half of their daily allotment of RD at 0700 and 1700 h, respectively. During the following 3 days (d 29–31 pp) they were fed again *ad lib* LD and 6 kg concentrates.

Milk sampling

During each milking, milk yield was recorded with electronic milk meters (Metatron P21, GEA WestfaliaSurge GmbH, Boenen, Germany) and stored electronically (DairyPlan C21, GEA WestfaliaSurge GmbH). Milk samples for analysis of milk components were taken at d 1 to 10, 12, 15, 17, 20 to 32, 36, 43, 50 and 57 pp. Proportional subsamples of total milk (~1 l) were obtained during morning and evening milking, depending on amount of milk and milk flow rate (Metatron P21, GEA WestfaliaSurge GmbH). To exclude variation of single morning or evening milking samples, as reported by Quist et al. (2008), aliquots of morning and evening milk were composited according to the morning and evening milk yield to obtain representative samples. Milk samples were stored at 4 °C (maximum 7 days) with a preservative (acidiol) until analysis. Milk composition (protein, fat, lactose) was analyzed in the laboratories of Milchpruefring Bayern e.V. (Wolnzach, Germany). Measurements of protein, fat and lactose

were done by infrared-spectrophotometry (Milko-Scan-FT-6000, VOSS GmbH, Rellingen, Germany).

Blood sampling

Blood samples were collected at d 1, 8, 15, 22, 26 to 29, 32, 43 and 57 pp. Jugular venipuncture was performed after milking and before feeding (0645 h) using 9-ml vacuum tubes (Vacuette, Greiner Bio-One, Kremsmuenster, Austria). After coagulation (maximum 1 h), serum was separated by centrifugation (2000 *g*, 15 min at 4 °C) and three aliquots (1.5 ml) were stored at −20 °C until analysis. Measurement of serum parameters was conducted at the laboratory of the Tieraerztliche Hochschule Hannover (Hannover, Germany) with an automated clinical chemistry analyzer (ABX Pentra 400, Horiba, Montpellier, France). Glucose concentrations were measured by hexokinase method (CV = 2.3%) and NEFA concentrations by colorimetric enzymatic reactions (CV = 6.2%; both Hoffmann La-Roche, Basel, Switzerland). Determination of beta-hydroxybutyric acid (BHBA) concentration was performed by spectrophotometric enzymatic analysis (CV = 7.1%; Sigma-Aldrich Diagnostics, Munich, Germany).

Cyclicity and health

Estrus activity was monitored by measurements of milk progesterone. Milk samples for analysis of progesterone were obtained twice a week throughout the experimental timeframe beginning at d 8 pp and stored at −20 °C until analysis. Progesterone was determined in the laboratories of Physiology Weihenstephan (Freising, Germany) with an enzyme immunoassay as described by Meyer et al. (1986). Day of first ovulation was defined as 3 days before first progesterone concentration was greater or equal 0.5 ng/ml. Cows were monitored daily for general condition and health status. Disease was defined as necessary veterinary intervention. During this study, retained placenta, ketosis, lameness and mastitis were diagnosed.

Liver tissue sampling

Liver biopsies were obtained within 24 h after calving and at d 15, 29 and 57 pp by blind percutaneous needle biopsy (Bard® Magnum™, Covington, USA). Biopsies were taken after milking and blood sampling, but before feeding (0650 h). An area of 15 cm × 15 cm on the right side of the cow was shaved, washed as well as degreased with 70% ethanol and subsequently disinfected with an iodine solution (Vet-Sept®, Albrecht GmbH, Aulendorf, Germany). Local anesthetic (7 ml procainehydrochloride, Procasel®, Selectavet, Weyarn, Germany) was used to desensitize skin and subcutaneous as well as intercostal muscle tissue. A small incision was made through the skin at the intersection of an imaginary line running from the tuber coxae to the shoulder joint with the 11th and 12th intercostal space (Pearson and Craig, 1980) and was barely the size of the trocar (2.7 mm). In total, approximate 100 mg of liver tissue were obtained and directly transferred into 1 ml RNA stabilization solution (RNA*later*®, Applied Biosystems, Darmstadt, Germany). After over-night incubation at 4 °C, the samples were stored at −80 °C until mRNA extraction.

RNA extraction and reverse transcription

The mRNA was extracted from 50 mg liver tissue by using peqGOLDTriFast™ (Peqlab, Erlangen, Germany) according to the manufacturer's protocol. RNA was dissolved in 50 μl sterile RNase-free water and quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg).

Constant amounts of 1 μg RNA were reversely transcribed to complementary DNA using 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 200 U of MMLV-H-reverse transcriptase (Promega, Regensburg, Germany). According to the manufacturer, reaction of reverse transcription was carried out in 60-μl volume, using a PCR thermocycler (Biometra, Goettingen, Germany) and was achieved by successive incubations at 21 °C for 10 min and 48 °C for 50 min, finishing with enzyme inactivation at 90 °C for 2 min. Reverse transcript products were stored at −20 °C.

Primer design

Gene sequences for primer design were obtained from the gene bank of the National Center for Biotechnology Information (NCBI). 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). Primers were synthesized at Eurofins MWG (Ebersberg, Germany). Primer sequences, accession numbers and product lengths for each gene are listed in Table 2.

Table 2 Primer sequences, accession numbers and product lengths

Function	Gene*	Sequence 5′–3′	GenBank accession no.	Length [bp]
Reference genes	ACTB for	AACTCCATCATGAAGTGTGAC	AY141970	202
	ACTB rev	GATCCACATCTGCTGGAAGG		
	GAPD for	GTCTTCACTACCATGGAGAAGG	U85042	197
	GAPD rev	TCATGGATGACCTTGGCCAG		
	H3F3A for	ACTTGCTACAAAAGCCGCTC	BT025472	183
	H3F3A rev	ACTTGCCTCCTGCAAAGCAC		
Lipid metabolism	ACACA for	CTCTTCCGACAGGTTCAAGC	AJ132890	248
	ACACA rev	ACCATCCTGGCAAGTTTCAC		
	ACADVL for	CGTACATGGTGAGTGCCAAC	BT030546	209
	ACADVL rev	GTCATTTGTCCCCTCGAAGA		
	CPT1A for	CCATACTCACATAATTGGTAGCC	XM_001789518	144
	CPT1A rev	GCAACTAGTGAAGCCTCTTATGAA		
	ECHS1 for	GCTGCTGTCAATGGCTATGC	BT021569	192
	ECHS1 rev	ACCAGTGAGGACCATCTCCA		
	GPAM for	TCTGACTGAAGATGGGGATG	AF469047	148
	GPAM rev	ATGGGGAATTTGCCGCTTAT		
Protein metabolism	CTSL for	CACTGGTGCTCTTGAAGGACA	BC102312	177
	CTSL rev	TAAGATTCCTCTGAGTCCAGGC		
	TAT for	ACCCTTGTGGGTCAGTGTTC	BT021798	165
	TAT rev	ACAGGATGGGGACTTTGCTG		
Carbohydrate metabolism	PC for	ATCTCCTACACGGGTGACGT	NM_177946	214
	PC rev	TGTCGTGGGTGTGGATGTGCA		
	PCK1 for	TTTGGCGTCGCTCCGGGAAC	AY145503	244
	PCK1 rev	GGCACTGGCTGGCTGGAGTG		
	PCK2 for	TACGAGGCCTTCAACTGGCGT	XM_583200	365
	PCK2 rev	AGATCCAAGGCGCCTTCCTTA		
Glucose transport	SLC2A2 for	GGACCTTGGTTTTGGCTGTC	BC149324	275
	SLC2A2 rev	CACAGACAGGGACCAGAACA		
Hormone receptor	INSR for	CCAACTGCTCAGTCATCGAA	XM_002688832	164
	INSR rev	GTTGGGGAACAAGTCCTTCA		
Ketogenesis	HMGCS2 for	CGCCCGGCGTCCCGTTTAAA	NM_001045883	294
	HMGCS2 rev	GGACCCGCCACACTTTCGGTC		
Translation	EIF4B for	CCACGCCGGGACATGGATCG	NM_001035028	164
	EIF4B rev	TCATAGCGGTCCCCGCCTCC		
Transcription regulation	HNF4A for	GCATGGCCAAGATCGACAA	AY318752	73
	HNF4A rev	TGGGCATGAGGTGCTTCAC		
	PPARA for	GGATGTCCCATAACGCGATTCG	BT020756	235
	PPARA rev	TCGTGGATGACGAAAGGCGG		
	SREBF1 for	CCAGCTGACAGCTCCATTGA	NM_001113302	67
	SREBF1 rev	TGCGCGCCACAAGGA		

*ACTB, actin beta; GAPD, glyceraldehyde-3-phosphate dehydrogenase; H3F3A, H3 histone family 3A; ACACA, acetyl-CoA carboxylase α; ACADVL, acyl-CoA dehydrogenase, very long chain; CPT1A, carnitine palmitoyltransferase 1A; ECHS1, enoyl CoA hydratase 1; GPAM, glycerol-3-phosphate acyltransferase; CTSL, cathepsin L; TAT, tyrosine aminotransferase; PC, pyruvate carboxylase; PCK1, phosphoenolpyruvate carboxykinase, cytosolic; PCK2, phosphoenolpyruvate carboxykinase, mitochondrial; SLC2A2, facilitated glucose transporter, member 2; INSR, insulin receptor; HMGCS2, 3-hydroxy-3-methylglutary-coenzyme A synthase 2; EIF4B, eukaryotic translation initation 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).

Quantitative PCR

Quantitative PCR was performed using MESA Green qPCR MasterMix plus for SYBR® Assay w/fluorescein (Eurogentec, Cologne, Germany) applying a standard protocol recommended by the manufacturer. All components necessary for real-time RT-qPCR were mixed in the reaction wells of semi-skirted twin.tec

PCR plate 96 (Eppendorf, Hamburg, Germany). The mastermix was prepared as follows: 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 plus 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: denaturation step (95 °C, 5 min), cycling program (95 °C, 3 s; primer specific annealing temperature, 60 s) and melting curve analysis.

Quantification of mRNA

Genes were selected as reference genes using GenEx Pro Software Version 5.2.7.44 (MultiD Analyses, Gothenburg, Sweden). The mean of the three housekeeping genes, actin beta (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPD) and H3 histone family 3A (H3F3A) was calculated for the reference index and used for normalization. Quantitative cycle (Cq)-values were calculated by Bio-Rad iQ5 Optical System Software Version 2.1 with the analysis mode 'PCR baseline substracted curve fit'. The ΔCq-values were calculated as $\Delta Cq = Cq_{target\ gene} - \text{mean } Cq_{refence\ genes}$. In order to avoid negative digits and to allow an estimation of a relative comparison between two time points, data were subtracted from the arbitrary value 10 (10-ΔCq). Thus, a high ΔCq-value resembles high transcript abundance (Livak and Schmittgen, 2001). An increase of one ΔCq represents a two-fold increase of mRNA transcripts.

Statistical analysis

Energy balance was calculated using the formula EB = (DMI diet × NE_L diet) + (DMI concentrates × NE_L concentrates) − (0.293 × body weight$^{0.75}$) − (0.38 × milk fat concentration) − (0.21 × milk protein concentration) + 0.95) × milk yield) as described by Kamphues et al. (2004). Energy-corrected milk yield was calculated using the formula ECM (kg/day) = (milk yield × 0.327) + (milk fat yield × 12.86) + (milk protein yield × 7.65).

Prior to performance of statistical analysis, repeated end point measurements (daily ECM yield and milk composition) were pooled to weekly means. From d 23 to 31 pp, measurements for ECM yield, milk composition, blood serum parameters, DMI and energy balance were summarized to 3-day intervals depending on different feeding levels. Data of milk, blood and gene expression in liver were analyzed using REML in the MIXED procedure in SAS (SAS Institute, 2002) including DIM and group and the interaction DIM × group as fixed effects. In this model, animal was used as repeated subject. All data are presented as least square means (LSM) ± standard error of means (SEM) and were considered to differ significantly at $p < 0.05$.

Results

Number and classification of cows

Out of 24 Holstein–Friesian cows which were included in the experimental trial from d 14 before expected calving to d 57 pp, one cow suffered recurrently from inflammations caused by a foreign body; consequently records from this animal were excluded from analyses. Remaining 23 cows were classified into the four groups according to mean ECM yield and mean milk protein concentration 3 days prior to FR (d 23, 24 and 25 pp; Table 3), i.e. six cows had high ECM yields (55.0 ± 2.72 kg/day) and high milk protein concentrations (3.28 ± 0.07%; MP-cows), five cows had low ECM yields (42.1 ± 1.08 kg/day) and low milk protein concentrations (2.84 ± 0.06%; mp-cows), seven cows had high ECM yields (51.3 ± 1.98 kg/day) and low milk protein concentrations (2.90 ± 0.06%; Mp-cows) and five cows had low ECM yields (41.7 ± 0.52 kg/day) and high milk protein concentrations (3.40 ± 0.05%; mP-cows). As intended, mean values of ECM yield 3 days prior to FR were different among groups ($p < 0.05$), except for MP- vs. Mp-cows ($p = 0.23$) and mp vs. mP-cows ($p = 0.90$). Furthermore, over the same time period mean milk protein concentrations also varied between groups ($p < 0.05$), except for mp- vs. Mp-cows ($p = 0.46$) and MP- vs. mP-cows ($p = 0.51$). Milk protein yield was higher in MP-cows (1381 ± 55 g/day) and Mp-cows (1313 ± 49 g/day) than in mp-cows (1045 ± 28 g/day; $p < 0.001$ and 0.01, respectively). Moreover, milk fat concentration was higher in MP- (5.66 ± 0.38%) than in Mp-cows (4.56 ± 0.25%; $p = 0.02$) and milk fat yield was highest in MP- (2387 ± 177 g/day) compared to mp- (1718 ± 82 g/day) and mP-cows (1716 ± 54 g/day; both $p < 0.001$). No differences could be found between groups in lactose concentrations. Milk lactose yield was similar among groups, except between MP- (2022 ± 73 g/day) and Mp-cows (2170 ± 66 g/day; $p = 0.22$), and between mp- (1748 ± 42 g/day) and mP-cows (1645 ± 40 g/day; $p = 0.45$). Parity of cows was comparable among all groups (2.8 ± 0.3, 2.4 ± 0.2, 2.3 ± 0.3, 2.8 ± 0.4 for MP-, mp-, Mp- and mP-cows, respectively).

Table 3 LSM ± SEM of energy-corrected milk yield, milk composition and blood serum parameters before (d 23–25 pp), during (d 26–28 pp) and after (d 29–31 pp) feed restriction

	Group*			
Item	MP	mp	Mp	mP
ECM† (kg/day)				
d 23–25 pp	55.0 ± 2.72^{a*}	42.1 ± 1.08^{b}	51.3 ± 1.98^{a}	41.7 ± 0.52^{b}
d 26–28 pp	51.3 ± 2.34^{a+}	39.5 ± 1.72^{b}	49.1 ± 1.85^{a}	39.6 ± 1.44^{b}
d 29–31 pp	53.4 ± 2.92^{a+*}	39.3 ± 1.23^{b}	47.4 ± 1.49^{a}	40.3 ± 1.51^{b}
Milk protein (%)				
d 23–25 pp	3.28 ± 0.07^{a}	2.84 ± 0.06^{b}	2.90 ± 0.06^{b}	3.40 ± 0.05^{a}
d 26–28 pp	3.19 ± 0.07^{ab}	2.89 ± 0.08^{a}	2.89 ± 0.04^{a}	3.28 ± 0.05^{b}
d 29–31 pp	3.02 ± 0.06	2.77 ± 0.11	2.91 ± 0.06	3.11 ± 0.08
Milk protein (g/day)				
d 23–25 pp	1381 ± 55^{a*}	1045 ± 28^{b}	1313 ± 49^{a}	1134 ± 37^{b}
d 26–28 pp	1250 ± 56^{a}	974 ± 57^{b}	1221 ± 40^{a}	1031 ± 4^{b}
d 29–31 pp	1215 ± 63^{a}	914 ± 66^{b}	1181 ± 32^{a}	987 ± 56^{b}
Milk fat (%)				
d 23–25 pp	5.66 ± 0.38^{a}	4.68 ± 0.26^{ab}	4.56 ± 0.25^{b}	5.17 ± 0.26^{ab}
d 26–28 pp	5.75 ± 0.29^{a}	4.88 ± 0.33^{ab}	4.78 ± 0.29^{b}	5.32 ± 0.26^{ab}
d 29–31 pp	5.99 ± 0.30^{a}	5.14 ± 0.40^{ab}	4.82 ± 0.28^{b}	5.49 ± 0.09^{ab}
Milk fat (g/day)				
d 23–25 pp	2387 ± 177^{a}	1718 ± 82^{b}	2060 ± 122^{ab}	1716 ± 54^{b}
d 26–28 pp	2250 ± 140^{ac}	1635 ± 98^{b}	2016 ± 115^{c}	1666 ± 75^{bc}
d 29–31 pp	2409 ± 167^{a}	1675 ± 90^{b}	1951 ± 93^{b}	1740 ± 56^{b}
Milk lactose (%)				
d 23–25 pp	4.80 ± 0.06	4.75 ± 0.06	4.80 ± 0.04	4.93 ± 0.04
d 26–28 pp	4.76 ± 0.04	4.67 ± 0.06	4.80 ± 0.05	4.87 ± 0.05
d 29–31 pp	4.77 ± 0.06	4.72 ± 0.04	4.78 ± 0.06	4.89 ± 0.04
Milk lactose (g/day)				
d 23–25 pp	2022 ± 73^{a*}	1748 ± 42^{b*}	2170 ± 66^{a*}	1645 ± 40^{b}
d 26–28 pp	1862 ± 58^{a+}	1575 ± 87^{b}	2031 ± 69^{a}	1530 ± 60^{b}
d 29–31 pp	1918 ± 86^{a+*}	1553 ± 71^{b}	1950 ± 79^{a}	1550 ± 56^{b}
Glucose (mmol/l)				
d 25 pp	3.63 ± 0.11	3.34 ± 0.24^{*+}	3.50 ± 0.30*	3.81 ± 0.14
d 26–28 pp	2.98 ± 0.17	2.62 ± 0.47*	2.87 ± 0.23^{+}	3.29 ± 0.30
d 31 pp	3.41 ± 0.26	3.84 ± 0.42^{+}	3.27 ± 0.25^{*+}	3.92 ± 0.17
NEFA (μmol/l)				
d 25 pp	730 ± 155	746 ± 238^{*+}	491 ± 37	491 ± 66
d 26–28 pp	1034 ± 125	1020 ± 292*	1078 ± 104*	924 ± 101*
d 31 pp	804 ± 181	591 ± 196^{+}	499 ± 60	455 ± 57
BHBA (mmol/l)				
d 25 pp	0.54 ± 0.06*	1.12 ± 0.42	0.52 ± 0.03*	0.48 ± 0.05
d 26–28 pp	1.87 ± 0.37^{a}	2.28 ± 0.68^{*ab}	1.80 ± 0.32^{ab}	1.42 ± 0.24^{b*}
d 31 pp	1.47 ± 0.67^{a}	1.14 ± 0.42^{ab}	1.40 ± 0.66^{a}	0.62 ± 0.14^{b}

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

$^{*+}$Means with figurative superscripts indicate differences between timepoints ($p < 0.05$).

*M represents cows with high ECM yield, m represents cows with low ECM yield, P represents cows with high milk protein concentration, and p represents cows with low milk protein concentration (6 MP-, 5 mp-, 7 Mp- and 5 mP-cows).

†Energy-corrected milk yield was calculated using formula ECM (kg/day) = (milk yield × 0.327) + (milk fat yield × 12.86) + (milk protein yield × 7.65).

ECM yield and milk composition during 57 days of lactation

During the first 9 weeks of lactation, MP-cows showed 50.3 ± 0.82 kg/day mean ECM yield per day, mp-cows 41.0 ± 0.85 kg/day, Mp-cows 49.5 ±0.62 kg/day and mP-cows 40.0 ± 0.62 kg/day, respectively. Mean milk yield was higher in MP- and Mp-cows compared to mp- and mP-cows ($p < 0.0001$). Milk yield had steepest increase during first 3 weeks in MP-cows, however initial milk yield in Mp-cows was highest among all groups (Fig. 1). Differences in

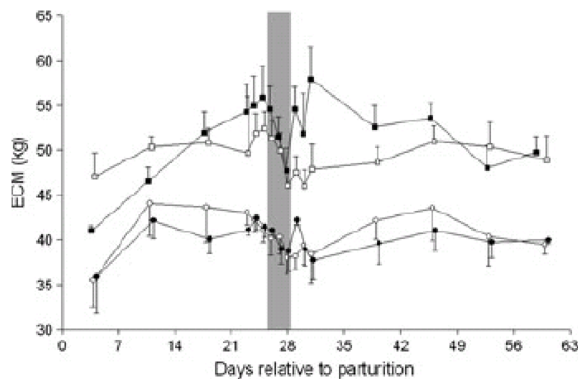

Fig. 1 Energy-corrected milk yield (kg) during the first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are presented as LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p < 0.01$, time × group: $p = 0.40$.

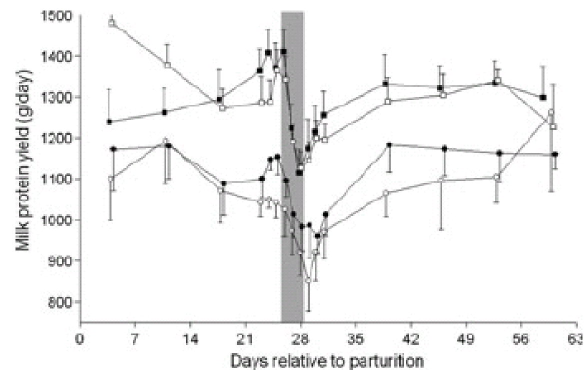

Fig. 3 Milk protein yield (g/day) during the first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p = 0.50$, group: $p = 0.07$, time × group: $p = 0.30$.

values between groups remained on a comparative level after FR, except for those of MP-cows which lowered.

During 57 days of lactation, mp- (3.24 ± 0.09%) and Mp-cows (3.19 ± 0.07%; $p = 0.67$) showed lower protein concentrations compared to MP- (3.55 ± 0.08%; $p < 0.05$) and mP-cows (3.73 ± 0.09% $p < 0.001$). All groups showed a marked decrease in milk protein during the first 3 weeks of lactation (Fig. 2). As a result of milk yield and milk protein concentration MP-cows produced 1297 ± 58 g mean

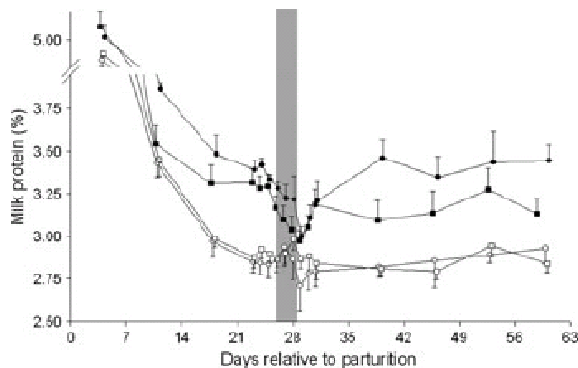

Fig. 2 Milk protein (%) during the first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares ■, mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p < 0.001$, time × group: $p = 0.16$.

milk protein yield per day, mp-cows 1127 ± 63 g/day, Mp-cows 1328 ± 53 g/day and mP-cows 1159 ± 63 g/day. Differences could only be found between mp- and Mp-cows ($p = 0.03$; Fig. 3).

Mean milk fat concentration during 57 days of lactation was 5.60 ± 0.27% in MP-, 4.73 ± 0.29% in mp-, 4.77 ± 0.25% in Mp- and 5.13 ± 0.29% in mP-cows, respectively. No differences could be observed between groups. Before FR, average milk fat concentrations in MP- and mP-cows were higher compared to mp- and Mp-cows. After FR, milk fat concentration was on highest levels in MP-cows, but approached values of other groups after approximately 7 weeks (Fig. 4).

Moreover, mean milk fat yield was higher in MP- (2076 ± 87 g/day; $p < 0.01$) compared to mp- (1636 ± 95 g/day) and mP-cows (1598 ± 95 g/day) and also higher in mP- (1985 ± 81 g/day) compared to mp- ($p = 0.01$) and mP-cows ($p < 0.01$).

During 57 days of lactation, mP-cows showed highest lactose concentration ($p < 0.01$). Lactose concentration in MP-cows was 4.70 ± 0.04%, in mp-cows 4.62 ± 0.04%, in Mp-cows 4.67 ± 0.03% and in mP-cows 4.86 ± 0.04% (Fig. 5).

Furthermore, mean lactose yield was in MP-cows 1808 ± 73 g/day, in mp- 1652 ± 80 g/day, in Mp- 1996 ± 68 g/day and in mP-cows 1546 ± 80 g/day, respectively. Mp-cows had higher lactose yields than mp- ($p < 0.01$) and mP-cows ($p < 0.001$) and MP-cows had higher lactose yields than mP-cows ($p = 0.03$).

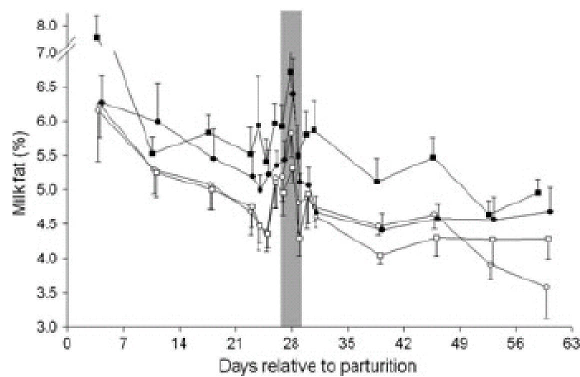

Fig. 4 Milk fat (%) during the first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p = 0.11$, time × group: $p = 0.02$.

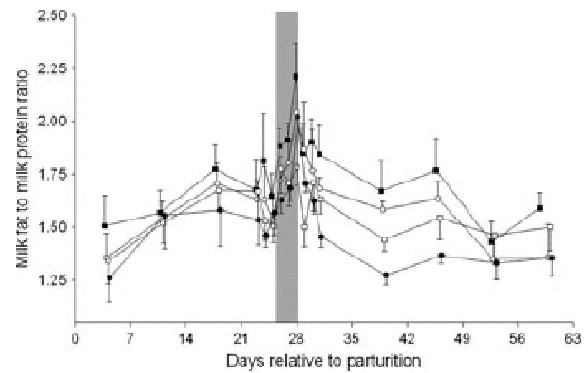

Fig. 6 Milk fat to milk protein ratio during the first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p = 0.25$, time × group: $p = 0.44$.

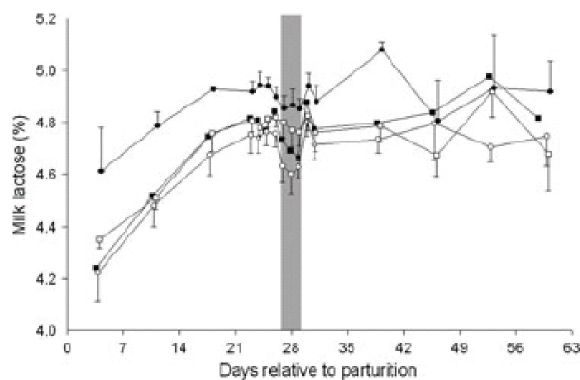

Fig. 5 Milk lactose (%) during the first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $P = 0.003$, time × group: $p = 0.86$.

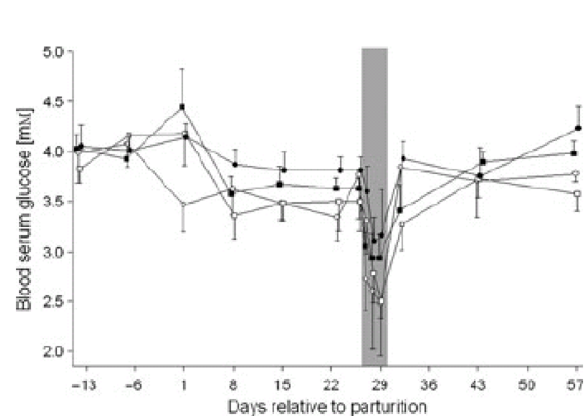

Fig. 7 Blood serum glucose levels (mmol/l) 2 weeks prior to expected calving through first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p = 0.59$, time × group: $p = 0.57$.

Regarding ratio of milk fat concentration to milk protein concentration (FPR), no differences could be seen between groups (Fig. 6). FPR of MP-cows was 1.6 ± 0.07, mp-cows showed FPR 1.48 ± 0.08, Mp-cows 1.52 ± 0.07 and mP-cows 1.39 ± 0.08.

Blood serum parameters

At calving, blood serum glucose levels of MP-cows (4.44 ± 0.38 mmol/l) were higher compared to mp-cows (3.46 ± 0.27 mmol/l; $p < 0.05$; Fig. 7). Before parturition, blood serum NEFA concentrations in MP-cows (455 ± 124 μmol/l) showed higher values compared to Mp-cows (162 ± 38 μmol/l; $p = 0.04$), but at d 15, 43 and 57 pp blood serum NEFA as well as BHBA levels were comparable among groups (Figs 8 and 9).

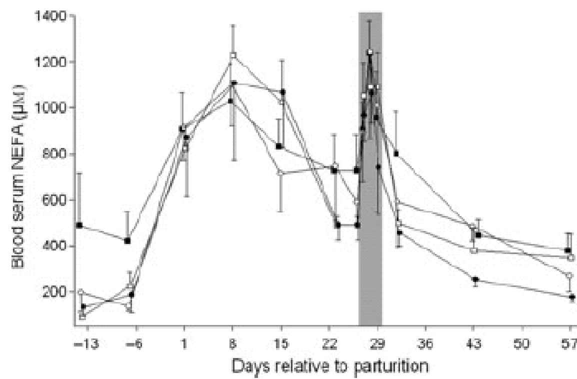

Fig. 8 Blood serum non-esterified fatty acid levels (µmol/l) 2 weeks prior to expected calving through first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p = 0.65$, time × group: $p = 0.75$.

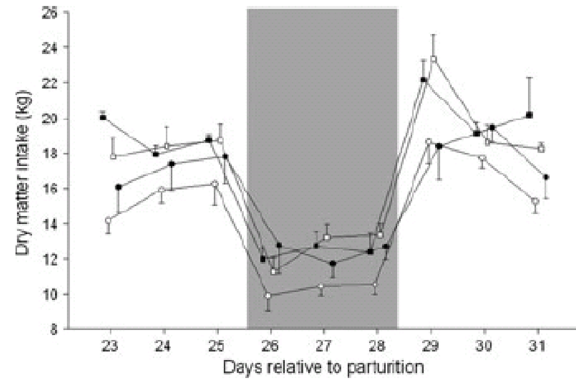

Fig. 10 Dry matter intake (kg) during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p = 0.06$, time × group: $p = 0.80$.

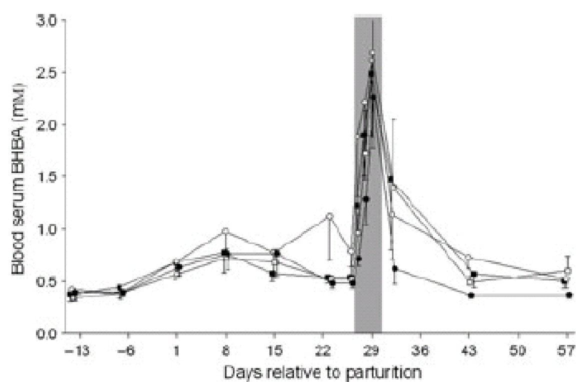

Fig. 9 Blood serum β-hydroxybutyric acid levels (mmol/l) 2 weeks prior to expected calving through first 9 weeks of lactation and during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p = 0.66$, time × group: $p = 0.89$.

Feed restriction during d 26–28 pp

Feed intake and energy balance

Before FR, dry matter intake (DMI) was lower in mp-cows (15.45 ± 0.58 kg/day) compared to MP- (18.91 ± 0.31 kg/day; $p = 0.01$) and Mp-cows (18.31 ± 0.94 kg/day; $p = 0.04$; Fig. 10). During FR, decrease of DMI was lower in mP-cows (−27.4%) compared to Mp- (−31.2%), mp- (−33.5%) and MP-cows (−34.6%). After FR, DMI was again lower in mp-cows (17.90 ± 0.68 kg/day) compared to MP- (20.67 ± 0.37 kg/day; $p = 0.04$) and Mp-cows (20.86 ± 0.89 kg/day; $p = 0.03$). At the first day of realimentation, compensatory effects of feed intake were measured in MP- (+25.1%; $p = 0.02$), mp- (+29.0%; $p = 0.01$) and Mp-cows (+35.3%; $p = 0.02$) compared to previously measured feed intake.

Before FR, EB was lower in MP-cows (−72.43 ± 6.25 MJ NE_L) compared to mP-cows (−46.38 ± 10.76 MJ NE_L; $p = 0.02$; Fig. 11). Decrease of EB was lowest in MP-cows (1.54-fold), and similar in mp- (1.61-fold), Mp- (1.63 fold) and mP-cows (1.68-fold). After FR, EB was lower in MP-cows (−55.53 ± 10.24 MJ NE_L) compared to mp- (−29.93 ± 4.13 MJ NE_L; $p = 0.04$) and mP-cows (−19.78 ± 9.22 MJ NE_L; $p < 0.01$).

Milk

During FR ECM yield decreased gradually in MP-cows (−3.7 kg; $p < 0.05$; Table 3; Fig. 1). While milk protein concentration seemed to remain on a constant level in all groups during FR, milk protein yield decreased in MP-cows (−131 g; $p = 0.02$; Table 3; Fig. 3). For all groups, milk fat percentage, fat yield and lactose concentration and FPR were not influenced by FR.

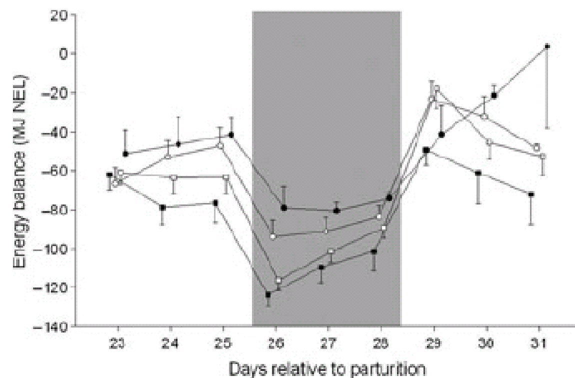

Fig. 11 Energy balance (MJ NEL) during 3 days of restricted feeding. M = high ECM yield, m = low ECM yield, P = high milk protein concentration and p = low protein concentration. MP-cows are shown as filled squares (■), mp-cows as empty circles (○), Mp-cows as empty squares (□) and mP-cows as filled circles (●). Grey bars show the 3 days of restricted feeding. Values are LSM ± SEM. Fixed effects in model: time: $p < 0.001$, group: $p < 0.01$, time × group: $p = 0.54$.

Blood

During FR blood serum glucose levels decreased in mp- (−1.14 mmol/l; $p < 0.01$) and Mp-cows (−0.63 mmol/l; $p < 0.05$; Table 3; Fig. 7). After FR blood serum glucose levels increased in mp- (1.22 mmol/l; $p < 0.01$) and mP-cows (0.63 mmol/l; $p = 0.02$). During the first 2 days of FR, blood serum concentrations of NEFA increased drastically in all cows, but significant only in Mp- (+587 μmol/l; $p < 0.01$) and mP-cows (+433 μmol/l; $p = 0.01$). After FR, blood serum concentrations of NEFA decreased in mp- (−429 μmol/l; $p = 0.01$), Mp- (−580 μmol/l; $p < 0.001$) and mP-cows (469 μmol/l; $p < 0.01$; Table 3; Fig. 8). During FR, blood serum BHBA levels steeply increased in all groups (+1.33, +1.16, +1.28 and +0.94 mmol/l for MP-, mp-, Mp- and mP-cows, respectively; $p < 0.001$). Three days after FR, blood serum BHBA levels decreased in mp- (−1.14 mmol/l; $p < 0.001$) and mP-cows (−0.80 mmol/l; $p < 0.001$; Table 3; Fig. 9).

Gene expression in liver

Lipid metabolism

At d 15 pp, lower mRNA abundance of ACADVL was measured in mp-cows (8.10 ± 0.26) compared to Mp- (8.85 ± 0.18; $p < 0.01$) and mP-cows (8.85 ± 0.12; $p = 0.02$; Table 4). At the same time, mRNA levels of CPT1A were lower in mp-cows (7.69 ± 0.30) compared to Mp- (8.46 ± 0.21; $p = 0.03$), MP- (8.56 ± 0.34; $p = 0.04$) and mP-cows (8.93 ± 0.33; $p < 0.01$). After FR higher mRNA abundance of ECHS1 was measured in Mp- (10.93 ± 0.19) compared with mp-cows (10.22 ± 0.28; $p = 0.02$). At the same time, mRNA encoding for GPAM showed higher abundance in Mp-cows (7.67 ± 0.23) compared with Mp- (6.49 ± 0.46; $p < 0.01$) and mp-cows (6.88 ± 0.26; $p < 0.05$). ECHS1, ACADVL, CPT1A and ACACA were affected by time; lower abundance of ECHS1 was measured at day of calving ($p < 0.001$), transcript abundances of ACADVL ($p < 0.05$) and CPT1A ($p < 0.05$) were highest after FR, and mRNA levels of ACACA were highest at d 57 pp ($p < 0.05$).

Protein metabolism

At calving, mRNA levels of CTSL were higher in mp-cows (10.78 ± 0.26) compared to MP-cows (9.95 ± 0.46; $p = 0.02$). In addition, at d 15 pp transcript abundance of CTSL was higher in mp-cows (10.91 ± 0.17) compared to MP-cows (10.17 ± 0.24; $p = 0.04$). The mRNA encoding for CTSL and TAT showed different patterns during lactation with highest abundance after FR.

Carbohydrate metabolism

At day of calving, mRNA encoding for PCK1 showed higher abundance in Mp-cows (11.13 ± 0.39) compared to mp-cows (9.52 ± 0.71; $p = 0.04$) and were generally lowest at day of calving ($p < 0.05$). At the same time, less mRNA abundance of PCK2 was measured in Mp-cows (2.24 ± 0.49) compared with mp- (5.43 ± 0.95; $p = 0.04$) and mP-cows (5.64 ± 1.02; $p = 0.04$). At d 15 pp transcript abundance of PC was higher in mP-cows (5.58 ± 0.26) compared to mp-cows (3.60 ± 0.54; $p = 0.04$). At d 57 pp mRNA abundance of PC was measured in MP-cows (2.75 ± 0.43) compared with Mp- (4.56 ± 0.68; $p = 0.04$) and mp-cows (4.76 ± 0.91; $p < 0.05$).

Ketogenesis

The mRNA encoding for HMGCS2 showed highest abundance after FR ($p < 0.001$; Table 4).

Glucose transport

Levels of transcript encoding for SLC2A2 were comparable among groups and during experimental timeframe.

Hormone receptor

Furthermore, transcript abundances of INSR were higher in mP-cows (7.28 ± 0.10) compared to Mp- (6.57 ± 0.09; $p < 0.05$) and MP-cows (6.34 ± 0.15; $p < 0.01$) at the day of calving.

Table 4 LSM ± SEM of mRNA abundance (log_2) of hepatic genes during early lactation and after 3-day feed restriction (d 29 pp)*

Gene‡	Group†			
	MP	mp	Mp	mP
ACACA§				
Calving	3.19 ± 0.86	4.01 ± 0.65	3.91 ± 0.23	4.87 ± 0.69
d 15 pp	3.25 ± 1.38	3.68 ± 0.47	3.49 ± 0.73	4.68 ± 0.96
d 29 pp	3.68 ± 0.90	3.98 ± 0.31	4.48 ± 0.46	3.91 ± 0.68
d 57 pp	4.85 ± 0.63	5.32 ± 0.63	5.04 ± 0.24	5.48 ± 0.48
ACADVL§				
Calving	8.61 ± 0.19	8.89 ± 0.16*	8.76 ± 0.17	8.72 ± 0.27
d 15 pp	8.69 ± 0.26^{ab}	8.10 ± 0.26^{a+}	8.85 ± 0.18^{b}	8.85 ± 0.12^{b}
d 29 pp	8.99 ± 0.20	8.63 ± 0.15^{*+}	9.09 ± 0.21	9.05 ± 0.23
d 57 pp	8.55 ± 0.25	8.28 ± 0.10^{*+}	8.42 ± 0.21	8.02 ± 0.25*
CPT1A§				
Calving	8.22 ± 0.27	7.91 ± 0.25^{*+}	8.12 ± 0.33*	8.09 ± 0.48
d 15 pp	8.56 ± 0.34^{a}	7.69 ± 0.30^{b*}	8.46 ± 0.21^{a*+}	8.93 ± 0.33^{a}
d 29 pp	8.56 ± 0.26	8.62 ± 0.16^{+}	9.04 ± 0.18^{+}	8.76 ± 0.41
d 57 pp	8.25 ± 0.33	8.26 ± 0.14^{*+}	8.37 ± 0.24*	8.30 ± 0.15
CTSL§, ¶				
Calving	9.95 ± 0.46^{a}	10.8 ± 0.26^{b}	10.4 ± 0.15^{ab*+}	10.1 ± 0.30^{ab}
d 15 pp	10.2 ± 0.24^{a}	10.9 ± 0.17^{b}	10.4 ± 0.19^{ab*}	10.6 ± 0.22^{ab}
d 29 pp	10.6 ± 0.27	11.1 ± 0.24	11.1 ± 0.21^{+}	10.7 ± 0.07
d 57 pp	10.4 ± 0.23	10.9 ± 0.07	10.7 ± 0.21^{*+}	10.7 ± 0.09
ECHS1§				
Calving	9.79 ± 0.33*	9.57 ± 0.28*	9.88 ± 0.02*	9.82 ± 0.20*
d 15 pp	10.4 ± 0.22^{*+}	10.1 ± 0.27^{*+}	10.5 ± 0.13^{*+}	10.6 ± 0.16^{+}
d 29 pp	10.7 ± 0.21^{+}	10.2 ± 0.28^{a+}	10.9 ± 0.19^{b+}	10.5 ± 0.22^{*+}
d 57 pp	10.4 ± 0.14^{*+}	10.6 ± 0.17^{+}	10.5 ± 0.27^{*+}	10.5 ± 0.08^{*+}
GPAM				
Calving	6.26 ± 0.30	6.35 ± 0.55*	6.77 ± 0.31*	7.08 ± 0.21
d 15 pp	7.12 ± 0.09	6.89 ± 0.34^{*+}	7.15 ± 0.18^{*+}	6.87 ± 0.17
d 29 pp	6.49 ± 0.46^{a}	6.88 ± 0.26^{a*+}	7.67 ± 0.23^{b+}	7.27 ± 0.22^{ab}
d 57 pp	7.12 ± 0.20	7.56 ± 0.29^{+}	6.93 ± 0.31*	7.38 ± 0.23
HMGCS2§				
Calving	9.41 ± 0.38	8.08 ± 0.56*	9.14 ± 0.35*	8.94 ± 1.24*
d 15 pp	11.1 ± 0.24	10.2 ± 0.66	10.1 ± 0.48^{*+}	9.91 ± 0.58^{*+}
d 29 pp	10.4 ± 1.26	10.6 ± 0.56	11.1 ± 0.57^{+}	11.6 ± 1.03^{+}
d 57 pp	11.1 ± 0.13	10.4 ± 0.39	9.55 ± 0.40*	10.4 ± 0.58^{*+}
HNF4A§				
Calving	8.49 ± 0.41	7.98 ± 0.45	7.74 ± 0.40*	8.87 ± 0.36
d 15 pp	8.42 ± 0.32	7.57 ± 0.40	8.22 ± 0.26*	8.13 ± 0.17
d 29 pp	8.94 ± 0.41	8.26 ± 0.24	9.15 ± 0.37^{+}	8.91 ± 0.70
d 57 pp	7.91 ± 0.25	8.36 ± 0.57	8.37 ± 0.16^{*+}	7.77 ± 0.27
INSR				
Calving	6.34 ± 0.15^{a}	6.80 ± 0.30^{ab}	6.57 ± 0.09^{a}	7.28 ± 0.10^{b}
d 15 pp	6.77 ± 0.06	6.75 ± 0.25	6.84 ± 0.10	7.11 ± 0.21
d 29 pp	6.88 ± 0.22	6.93 ± 0.13	7.45 ± 0.33^{+}	7.10 ± 0.31
d 57 pp	6.68 ± 0.17	7.14 ± 0.37	6.85 ± 0.13	6.67 ± 0.24
PC§				
Calving	5.01 ± 0.68*	6.64 ± 0.76*	5.16 ± 0.90^{*+}	6.86 ± 0.85*
d 15 pp	4.42 ± 0.50^{ab*+}	3.60 ± 0.54^{a+}	4.86 ± 0.60^{ab*+}	5.58 ± 0.26^{b*+}
d 29 pp	5.00 ± 0.41^{*+}	5.35 ± 0.47^{*+}	6.24 ± 0.27*	5.47 ± 0.80^{*+}
d 57 pp	2.75 ± 0.43^{a+}	4.76 ± 0.91^{b+}	4.56 ± 0.68^{b+}	3.53 ± 0.65^{ab+}
PCK1§				
Calving	10.2 ± 0.29^{ab*}	9.52 ± 0.71^{a*}	11.1 ± 0.39^{b}	10.9 ± 0.64^{ab}
d 15 pp	11.9 ± 0.19^{+}	10.8 ± 0.81^{*+}	11.5 ± 0.69	12.2 ± 0.17
d 29 pp	11.5 ± 0.59^{*+}	11.6 ± 0.47^{+}	11.6 ± 0.46	11.0 ± 0.29
d 57 pp	12.4 ± 0.37^{+}	12.2 ± 0.61^{+}	12.1 ± 0.30	12.0 ± 0.19

Table 4 (Continued)

Gene[‡]	Group[†]			
	MP	mp	Mp	mP
PCK2				
Calving	2.78 ± 0.67[ab]	5.43 ± 0.95[a*]	2.24 ± 0.49[b]	5.46 ± 1.02[a]
d 15 pp	3.07 ± 1.23	1.08 ± 1.01[+]	2.65 ± 1.05	3.29 ± 0.39
d 29 pp	2.49 ± 2.35	1.77 ± 1.04[+]	4.06 ± 0.59	3.29 ± 0.28
d 57 pp	3.22 ± 0.66	4.60 ± 1.01[*]	3.24 ± 0.88	4.11 ± 0.88
PPARA[§]				
Calving	8.60 ± 0.26	8.17 ± 0.24[*]	8.44 ± 0.14	8.72 ± 0.05
d 15 pp	8.47 ± 0.08	8.31 ± 0.17[*+]	8.70 ± 0.09	8.72 ± 0.23
d 29 pp	8.75 ± 0.24	8.58 ± 0.20[*+]	8.99 ± 0.28	8.74 ± 0.30
d 57 pp	8.90 ± 0.29	8.89 ± 0.20[+]	8.98 ± 0.15	9.06 ± 0.25
SREBF1[§]				
Calving	1.63 ± 0.56[a*]	2.13 ± 0.58[ab*]	1.26 ± 0.57[a*]	3.01 ± 0.13[b]
d 15 pp	3.54 ± 0.29[+]	2.53 ± 0.52[*+]	3.17 ± 0.43	3.41 ± 0.31
d 29 pp	2.78 ± 0.14[ab*+]	2.10 ± 0.53[a*]	3.28 ± 0.29[b]	2.05 ± 0.49[ab]
d 57 pp	2.94 ± 0.34[*+]	3.98 ± 0.57[+]	3.24 ± 0.48	3.21 ± 0.24
TAT				
Calving	11.0 ± 0.41	11.0 ± 0.31	10.4 ± 0.28	10.7 ± 0.42
d 15 pp	10.5 ± 0.14	10.3 ± 0.27	10.5 ± 0.19	10.4 ± 0.26
d 29 pp	11.0 ± 0.69	10.7 ± 0.31	11.5 ± 0.23[+]	10.9 ± 0.41
d 57 pp	10.6 ± 0.15	10.9 ± 0.33	10.6 ± 0.30	10.9 ± 0.43

*mRNA abundance was calculated relative to the expressions of the three references genes, actin beta, glyceraldehyde-3-phosphate dehydrogenase and H3 histone family 3A.

[†]M represents cows with high ECM yield, m represents cows with low ECM yield, P represents cows with high milk protein concentration, and p represents cows with low milk protein concentration (6 MP-, 5 mp-, 7 Mp- and 5 mP-cows).

[‡]ACACA, acetyl-CoA carboxylase α; ACADVL, acyl-CoA dehydrogenase, very long chain; CPT1A, carnitine palmitoyltransferase 1A; ECHS1, enoyl CoA hydratase 1; EIF4B, eukaryotic translation initation factor 4B; GPAM, glycerol-3-phosphate acyltransferase; HMGCS2, 3-hydroxy-3-methylglutary-coenzyme A synthase 2; HNF4A, hepatocyte nuclear factor-4A (Loor et al., 2005); IGF1, insulin-like growth factor 1; INSR, insulin receptor; PC, pyruvate carboxylase; PCK1, phosphoenolpyruvate carboxykinase, cytosolic; PCK2, phosphoenolpyruvate carboxykinase, mitochondrial; PPARA, peroxisome proliferator activated receptor-α (Sigl et al., 2010); SREBF1, sterol regulatory element binding transcription factor 1 (Van Dorland et al., 2009); TAT, tyrosine aminotransferase; TNFA, tumor necrosis factor α.

[§] Time effect ($p < 0.05$).

[¶] Group effect ($p < 0.05$).

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

[*+]Means with figurative superscripts indicate differences between timepoints ($p < 0.05$).

Transcriptional regulation and translation initiation

At the day of calving, transcript abundance of SREBF1 was higher in mP-cows (3.01 ± 0.13) compared with MP- (1.63 ± 0.56; $p < 0.05$) and Mp-cows (1.26 ± 0.57; $p = 0.02$). After FR transcript abundance of SREBF1 was higher in Mp-cows (3.28 ± 0.29) compared with mp-cows (2.10 ± 0.53; $p < 0.05$). Furthermore, higher mRNA abundance of HNF4A was measured after FR ($p < 0.05$). The mRNA encoding for PPARA showed higher abundance at d 57 pp ($p < 0.05$). Transcript abundances for EIF4B were comparable among groups and during experimental timeframe.

Cyclicity and health

Three cows (one mp- and two Mp-cows) had to be treated because of ovarial cysts and were not considered for statistical health analyses. Day of first ovulation did not differ among groups (24 ± 4, 24 ± 6, 31 ± 4 and 22 ± 3 d pp for MP-, mp-, Mp- and mP-cows, respectively; $p = 0.44$; Table 5). Prior to d 30 pp, 67% of MP-, 75% of mp-, 40% of Mp- and 100% of mP-cows ovulated. During the experimental timeframe, 11 cows showed clinical signs of lameness (3 MP-cows, 1 mp-cow, 4 Mp-, and 3 mP-cows), 7 of retained placenta (3 MP-, 1 mp-, 2

Table 5 Cyclicity and occurrence of health disorders with first day of diagnosis* in the four groups

	Group†			
	MP	mp	Mp	mP
Cyclicity				
Ovarial cycsts (%)		20	29	
First ovulation (days)	24 ± 4	24 ± 6	31 ± 4	22 ± 3
First ovulation < d 30 pp (%)	67	75	40	100
Diagnosis				
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)

*Data in parentheses indicate the first day of diagnosis relative to calving.
†M represents cows with high ECM yield, m represents cows with low ECM yield, P represents cows with high milk protein concentration, and p represents cows with low milk protein concentration.

Mp- and 1 mP-cow) and one mP-cow suffered on mastitis. One Mp-cow recommended veterinary intervention because of a clinical ketosis (inappetence, urinary ketone bodies) after parturition.

Discussion

It is widely recognized that NEB leads to a marked decrease in milk protein concentration in the immediate postpartum period of dairy cows and therefore to an undesirable loss in average 305-day milk protein yield (DePeters and Cant, 1992; Murphy and O'Mara, 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 our experimental trial to evaluate those physiological adaptive responses in cows with significantly different milk protein concentrations during early lactation and concomitant significantly varying ECM yield under same housing and feeding conditions.

Classification of cows

As expected, classification of the 23 Holstein–Friesian cows in four groups according to ECM yield and milk protein concentration also affected yields of milk fat and milk lactose as well as milk fat concentration. Nevertheless, serum metabolites were comparable among groups during the first 9 weeks of lactation. However, we found higher blood serum glucose levels in MP- compared to mp-cows at the day of calving. Because average time of sampling tended to be earlier in MP- (6 h after parturition) than in mp-cows (12.5 h after parturition), the results of blood sampling within 24 h after parturition could be influenced by the physiological high blood glucose level during calving. Furthermore, 2 weeks before expected calving, MP-cows showed higher blood serum concentrations of NEFA compared to Mp-cows which might reflect a higher energy deficit in those cows. In the present study, ovarian cycle activity was not influenced by milk volume and milk protein concentration during early lactation. Nevertheless, three cows were excluded from statistical analysis of cyclicity because of ovarial cysts. Of those three animals, one mp-cow suffered inflammation of uterus (retained placenta), whereas the other two cows had the highest ECM yield during early lactation (52.6 ± 0.73 kg/day). Infectious diseases of genital tract and risk of metabolic imbalances over the course of periparturient period due to high milk yield are the two main reasons leading to decreased fertility (Walsh et al., 2011). Crowe (2008) reported that dairy cows in good nutritional state ovulate around 15 days pp. In the present study, cows ovulated at d 23 ± 2 pp but within the physiological timeframe approximately up to 30 days pp. Incidence of lameness and retained placenta was highest in dairy cows with high ECM yields during early lactation whereas mp-cows had lowest incidence of clinical diseases. Previous studies confirmed that high-yielding dairy cows are more susceptible to diseases (Mallard et al., 1998; LeBlanc, 2010).

Feeding, milk and blood serum parameters during FR

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. The average DMI of all animals was 16.3 ± 0.75 kg from d 23 until 25 pp which was slightly lower compared with previously reported DMI for multiparous cows on d 24 pp (approximately 17 kg; Ingvartsen and Andersen, 2000). As expected, mp-cows showed lowest *ad lib* 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 23–25 pp, which was roughly met only by mP-cows (66.9%). DMI of MP-, mp-, and Mp-cows during restriction was still lower (63.6%, 62.0% and 59.5% for Mp-, mp- and Mp-Cows, respectively) due to a marked decrease during the first day of restriction associated with slower adaption to straw-supplemented RD. Moreover, hyperketonemia could have decreased feed intake, but this metabolic challenge had to be faced by all animals during FR.

Our results are supported by the well-known fact, that during early lactation nutrient energy intake regularly lags behind milk-production related energy demands leading to a NEB (Bell, 1995; Drackley, 1999). All cows, regardless of group membership, experienced a NEB in the third week of lactation (average −64.9 ± 5.7 MJ NE_L) but NEB was even lower compared to Kessel et al. (2008; approximately −35 MJ NE_L). On the first day of FR, a severe decline of EB to values below −110 MJ NE_L in MP- and Mp-cows was found. Subsequently, in those animals EB increased slightly during the following two restriction days. These findings reflect the fast metabolic adaptation of high-yielding animals to increased NEB. In low-yielding cows, the decline in EB was more moderate and remained on the level of the first day of FR during remaining FR period. In agreement with Nielsen et al. (2003) and Agenäs et al. (2003) milk protein concentration of all cows was unaffected by FR. Agenäs et al. (2003) illustrated a distinct decline in milk protein concentration during subsequent first 2 days of realimentation. In our study, milk protein concentration also declined to a nadir 1 day after FR, particularly in mP-cows. As expected due to the decreased milk yield and milk protein concentration, milk protein yield also declined over the course of FR. Moreover, in all groups milk fat concentration and yield did not vary during FR. 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 a decrease in milk fat yield (Velez and Donkin, 2005). Throughout our experiment, milk lactose concentration was constant in all cows which can be explained by the osmotic role of lactose and the fact that milk volume is mainly depending on lactose synthesis (Peaker, 1978). As shown before, lowest level of protein content was reached on the first day of refeeding, while milk fat and lactose content were not responsive to the reduced feeding level. The subsequent minimum of protein content in FR is not unusual, as fat and lactose synthesis have top priority due to breeding preferences over the last decades. Also, fat mobilization 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). Almost all cows in our experiment, regardless of classification, showed higher FPR values before and during FR. As previously shown, blood serum glucose levels decreased and blood serum NEFA concentrations increased in all groups and reached the initial level during subsequent *ad lib* feeding (Nielsen et al., 2003; Loor et al., 2007). The steep decrease of glucose far below the basal level of 3.0 mmol/l (Rosenberger, 1990) in high-yielding dairy cows could be explained by the largely distribution of blood glucose to milk synthesis. Insufficient energy supply results in lipolysis of adipose tissue (Mashek and Grummer, 2003). This is indicated by the higher concentration of circulating NEFAs in blood which are supplied to gluconeogenesis and β-oxidation in hepatocytes. In the present study, average blood serum NEFA levels rose in all groups above threshold levels of 1000 μmol/l during restricted feeding, except in mp-cows. Lower serum NEFA levels in early lactation for mp-cows suggested a more stable metabolic status together with a sustained physiological serum glucose concentration. Also, due to deficiency of glucose, product of β-oxidation Acetyl-CoA is not metabolized in citrate-cycle and induces ketogenesis during NEB (Zammit, 1983). Blood serum BHBA levels increased drastically up to the third day of FR above threshold values for subclinical ketosis of 1.2–1.4 mmol/l in all groups (LeBlanc, 2010). However, our 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. Therefore, individual regulation of these metabolic adaptations requires further investigations.

Hepatic gene expression

One of the principal aims of our study was to analyze gene networks related to hepatic metabolism in early lactation and particularly after an induced FR (d 29 pp). Due to the enormous increase of blood serum concentrations of NEFA and BHBA during FR, we suggested that our FR model was appropriate to further enhance hepatic fatty acid oxidation, gluconeogenesis and ketogenesis, diminished fatty acid synthesis and modified amino acid catabolism during early lactation. The nuclear protein PPAR-α is a mediator of NEFA to mitochondrial fatty acid oxidation and upregulates genes involved in ketogenesis (Mandard et al., 2004). However, mRNA levels of PPARA were not correlated to levels of transcripts encoding for ACADVL and CPT1A in contrast to previous findings (Drackley, 1991). At d 15 and 29 pp mRNA levels of genes related to β-oxidation (ACADVL and CPT1A, ECHS1 and GPAM, respectively) were lowest in mp-cows. Based on higher milk fat yields in Mp-cows, those cows were expected to experience increased lipolysis of body fat tissue. Concerning the de novo fatty acid synthesis, mRNA levels of ACACA were lower after calving, at d 15 pp and after FR in all animals, regardless the classification. These results go in line with Loor et al. (2007), who found down regulation of ACACA associated with FR and ketosis in high-yielding dairy cows.

NEFA blood levels and its uptake in liver tissue influence expression of transcription factors PPARA and HNF4A as well as expression of liver triacylglycerol content-related genes SREBF1 and GPAM (Loor et al., 2005). It is well established, that transcript abundances for SREBF1 and GPAM increase gradually, yet markedly throughout early lactation (Loor et al., 2005). Kim et al. (2004) and Romics et al. (2004) reported that upregulation of SREBF1 in mice was followed by upregulation 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. However, only in Mp-cows a further increase was measured during FR in both genes which might suggest a higher risk of liver-related disorders. HNF4A plays an important role for PPARA in fatty acid oxidation and gluconeogenesis (Odom et al., 2004), through binding to the promoter region of ACADVL and PCK1 (Loor et al., 2005). Our results indicated higher mRNA levels of HNF4A which was associated with increased fatty acid oxidation. As lactation advanced, mRNA levels of PCK1 increased from d 15 pp through d 57 pp. Comparable results were reported by Greenfield et al. (2000). The upregulation of PCK1 during early lactation in our study is associated with the large demand of glucose for milk synthesis and describes increased gluconeogenesis initiated by FR. During calving, mRNA levels of PCK2 were lower in Mp-cows compared to mp- and mP-cows. Therefore, the absence of increased mRNA levels of PCK2 around calving (Loor, 2010) was associated with the previously reported higher risk for liver-related disorders in Mp-cows. With onset of lactation, increasing milk yield and demand for lactose is associated with upregulation of mRNA levels of PC (Greenfield et al., 2000). At d 15 pp transcript abundances of PC were lowest in mp-cows and highest in mP-cows. Due to the high milk production in high yielding dairy cows, higher transcript abundances of PC were presumed. During periparturient period, hepatic ketogenesis occurs frequently in dairy cows with HMGCS2 acting as a controlling enzyme (Voet and Voet, 2004; Loor et al., 2005). Present results showed higher mRNA levels of HMGCS2 after FR which are supported by Hegardt (1999) who 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. Liver TAT and CTSL are proteinases associated with amino acid catabolism which is diminished during periparturient period (Loor et al., 2005). Our data showed downregulation of CTSL in MP-cows at d 1 and 15 pp, whereas CTSL was upregulated in mp-cows. Additionally, we measured highest mRNA levels of TAT and CTSL after FR. Enhanced activities of TAT and CTSL are associated with increased amino acid fragments, partly used for gluconeogenesis or ketogenesis.

In conclusion, high yielding dairy cows (Mp- and MP-cows) resulted in comparable milk protein yields during first 9 weeks of lactation. Simultaneously, physiological adaptation to a metabolic challenge seemed to be in part diminished in cows with high milk yield and low milk protein concentration (Mp-cows). Therefore, efforts to increase milk protein concentration in high yielding dairy cows during early lactation could be not only a lucrative and sustainable but also an animal-appropriate management tool to increase dairy economic outcome and to reduce metabolic imbalances at the same time.

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Gene expression of six major milk proteins in primary bovine mammary epithelial cells isolated from milk during the first twenty weeks of lactation

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ABSTRACT: The objective of the present study was to refine a previously developed method to isolate primary bovine mammary epithelial cells (pBMEC) from fresh milk. Using this method, it was tested whether the number of pBMEC and the relation of recovered pBMEC to total somatic cell count vary within the individual lactation stages. Furthermore, the expression levels of the milk protein genes during the first twenty weeks of lactation were determined by quantitative PCR method. A total number of 152 morning milk samples were obtained from twenty-four Holstein-Friesian cows during the first 20 weeks of lactation (day 8, 15, 26, 43, 57, 113, and 141 postpartum). Numbers of extracted pBMEC were consistent at all time-points (1.1 ± 0.06 to 1.4 ± 0.03 $\times 10^3$/ml) and an average value of RNA integrity number (RIN) was 6.3 ± 0.3. Percentage of pBMEC in relation to total milk cells (2.0 ± 0.2 to 6.7 ± 1.0%) correlated with milk yield. Expression patterns of the casein genes alpha $(\alpha)_{S1}$, $(\alpha)_{S2}$, beta (β), and kappa (κ) (CSN1S1, CSN1S2, CSN2, CSN3, respectively) and the whey protein genes α-lactalbumin (LALBA) and progestagen-associated endometrial protein (PAEP; known as β-lactoglobulin) were shown to be comparable, i.e. transcripts of all six milk protein genes were found to peak during the first two weeks of lactation and to decline continuously towards mid lactation. However, mRNA levels were different among genes with CSN3 showing the highest and LALBA the lowest abundance. We hypothesized that milk protein gene expression has a pivotal effect on milk protein composition with no influence on milk protein concentration. This paper is the first to describe milk protein gene expression during lactation in pBMEC collected in milk. Future studies will be needed to understand molecular mechanisms in pBMEC including regulation of expression and translation throughout lactation.

Keywords: dairy cow; immunomagnetic cell separation; mammary gland; milk protein gene expression

Six bovine milk proteins, comprising 95% of the total protein, have previously been classified into the four caseins (α_{S1}, α_{S2}, β, and κ), and the two major whey proteins (α-lactalbumin and β-lactoglobulin) (Threadgill and Womack, 1990). All major milk proteins are synthesized in the mammary epithelial cells (MEC). During the milking process, the MEC detach from the alveolar epithelium and are continuously shed into milk during the entire lactational period comprising approximately 2% of total somatic cells (Boutinaud and Jammes, 2002).

The number of mammary secretory cells and their secretory activity are mainly responsible both for the daily produced amount of milk and the fast increase in milk yield during the first weeks of lactation (Boutinaud et al., 2004). After peak lactation, the number of the secretory cells in the mammary gland declines gradually by 8% between days 90 and

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240 of lactation accounting for lower milk yields at the end of lactation (Capuco et al., 2003).

Consequently, typing of different protein variants as well as knowledge about the regulation of expression of the different milk protein genes during lactation is crucial for the genetic improvement of milk composition and milk yield (Groenen and van der Poel, 1994). To study the cellular mechanism responsible for synthesis of milk constituents, especially protein synthesis, and to better understand these molecular events, pBMEC samples need to be harvested either by invasive mammary gland biopsies or by purification from milk. Boutinaud et al. (2008) refined the isolation of viable BMEC directly from fresh milk. Therefore, gene expression studies in pBMEC are frequently repeatable during lactation cycle.

As concerns recent investigations of the expression of the milk protein genes, Bionaz and Loor (2011) determined the expression of the milk protein genes LALBA and CSN3 in bovine mammary gland tissue during lactation cycle. They found the highest expression levels for both on day 60 postpartum (pp) (Bionaz and Loor, 2007, 2011, respectively). In lactating dairy ewe mammary gland expression levels of the caseins (CSN1S1, CSN1S2, CSN2, CSN3) did not change, but during involution and late pregnancy the expression levels lowered (Colitti and Pulina, 2010). Bevilacqua et al. (2006) investigated the expression levels and the translation efficiency of the four caseins in goat, ewe, and cow. In cows, transcripts seemed to be at the same level of abundance.

Research into the molecular mechanisms of milk protein synthesis may also help the improvement of strategies and technologies for enhancing milk protein production of the dairy cow.

MATERIAL AND METHODS

Cows, housing, and feeding

Milk was collected from twenty-four multiparous German Holstein-Friesian cows (parity 2.5 ± 0.1, calving throughout the year). After parturition, cows were machine-milked twice daily (at 4.20 a.m. and 3.40 p.m.). During each milking, milk yield was recorded with electronic milk meters (Metatron P21) and stored electronically (DairyPlan C21; both GEA WestfaliaSurge GmbH, Bönnen, Germany). The partly mixed feed ration was based on corn and grass silage and mixed with concentrates to meet the energy requirements of cows on the basis of a total daily milk production of 22 kg per day. The diet was offered once daily in sufficient amounts to secure ad libitum intake (> 5% residual feed). Additional concentrates were given individually according to the day of lactation. Water was freely available at all times.

Sampling

A total number of 168 morning milk samples was collected. Samples were obtained from each cow at days 8, 15, 26, 43, 57, 113, and 141 after parturition. Whole morning milk samples were separated during milking into a sterile bucket and milk yield was determined with a spring scale. 1800 ml of the total morning milk was filled in autoclaved glass bottles and used for cell isolation immediately. One aliquot was stored at 4°C for a maximum of 7 days with a preservative (acidiol) until analyses of milk composition.

Milk composition analysis

Milk protein, fat, and lactose were analyzed by infrared-spectrophotometric technique – infrared absorption measurement evaluated by Fourier transform (MilkoScan FT6000) and somatic cell count was determined by a fluorescence-optical counting system (Fossomatic FC; both Foss, Hillerød, Denmark) in the laboratories of Milchpruefring Bayern e.V. (Wolnzach, Germany).

Cell isolation

Milk (1800 ml) was defatted by centrifugation at 1800 *g* at 4°C for 30 min in four 450-ml corning tubes and skim milk was removed. Remaining total cell pellets were resuspended in 25 ml of phosphate buffered saline (PBS) and pooled in pairs. After a second centrifugation step (1850 *g*, 15 min at 4°C) the two total cell pellets were resuspended and pooled in 1 ml of PBS containing 1% bovine serum albumin (BSA). Purification of pBMEC was performed applying an immunomagnetic-bead based separation technique. Cell suspension was first incubated for 10 min on a rotary mixer at 4°C with a primary mouse monoclonal antibody against cytokeratin 8 antibody (clone C-43, EXBIO, Prague, Czech Republic), which is specific to bovine epithe-

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lial cells. Unbound antibodies were removed from the cell-antibody complex by 8 min of centrifugation at 300 *g* at 4°C. After discarding the supernatant cell-antibody complex was resuspended in 1 ml of 1% BSA-PBS. Dynabeads (25 µl) (PanMouse IgG, Invitrogen, Dynal AS, Oslo, Norway) were added and the suspension was incubated for 20 min on a rotary mixer at 4°C. Antibody-bound cells were collected by placing the sample vials into the Dynal MPC-L (Dynal AS, Oslo, Norway) for 2 min and withdrawing of the supernatant. A second washing including a magnetic separation step was performed with 1 ml of 1% BSA-PBS followed by a suspension of pBMEC in 1 ml of 1% BSA-PBS. A 7 µl aliquot was removed to perform a hematocytometer cell count and a 10 µl aliquot was collected to stain pBMEC immunohistochemically. Purified MEC were obtained by centrifugation of tubes at 1800 *g* at 4°C for 5 min, resuspended in 700 µl Qiazol (Qiagen GmbH, Hilden, Germany), and stored at −80°C until RNA extraction.

Immunohistochemical detection of pBMEC

For immunohistochemical studies, 10 µl of the cell suspension was spread on an object slide, treated with 7 µl of poly-L-Lysine solution (Science Services, Munich, Germany), and cells were fixed with 100% ethanol for 10 min. Addition of methanol (99.8%) for 5 min permeabilized cell surfaces. Thereafter, samples were washed twice with PBS for 5 min. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide (H_2O_2) for 20 min. Following blocking with 10% goat serum, samples were incubated with cytokeratin 8 antibody at 37°C for 45 min. After washing with PBS (twice for 10 min), samples were incubated with a secondary anti-mouse IgG peroxidase-conjugated antibody (2.5 mg/ml, Sigma-Aldrich, Munich, Germany). Next, samples were washed twice with PBS. Binding of antibody was detected by incubation with PBS containing 0.01% diaminobenzene and 0.01% H_2O_2 for 15 min. Cells were counterstained using Mayer's Haemalaun (Carl Roth GmbH, Karlsruhe, Germany).

RNA extraction and reverse transcription

Total RNA was extracted from the purified MEC applying the miRNeasy MiniKit (QIAgen GmbH, Hilden, Germany). In brief, samples, frozen in 700 µl QIAzol were thawed at room temperature (RT) and homogenized by vortexing. To each sample, 140 µl of chloroform was added and the samples were vortexed vigorously for 15 s. After a 2 min incubation at RT, the mixture was centrifuged at 1.5×10^4 *g* for 15 min at 4°C. The aqueous supernatant containing total RNA was recovered and mixed with 1.5 volumes of 100% ethanol. After vortexing, up to 700 µl were pipetted into an RNeasy Mini spin column and centrifuged at 10^4 *g* for 15 s at RT. This step was repeated with the remainder of the sample. To wash the column, 700 µl of Buffer RWT was added and centrifuged at 10^4 *g* for 15 s at RT. Washing was performed twice by adding 500 µl of Buffer RPE followed by centrifugation at 10^4 *g* for 15 s at RT. Preceding dissolving of RNA in 30 µl sterile RNase-free water, the RNeasy Mini spin column membrane was dried by centrifugation (10^4 *g* for 2 min at RT). RNA was quantified by spectrophotometry (BioPhotometer; Eppendorf, Hamburg, Germany). Integrity of the RNA (RNA integrity number; RIN) was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) connected to the RNA 6000 Nano Assay. Accurate amounts of 250 ng RNA were reversely transcribed to complementary DNA adding the following reverse transcription master mix: 12 µl 5 × Buffer (Promega, Mannheim, Germany), 3 µl Random Hexamer Primers (50mM) (Invitrogen, Carlsbad, USA), 3 µl dNTP Mix (10mM) (Fermentas, St Leon-Rot, Germany), and 200 U of MMLV-H-reverse transcriptase (Promega, Regensburg, Germany). The reverse transcription reaction was carried out according to the manufacturer with a 60 µl reaction volume in a PCR thermocycler (Biometra, Göttingen, Germany) by successive incubations at 21°C for 10 min and at 48°C for 50 min, finishing with enzyme inactivation at 90°C for 2 min. Reverse transcription products were stored at −20°C.

Selected target genes and primer design

The two fractions of milk proteins synthesized in the pBMEC, comprising 95% of the total protein, are namely caseins (80%) and whey proteins (20%). The four caseins are classified into α_{S1}, α_{S2}, β, and κ, and the two major whey proteins into α-lactalbumin (LA) and β-lactoglobulin (LG). Consequently, the genes encoding for the six major milk proteins were in main focus.

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Gene sequences for primer design were obtained from the gene bank of the National Center for Biotechnology Information (NCBI). Exon-spanning primers were designed with the help of the NCBI primer tool and synthesized at Eurofins MWG (Ebersberg, Germany). PCR products of primers were sequenced at LGC Genomics (Berlin, Germany). Primers, accession numbers, and product lengths for each gene are listed in Table 1.

Quantitative PCR and PCR amplification efficiency

Quantitative PCR was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/fluorescein (Eurogentec, Cologne, Germany) applying a standard protocol recommended by the manufacturer. All components necessary for real-time qPCR were mixed in the reaction wells. The mastermix was prepared as follows: 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 µl RNase free water. Per well, 13.5 µl mastermix plus 1.5 µl cDNA was added. The plate was sealed, placed in the iQ5 Cycler (Bio-Rad, Munich, Germany), and the following PCR protocol was started: denaturation step (95°C, 5 min), cycling program (95°C, 3 s; primer specific annealing temperature, 60 s) and melting curve analysis.

Afterwards, the qPCR assays were evaluated by the generation of a standard curve. Calibration curves for each gene were done on the Bio-Rad iQ5 with eight 10-fold serial dilutions (in triplicates) and were calculated by Bio-Rad iQ5 Optical System Software (Version 2.1) with the analysis mode "PCR

Table 1. Primer pairs, product sizes, and accession numbers

Gene[1]	Sequence (5' → 3')	Product size (bp)	GeneBank accession No.	E* (%)
Major milk protein genes				
CSN1S1 for	ATGAAACTTCTCATCCTTACCTGTCTT	179	NM_181029.2	98
CSN1S1 rev	CCAATATCCTTGCTCAGTTCATT			
CSN1S2 for	AGCTCTCCACCAGTGAGGAA	150	NM_174528.2	90
CSN1S2 rev	GCAAGGCGAATTTCTGGTAA			
CSN2 for	GTGAGGAACAGCAGCAAACA	233	NM_181008.2	85
CSN2 rev	AGGGAAGGGCATTTCTTTGT			
CSN3 for	TGCAATGATGAAGAGTTTTTTCCTAG	150	NM_174294.1	87
CSN3 rev	GATTGGGATATATTTGGCTATTTTGT			
LALBA for	CTCTCTGCTCCTGGTAGGCAT	247	NM_174378.2	96
LALBA rev	GTGAGGGTTCTGGTCGTCTT			
PAEP for	AGAAGGTGGCGGGGACTTGG	375	NM_173929.3	100
PAEP rev	TGTCGAATTTCTCCAGGGCCT			
Marker of epithelial cells				
KRT8 for	GCTACATTAACAACCTCCGTC	237	NM_001033610.1	97
KRT8 rev	TCTCATCAGTCAGCCCTTCC			
References genes				
GAPD for	GTCTTCACTACCATGGAGAAGG	197	NM_001034034.1	100
GAPD rev	TCATGGATGACCTTGGCCAG			
H3F3A for	ACTCGCTACAAAAGCCGCTCG	232	NM_001014389.2	94
H3F3A rev	ACTTGCCTCCTGCAAAGCAC			
RPS9 for	CCTCGACCAAGAGCTGAAG	64	NM_001101152.1	100
RPS9 rev	CCTCCAGACCTCACGTTTGTTC			

[1]CSN1S1 = α_{S1}-casein, CSN1S2 = α_{S2}-casein, CSN2 = β-casein, CSN3 = κ-casein, GAPD = glyceraldehyde-3-phosphate dehydrogenase, H3F3A = H3 histone family 3A, KRT8 = keratin 8, LALBA = α-lactalbumin, PAEP = progestagen-associated endometrial protein, better known as β-lactoglobulin, RPS9 = ribosomal protein S9

*efficiency was calculated by the slope of the standard curve by the equation: $E = 10^{(-1/slope)}$

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base line substracted". Amplification efficiency (E) of qPCR reactions was calculated with the slope of the log-linear portion of the calibration curve according to the equation: $E = 10^{(-1/slope)}$ (Rasmussen, 2001; Bustin et al., 2009).

Quantification of mRNA

Genes were selected as reference genes using GenEx Pro Software Version 5.2.7.44 (MultiD Analyses, Gothenburg, Sweden). In the present study, the NormFinder algorithm was used. The mean of the three housekeeping genes, glyceraldehyde-3-phosphate dehydrogenase (GAPD), H3 histone family 3A (H3F3A), and ribosomal protein S9 (RPS9) was calculated for the reference index and used for normalization. Quantitative cycle (Cq)-values 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 $\Delta Cq = Cq_{target\ gene} - meanCq_{refence\ genes}$ (Pfaffl, 2001). In order to avoid negative digits while allowing an estimation of a relative comparison between two time points, data are presented as least square means (LSM) ± standard error of means (SEM) subtracted from the arbitrary value 2 (2 – ΔCq). Thus, a high ΔCq-value resembles high transcript abundance. An increase of one ΔCq represents a two-fold increase of mRNA transcripts.

Statistical analysis

The statistical analysis of the data for milk yield, milk composition, and number of somatic and epithelial cells was assessed by repeated measurements ANOVA using the MIXED Procedure of SAS (Statistical Analysis System, Version 9.1, 2002). The ΔCq-values were normalized individually in relation to the housekeeping gene index of GAPD, H3F3A, and RPS9 before using the MIXED Procedure of SAS with repeated measurements. The ANOVA models used contained the fixed effect DIM and a random cow effect. Results are represented as LSM ± SEM.

RESULTS

Milk yield and milk composition

Detailed findings are presented in Table 2. In summary, the results reflected the well established course of the morning milk yield with peak yields of 21.7 ± 0.7 kg on days 43 and 57 pp. Milk protein concentration decreased to a nadir of 2.99 ± 0.06% on day 43 pp and was followed by an increase until day 141 pp to a value of 3.44 ± 0.09%. Milk fat concentration started with the highest concentrations on day 8 pp (5.48 ± 0.22%), reached the lowest concentrations on day 57 pp (3.94 ± 0.15%), and afterwards it was gradually rising.

Cell isolation

The immunomagnetic cell binding technique using cytokeratin 8 coated antibodies was applicable for specific binding of pBMEC in fresh milk. Quality of extracted mRNA was also sufficient for gene expression studies. An average RIN-value of 6.3 ± 0.3 was obtained.

Table 2. Morning milk yield and daily concentrations of protein, fat, and lactose[1] during the first twenty weeks of lactation

	Day of sampling postpartum						
	8	15	26	43	57	113	141
Milk yield (kg)	17.8 ± 0.6[a]	18.7 ± 0.8[bf]	20.2 ± 0.7[ce]	21.7 ± 0.7[d]	21.7 ± 0.7[cd]	19.1 ± 0.7[be]	16.9 ± 0.9[af]
Protein (%)	3.80 ± 0.07[a]	3.34 ± 0.07[bde]	3.08 ± 0.06[bc]	2.99 ± 0.06[c]	3.10 ± 0.06[cd]	3.35 ± 0.07[de]	3.44 ± 0.09[e]
Protein (g)	676 ± 24[a]	620 ± 25[b]	623 ± 23[ab]	646 ± 21[bd]	669 ± 19[c]	635 ± 21[bd]	575 ± 27[cd]
Fat (%)	5.48 ± 0.22[a]	4.77 ± 0.28[b]	4.98 ± 0.23[b]	4.68 ± 0.21[bc]	3.94 ± 0.15[ac]	4.48 ± 0.16[bd]	4.42 ± 0.23[d]
Fat (g)	969 ± 45[ab]	884 ± 57[ad]	1 006 ± 56[c]	1 024 ± 65[c]	847 ± 36[d]	859 ± 48[d]	664 ± 40[be]
Lactose (%)	4.62 ± 0.03[a]	4.82 ± 0.03[ab]	4.83 ± 0.03[ab]	4.79 ± 0.03[a]	4.88 ± 0.03[b]	4.79 ± 0.03[a]	4.76 ± 0.03[a]

[1]values are presented as least square means ± SEM

[a–f]means with different letters within the same row are significantly different

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Table 3. Number of samples and least square means ± SEM, number of total milk cells, number of separated MEC, RNA quantity, and cytokeratin mRNA levels

	Day of sampling postpartum						
	8	15	26	43	57	113	141
Milk samples (*n*)	21	22	22	23	22	21	21
No. of total milk cells, $\times 10^3$/ml of milk	83 ± 18[ac]	89 ± 34[ac]	77 ± 42[ac]	38 ± 8[b]	48 ± 17[b]	123 ± 80[c]	108 ± 41[a]
No. of recovered MEC, $\times 10^3$/ml of milk	1.2 ± 0.04	1.3 ± 0.04	1.1 ± 0.06	1.2 ± 0.04	1.2 ± 0.03	1.4 ± 0.03	1.1 ± 0.06
Recovered pBMEC (%) of total milk cells	2.0 ± 0.2[a]	3.4 ± 0.4[b]	3.6 ± 0.3[b]	5.6 ± 0.8[c]	6.7 ± 1.0[c]	4.9 ± 0.9[c]	2.2 ± 0.3[a]
RNA quantity (μg)	4.1 ± 0.7	3.3 ± 0.3	2.5 ± 0.5	3.6 ± 0.9	2.6 ± 0.5	3.6 ± 0.6	4.5 ± 0.7
KRT 8 mRNA level, arbitrary value	6.8 ± 0.3	6.7 ± 0.4	6.9 ± 0.4	7.0 ± 0.5	6.6 ± 0.4	7.7 ± 0.5	7.6 ± 0.5

MEC = mammary epithelial cells, pBMEC = primary bovine MEC
[a–c]means with different letters within the same row are significantly different

Ten out of 168 milk samples had a high somatic milk cell count (> 2×10^5 cells/ml) and were discarded without isolation of pBMEC. In addition, total amounts of RNA from six samples were too low for reverse transcription (< 250 ng) and those samples were also excluded from further analysis. The number of total milk cells in the 152 analyzed samples tended to be the lowest on day 43 pp ($38 \pm 8 \times 10^3$ cells/ml milk) and highest on day 113 pp ($123 \pm 80 \times 10^3$ cells/ml milk; Table 3). Totals of pBMEC ($1.2 \pm 0.04 \times 10^3$ cells/ml milk) were comparable at all time points, whereas the percentage of pBMEC in relation to total milk cells differed during lactation ($P < 0.001$) (Table 3). Percentage of pBMEC increased from day 8 pp (2.0 ± 0.2%) to day 43 pp (5.6 ± 0.8%, $P < 0.001$) and day 57 pp (6.7 ± 1.0%). Afterwards, fractions of pBMEC decreased to day 141 pp (2.2 ± 0.3%, $P < 0.001$) (Table 3). Percentage of pBMEC and milk yield were correlated during the first 20 weeks of lactation ($R = 0.79$, $P < 0.05$). Extracted quantity of pBMEC mRNA did not vary during experimental timeframe (Table 3). Expression levels of keratin 8 (KRT8) were used as a marker for epithelial cells. Transcript abundance of this marker was constant at all time points (Table 3).

PCR amplification efficiencies and linearity

Investigated transcripts showed high PCR efficiency rates with high linearity (Pearson's correlation coefficient $r > 0.90$). The calculated average PCR efficiency for the ten genes was 94.7 ± 1.76% and varied between 85 to 100% (Table 1). Since accuracy of qPCR depends highly on PCR efficiency, efficiency should be at least 80% (Ma et al., 2006).

Milk protein gene expression

Transcript abundances of all investigated milk protein genes were different at sampled time points ($P < 0.001$), but all protein genes showed similar expression patterns during the first 20 weeks of lactation (Figure 4). Levels of CSN1S1-mRNA increased by 1.9 fold from day 8 to day 15 pp ($P = 0.03$). After that, transcripts of CSN1S1 decreased gradually by 80% to day 141 pp ($P < 0.01$). Likewise, mRNA levels of CSN1S2 decreased successively after a peak on days 8 and 15 pp, respectively to 0.27 fold on days 113 and 141 pp ($P < 0.01$). Transcripts of CSN2 doubled from days 8 to 15 pp ($P = 0.04$) and lessened after day 15 pp by 80% to day 141 pp ($P < 0.01$). Expression of CSN3-mRNA was the highest on days 8 and 15 pp, respectively and decreased to 2.6 fold until days 113 and 141 pp ($P < 0.001$). The mRNA levels of LALBA were lower compared with those of the other milk proteins. The transcription of LALBA was similar on days8 and 15 pp and declined by 86% to days 113 and 141 pp ($P < 0.001$). After an early peak on day 8 pp, mRNA levels of PAEP decreased subsequently by 0.16 fold until days 113 and 141 pp, respectively ($P < 0.001$).

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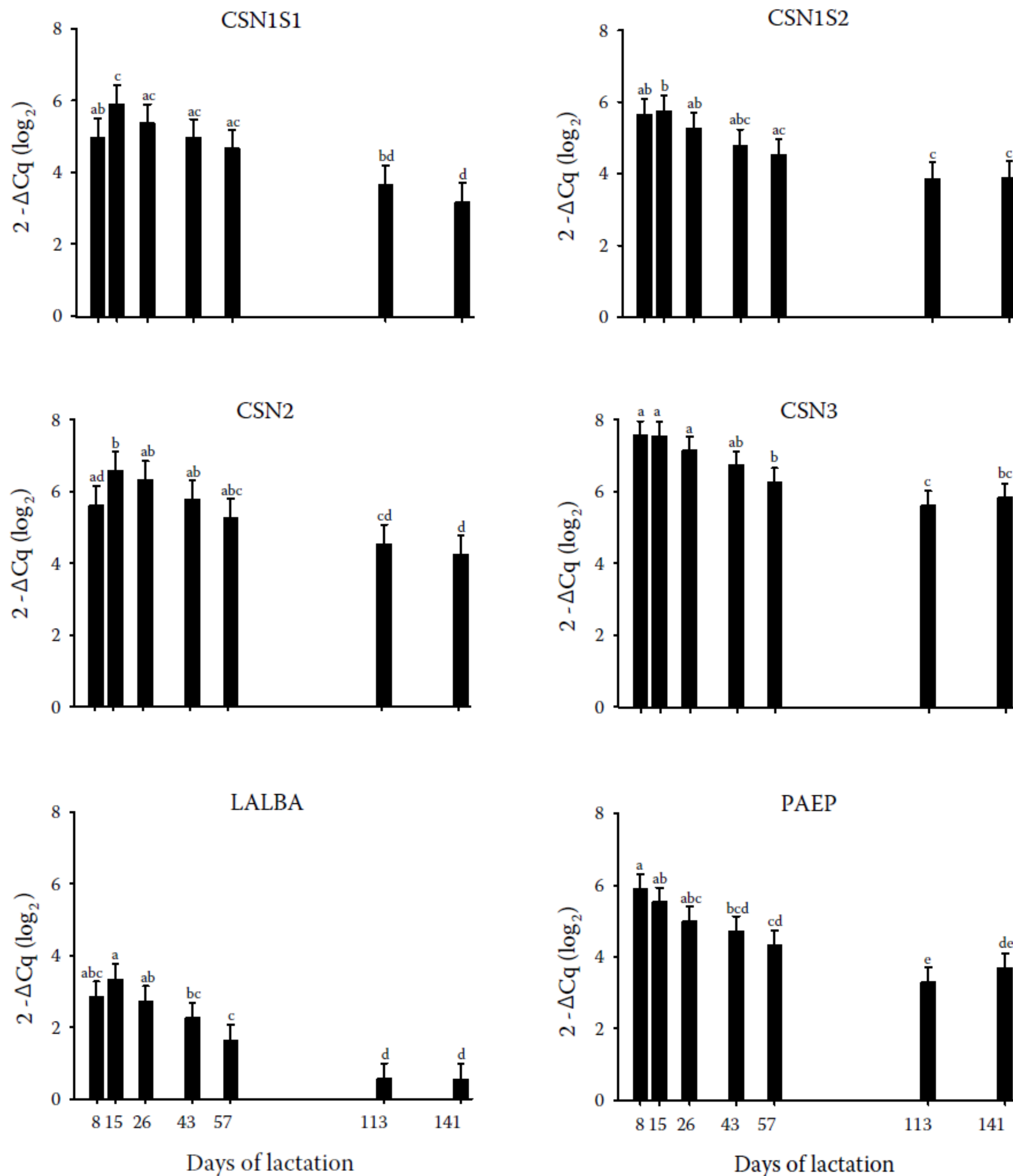

Figure 1. Transcript abundance of α_{S1}-, α_{S2}-, β-, κ-casein, α-lactalbumin, and β-lactoglobulin during the first 20 weeks of lactation in primary bovine mammary epithelial cells purified from milk. ΔCq was calculated as $Cq_{target\ gene}$ – $meanCq_{refence\ genes}$. Results are shown as 2 – ΔCq ± SEM. Letters indicate significant differences ($P < 0.05$)

DISCUSSION

Milk yield and milk composition

Milk yield increased rapidly during the first six weeks, plateaued and lowered towards the end of the experimental period roughly around mid lactation. As a result, although only morning milk yield which is higher than evening milk yields (Quist et al., 2008) was evaluated, the estimated shape of lactation curves during the first 20 weeks of lactation were consistent with those reported by numerous previous studies (e.g. Wood, 1969; Walsh et al., 2007). Decline of milk protein and milk fat concen-

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tration during the first two months after parturition followed by a respective increase of values is in agreement with findings of Friggens et al. (2007). The authors examined milk composition of Danish Holstein-Friesian cows during 301-day lactation periods and found that milk protein and fat concentration reached their lowest points approximately on days 40 and 60 pp, respectively. In the present study, proportion of milk lactose was constant during the first 20 weeks of lactation, but tended to be higher on day 57 pp. These results coincide with data from Gáspárdy et al. (2004) which show that lactose concentration of Israeli Holstein-Friesian cows peaked on day 66 of lactation. Total somatic cell count was constant during the experimental timeframe but tended to be higher at the end of the study. Our results agree with those from Sheldrake et al. (1983) and Hagnestam-Nielsen et al. (2009), who reported a constant amount of somatic cells during early and mid lactation.

Cell isolation

In numerous previous studies on mRNA expression, mammary gland tissue was obtained at one or at few time points via intricate biopsies (Farr et al., 1996; Finucane et al., 2008) or once after slaughtering of precious experimental animals (Capuco et al., 2001; Colitti and Pulina, 2010). In those samples, mRNA was extracted directly from all cells or after a preceding step of pBMEC cultivation (Talhouk et al., 1990; Griesbeck-Zilch et al., 2008). Also, techniques to culture pBMEC from milk were described (Buehring, 1990). Next to the disadvantage of a potential influence of cell culture condition on pBMEC mRNA expression, cell cultivation from mammary gland tissue partly resulted in samples that included a large fraction of non-pMEC, like fibroblasts and adipocytes. To circumvent that drawback, Gomm et al. (1995) described the isolation of pure pMEC from human mammary tissue applying an immunomagnetic separation technique. Boutinaud et al. (2008) refined that method further to extract pBMEC directly from milk. During milking, the pBMEC detach from the alveolar epithelium and discard the mammary gland within the milk. Moreover, milk is a noninvasive source of viable pBMEC (Boutinaud and Jammes, 2002). The number of pBMEC (2.1×10^6) purified from a similar volume of milk (1800 ml) did not vary among sampled time points in our study reflecting a constant renewal during lactation. Boutinaud et al. (2008) isolated approximately 162 days pp comparable 2.7×10^6 pBMEC from 1750 ml of milk of Holstein-Friesian cows which comprised 2% of total milk cells. Despite the constant discharge of pBMEC, it is well established that fraction of pBMEC of total milk cells is low (Miller et al., 1991; Boutinaud and Jammes 2002). In our study, pBMEC represented about 2–6.7% of total milk cells and that proportion was the highest during peak lactation. These results are supported by Capuco et al. (2001) who found a peak of MEC number in the udder during early lactation with a subsequent decrease during the following lactation. They concluded that the proportion of pBMEC is influenced by the stage of lactation. However, milk somatic cell count (SCC) depends mainly on immune status of the udder and only cows with a total somatic cell count below 2×10^5/ml were included in the study. Therefore, a varying proportion of pBMEC in the milk is expected in cases of clinical mastitis due to increased number of immune cells with or without increased shedding of pBMEC. Contrary to that of SCC, the number of pBMEC depends predominantly on the structure of the mammary epithelium, stage of lactation, and milking methods (Boutinaud and Jammes, 2002).

Contrary to the direct cell isolation method described by Boutinaud et al. (2008), a method of indirect cell purification was established in the present work. Total cells were first coated with the monoclonal antibody directed against cytokeratin 8, and afterwards cells-antibody complexes were incubated with the immunomagnetic particles resulting in a comparable number of recovered MEC. Previously, it was postulated that milk yield depended primarily on the size of the mammary gland (Linzell, 1966; Sorensen et al., 1998). However, it has been demonstrated more recently that milk yield is regulated by the quantity of mammary secretory cells and their secretory activity (Capuco et al., 2001). According to this, in our study the ratios of pBMEC of total milk cells and milk yield were found to be correlated during the experimental timeframe ($R = 0.79$), whereas correlation of milk yield and total somatic milk cells was lower ($R = 0.62$). Earlier studies revealed that the number of pBMEC found in milk is correlated with milk yield. Annen et al. (2007) supported the hypothesis that increased milk yield during early lactation is associated with an increased accumulation of new pBMEC during late gestation and increased pBMEC shedding during early lactation.

Milk protein gene expression

The relative expression of the six major milk protein genes (CSN1S1, CSN1S2, CSN2, CSN3, LALBA, and PAEP) showed similar patterns during the first 20 weeks of lactation. Maxima of mRNA abundances were reached during the first two weeks of lactation followed by respective declines towards the end of the experimental period. Due to applying the immunomagnetic isolation method of pBMEC from fresh milk, it was possible for the first time to determine milk protein gene expression profiles in the very same animal over the course of lactation. Nonetheless, major milk protein gene expression patterns in mammary tissue of other species like common brushtail possum or mouse during pregnancy, lactation, and dry period did exist (Demmer et al., 1998; Stein et al., 2004; Anderson et al., 2007). Colitti and Farinacci (2009) examined gene expression of LALBA in mammary tissue in dairy ewes, collected after slaughtering, during peak (day 30 pp), mid (day 60 pp), and end of lactation (day 150 pp). In contrast to our findings in lactating cows, the relative expression level of LALBA in ewes reached the highest value only at the end of lactation. Furthermore, Colitti and Pulina (2010) analyzed transcripts of the four caseins CSN1S1, CSN1S2, CSN2, and CSN3 in mammary tissue after slaughter in dairy ewes. Respective to the study of Colitti and Pulina (2010), gene expression of the four caseins was up-regulated during peak, mid-, and late lactation but down-regulated during pregnancy and involution. Those findings correspond to ovine milk protein composition during lactation. Concentrations of caseins, total albumins, and β-lactoglobulin in whole milk increased significantly over the course of lactation (Poulton and Ashton, 1970). No milk protein fractions were analyzed in the present study, but previous studies in dairy cows showed different composition during lactation by contrast with ewes. Early reports stated peak concentrations of total caseins and serum proteins approximately five days after calving followed by a decline during the remaining 310-day lactation period, except for a slight increase during the time of peak yield (Larson and Kendall, 1957). In contrast, Ng-Kwai-Hang et al. (1987) determined a decline in concentrations of major milk proteins only between days 30 and 90 pp during peak milk yield followed by a marked increase until day 365 pp concomitant to lowering volumes of milk. Nevertheless, the highest total protein production was found, as described previously, during the first months of lactation (Friggens et al., 2007). The ratio of total caseins to whey proteins does not vary depending on the stage of lactation, reflecting no changes in the rates of synthesis for both main fractions (Coulon et al., 1998). Yet, relations between specific caseins differed depending on the stage of lactation (Kroeker et al., 1985; Çardak, 2009). During the first two months a marked decrease of α-casein and a reciprocal systematical increase of β-casein as a proportion of the casein fraction were demonstrated. The relative amount of κ-casein remained constant during the whole lactation cycle. Concentrations of β-lactoglobulin were on their minimum level during the second month of lactation, whereas proportions of α-lactalbumin, which is involved in milk lactose synthesis, decreased with progress in lactation as a result of lowering milk yields. In the present study, transcripts of all six milk protein genes were found to peak during the first two weeks of lactation and to decline continuously towards mid lactation. We hypothesized that milk protein gene expression has a pivotal effect on milk protein composition whereas milk protein concentration was not influenced. This assumption is confirmed by Bionaz and Loor (2007).

In this context, the translational efficiency of milk protein transcripts also has to be taken into account. Bevilacqua et al. (2006) measured equal proportions of casein gene transcripts which is roughly comparable to our findings. However, the four casein mRNAs were not translated with the same efficiency. They showed that CSN1S1 and CSN2 were translated 3 to 4-fold more efficiently in comparison with CSN1S2 and CSN3 and explained their findings with differences in the mRNA leader region. Due to those differences in translational efficiency, the differences in quantities of milk proteins could be explained. Milk proteins α_{S1}- and β-casein account for the major part of milk proteins (15 and 11 g/l, respectively), whereas α_{S2}- and κ-casein represent only a minor part (both 4 g/l) in skim milk (Farrell et al., 2004). In addition, whey proteins only amount to 5.5 g/l (1.5 g/l for α-lactalbumin and 4 g/l for β-lactoglobulin). However, no data are available on translational efficiency throughout lactation which could be influenced by different factors such as genetics, epigenetics, nutrition, milking frequency, hormonal status, or diseases.

Furthermore, milk protein synthesis may be regulated at multiple levels within the mammary epithelial cells including transcription, post-transcription,

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translation, and amino acid supply (Menzies et al., 2009). The genes encoding these proteins are regulated by the complex interplay of peptide and steroid hormones, predominantly the lactogenic hormones prolactin, insulin, and hydrocortisone, and cell-cell and cell-substratum interactions. Moreover, the uptake of amino acids from feed and their metabolic conversion are important preludes to milk protein synthesis. Therefore, Shennan and Peaker (2000) reported that the transport rate of amino acids seems to be the limited factor for milk protein synthesis. In this context, many signalling pathways in the lactating pBMEC are known, i.e. the janus kinase/signal transducer and activator of transcription cascade (Darnell, 1997), the growth hormone effects in the mammary mammalian target of rapamycin signalling pathway (Cui et al., 2003), the interaction of insulin and the major milk protein transcription factor E74-like factor 5 (Menzies et al., 2009), and the amino acids and glucose transporters (Zhao et al., 1996, 2005). Future research in that field could provide valuable information on improved lactation performance of dairy cows.

CONCLUSION

The indirect immunomagnetic bead-based method was appropriate to isolate pBMEC directly from fresh milk for further quantitative PCR analysis. The percentage of shed pBMEC in relation to somatic milk cells was highly correlated to milk yield. Expression patterns of the six major milk protein genes in twenty-four Holstein-Friesian cows were comparable during the first 20 weeks of lactation and respective proportions were comparable to previous findings on casein and whey protein concentrations in milk. Milk proteins are of great importance to the dairy industry. Therefore, further studies are likely to include investigations on regulation of milk protein gene expression and translation efficiency during the course of lactation.

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ORIGINAL ARTICLE

Gene expression analysis of protein synthesis pathways in bovine mammary epithelial cells purified from milk during lactation and short-term restricted feeding

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Summary

The objective of the study was to investigate selected key regulatory pathways of milk protein biosynthesis in primary bovine mammary epithelial cells (MECs) of dairy cows during the first 155 days of lactation. In addition, cows were exposed to feed restriction for a short period (FR) during different stages of lactation (week 4 and 21 pp) to study adjustment processes of molecular protein biosynthesis to metabolic challenge. Morning milk samples from twenty-four Holstein–Friesian cows were collected throughout the experimental period (n = 10 per animal). MEC from raw milk were purified using an immunomagnetic separation technique and used for real-time quantitative PCR analyses. As was seen in transcript abundances of all major milk proteins, mRNA levels of *E74-like factor 5* (*ELF5*), an enhancer of signal transducer and activator of transcription (STAT) action, concomitantly decreased towards mid-lactation. Expression of *ELF5* as well as of all milk protein genes showed a similar increase during FR in early lactation. Occasional changes in expression could be seen in other Janus kinase (JAK)/STAT factors and in mammalian target of rapamycin (mTOR) pathway elements. Amino acid transfer and glucose transporter and the β-casein expression were also partially affected. In conclusion, our findings suggest a pivotal role of the transcription factor *ELF5* in milk protein mRNA expression with complementary JAK/STAT and mTOR signalling for the regulation of protein biosynthesis in the bovine mammary gland.

Keywords cell signalling, gene expression, JAK/STAT, mTOR, primary mammary epithelial cells, transcription factor

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Introduction

During the last decades, protein has become a highly valued constituent of cows' milk and milk protein yield has gained a strong focus in modern breeding programmes (Lipkin et al., 2008). For a genetic, nutritional and managerial improvement in milk composition, both the typing of different protein variants and a detailed knowledge of the molecular mechanisms of milk protein biosynthesis pathways are of importance (Groenen and Vanderpoel, 1994). Milk proteins are synthesized and excreted by mammary epithelial cells (MECs) during lactation. Milk is a non-invasive source of viable MEC (Boutinaud and Jammes, 2002), and cells purified from fresh milk can be used to analyse mammary mRNA levels of milk protein (Boutinaud et al., 2004; Sigl et al., 2012b). In MEC, transcripts of all six major milk protein genes have been found to peak during the first 2 weeks of lactation and to decline continuously towards mid-lactation (Sigl et al., 2012b). This protein synthesis within the MEC may be regulated at multiple levels including transcription, post-transcription, translation and amino acid (AA) supply (Menzies et al., 2010; Bionaz and Loor, 2011). Protein production is controlled by the complex interplay of peptide and steroid hormones, predominantly the lactogenic hormones – prolactin, insulin and hydrocortisone – and cell–cell and cell–substratum interactions. It has been shown that insulin plays a major role in a coordinated induction of milk protein synthesis via induction of the central transcription factors E74-like factor (*ELF5*) and signal transducer and activator of transcription 5A (*STAT5A*; Menzies et al., 2010; Bionaz and Loor, 2011). In lactating cows, in addition to endocrine signals, the mammary availability of AA in particular as well as the cell energy status is the key regulating factors of protein synthesis mediated by the mammalian target

of rapamycin (mTOR) signalling (Proud, 2007; Yang et al., 2008; Burgos et al., 2010). Their supply is strongly influenced by the cows' diet. Feed restriction (FR) during early lactation (60% of *ad libitum* intake for 3 days) has been shown to alter the hepatic expression of genes encoding for enzymes involved in catabolism of AA, β-oxidation, gluconeogenesis and ketogenesis (Sigl et al., 2012a). In agreement with those findings, nutrition-induced ketosis (50% of *ad libitum* intake until occurrence of clinical signs of ketosis) has been shown to lead to massive changes in gene expression of the key regulatory hepatic enzyme important for energy distribution (Loor et al., 2007). An energy deficit for 3 weeks during mid-lactation (around 100 DIM; 70% of calculated requirements) has been found to increase the mRNA abundance of genes of the somatotropic axis and the insulin system (Gross et al., 2011). Other studies have reported that in purified MEC, expression levels of type 1 glucose transporter (*SLC2A1*) are roughly halved during FR (70% of requirements), whereas other genes involved in the milk biosynthesis are not influenced (Boutinaud et al., 2008). The authors of this study suggested a pivotal role of glucose transporter transcription in the regulation of glucose uptake and main milk constituents. In supplementation, genes involved in lipid metabolism and molecular transport were up-regulated, whereas several genes associated with cell growths, proliferation and deaths were down-regulated in mammary biopsies of cows with a negative energy balance (NEB; Moyes et al., 2011).

The objective of this investigation was to determine the key regulating factors responsible for milk protein biosynthesis in bovine MEC. To evaluate the role of genes in the network, central gene expression pathways for milk protein biosynthesis were selected and studied in MEC from dairy cows in the first 155 day of lactation and under the influence of short-term FR during weeks 4 and 21 of lactation.

Materials and methods

Cows, housing and feeding

Twenty-four multiparous Holstein–Friesian dairy cows were housed and fed in free-stall barns fitted with rubber mats. Lactation and feeding of restriction diet were carried out as described in Sigl et al.'s study (2012a). In short, cows were offered a lactation diet (LD; based on 22 kg milk) *ad libitum* starting 2 weeks before expected calving. The LD contained 60% corn silage, 23% grass silage, 4% hay, 12% concentrates and 1% mineral mix. Additional concentrates (7 MJ NE_L/kg; Raiffeisen Kraftfutterwerke Sued, Wuerzburg, Germany) were fed in automated feeding stations according to day of lactation (2–6 kg). Water was available at all times. After parturition, the cows were milked twice daily (0420 and 1540 h).

From day 23 until 31 pp and day 138 until 147 pp, the cows were moved to a tie stall with separated feed troughs and free access to water. During the first 3 days (day 23–25 and 138–140 pp), the cows were fed *ad libitum* with LD and additional concentrates (6 and 5 kg during early and mid-lactation respectively; Raiffeisen Kraftfutterwerke Sued, Wuerzburg, Germany). From day 26 to 28 and 141 to 143 pp, the cows received a restrictive diet (56.4% corn silage, 21.6% grass silage, 3.8% hay, 11.3% concentrates, 0.9% mineral mix and 6.0% straw) and no additional concentrates (RD, 70% of *ad libitum* energy intake; Sigl et al., 2012b). Fresh feed was mixed daily, and the cows were fed half of their daily allotment of RD at 0700 and 1700 h. The following 3 days (day 29–31 and 144–147 pp), animals were refed again with *ad libitum* LD and defined amounts of additional concentrates.

Experimental design

Milk samples (n = 10 per animal) were collected for cell isolation during the first 155 days of lactation. Whole-morning milk samples were obtained from a sterile bucket at day 8, 15, 26, 29, 43, 57, 113, 141, 144 and 155 pp. Milk yield was determined with a spring scale, and one aliquot (40 ml) was stored at 4 °C for a maximum of 7 days with a preservative (acidiol) for analysis of milk composition. Milk sample volumes of 3.6 l were filled into autoclaved glass bottles and used for immediate immunomagnetic cell isolation.

Milk composition analysis

Milk composition (protein, fat and lactose) was analysed by infrared spectrophotometric technique (infrared absorption measurement evaluated by Fourier transform, MilkoScan FT6000, Foss, Hillerød, Denmark), and somatic cell count was determined by a fluorescence-optical counting system (Fossomatic FC, Foss) in the laboratories of Milchpruefring Bayern e.V. (Wolnzach, Germany).

Cell isolation

Isolation of milk MEC was performed using an immunomagnetic separation technique as described by Sigl et al. (2012b). In short, two aliquots of milk samples (1.8 l each) were centrifuged, the supernatant was

removed, and the cell pellet was resuspended in phosphate-buffered saline (PBS). After a second centrifugation step, the cell pellet was re-suspended in PBS containing 1% bovine serum albumin (BSA) and incubated on a rotary mixer with a primary mouse monoclonal antibody against cytokeratin-8 antibody (clone C-43, Exbio antibodies). Unbound antibodies in the supernatant were removed from the mixture by centrifugation. The cell–antibody complex was re-suspended in 1 ml of 1% BSA–PBS. Dynabeads (25 μl; PanMouse IgG, Dynal Biotech, Invitrogen) were added, and the suspension was incubated on a rotary mixer. Specifically bound cells were collected by placing the sample vials in the Dynal MPCTM-L (2 min) after aspiration of the supernatant. A second washing was performed with 1 ml of 1% BSA–PBS. A 7-μl aliquot was collected for cell count determination under the microscope. Purified MECs were re-suspended in 700 μl Qiazol (Qiagen GmbH, Hilden, Germany) and stored at −80 °C until RNA extraction.

RNA extraction and reverse transcription

The total RNA was extracted from the purified MEC, applying the miRNeasy MiniKit (Qiagen GmbH) as described by Sigl et al. (2012b). In brief, after thawing and homogenizing of samples, chloroform was added to each sample. Subsequently, the mixture was vortexed vigorously, shortly incubated and centrifuged. The aqueous supernatant containing total RNA was recovered and mixed with 1.5 volumes of 100% ethanol. After vortexing, up to 700 μl of mixture was pipetted into an RNeasy Mini spin column and repeatedly centrifuged. To wash the column, buffer RWT (700 μl) was added, and samples were centrifuged. Washing was performed twice by adding buffer RPE (500 μl) followed each by a short centrifugation procedure. The RNeasy mini spin column membrane was dried by a 2-min centrifugation; the extracted RNA was dissolved in sterile RNase-free water (30 μl) and quantified by spectrophotometry (BioPhotometer; Eppendorf, Hamburg, Germany). The integrity of the RNA (RNA integrity number; RIN) was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) connected to the RNA 6000 Nano Assay. Accurate amounts of 250 ng RNA were reverse-transcribed to complementary DNA adding the following reverse transcription master mix: 5× Buffer (12 μl; Promega, Mannheim, Germany), Random Hexamer Primers (50 mM; 3 μl; Invitrogen, Carlsbad, USA), dNTP Mix (10 mM; 3 μl; Fermentas, St Leon-Rot, Germany) and MMLV-H$^-$ reverse transcriptase (200 U; Promega, Regensburg, Germany). The reverse transcription reaction was carried out according to the manufacturer's instruction with a 60-μl reaction volume in a PCR thermocycler (Biometra, Goettingen, Germany) by successive incubations at 21 °C for 10 min and at 48 °C for 50 min, finishing with enzyme inactivation at 90 °C for 2 min. The reverse transcription products were stored at −20 °C.

Primer design

Gene sequences for primer designs were obtained from the gene bank of the National Center for Biotechnology Information (NCBI). Exon-spanning primers were designed with the help of the NCBI primer tool and synthesized at Eurofins MWG (Ebersberg, Germany). All newly designed primers were tested for their optimal amplification temperature, and PCR products were sequenced at LGC Genomics (Berlin, Germany). The primer sequences and abbreviations of measured genes are listed in Table 1.

Real-time reverse transcription quantitative PCR

Quantitative PCR was performed using MESA Green qPCR MasterMix plus for SYBR Assay w/fluorescein (Eurogentec, Cologne, Germany) with a standard protocol recommended by the manufacturer's instructions. All components necessary for real-time RT-qPCR were mixed in the reaction wells of semi-skirted twin.tec PCR plate 96 (Eppendorf). The mastermix was prepared as follows: MESA Green 2× qPCR MasterMix (7.5 μl), forward primer (1 μM), reverse primer (1 μM) and RNase-free water (3.0 μl). Mastermix (13.5 μl) and cDNA (1.5 μl) were added to each well. The plate was sealed and placed in the iQ5 Cycler (Bio-Rad, Munich, Germany), and the following PCR protocol was started: denaturation step (95 °C, 5 min), cycling programme (95 °C, 3 s; primer-specific annealing, 60 s) and one cycle to obtain a melting curve to ensure integrity of amplification (60 °C–95 °C with a heating rate of 0.1 °C/s). Quantitative PCR efficiency varied from E = 85% to E = 100%.

Statistical analysis

Genes were selected as reference genes using GenEx Pro Software version 5.2.7.44 (MultiD Analyses, Gothenburg, Sweden). The mean of the resulting three reference genes *GAPD*, *H3F3A* and ribosomal protein *S9 (RPS9)* was calculated and used for normalization (SD = 0.6). Cq values were calculated by Bio-Rad iQ5 Optical System Software version 2.1 with the analysis mode 'PCR base line subtracted

Table 1 Primer sequences, accession numbers and product lengths for genes measured in mammary epithelial cells

Gene*	Sequence (5′ → 3′)	Product size (bp)	GenBank accession no.
Major milk protein genes			
CSN1S1 for	ATGAAACTTCTCATCCTTACCTGTCTT	179	NM_181029.2
CSN1S1 rev	CCAATATCCTTGCTCAGTTCATT		
CSN1S2 for	AGCTCTCCACCAGTGAGGAA	150	NM_174528.2
CSN1S2 rev	GCAAGGCGAATTTCTGGTAA		
CSN2 for	GTGAGGAACAGCAGCAAACA	233	NM_181008.2
CSN2 rev	AGGGAAGGGCATTTCTTTGT		
CSN3 for	TGCAATGATGAAGAGTTTTTTCCTAG	150	NM_174294.1
CSN3 rev	GATTGGGATATATTTGGCTATTTTGT		
LALBA for	CTCTCTGCTCCTGGTAGGCAT	247	NM_174378.2
LALBA rev	GTGAGGGTTCTGGTCGTCTT		
PAEP for	AGAAGGTGGCGGGGACTTGG	375	NM_173929.3
PAEP rev	TGTCGAATTTCTCCAGGGCCT		
Marker of epithelial cells			
KRT8 for	GCTACATTAACAACCTCCGTC	237	NM_001033610.1
KRT8 rev	TCTCATCAGTCAGCCCTTCC		
Receptors			
IGF-1R for	CCCAAAACCGAAGCTGAGAAG	200	XM_606794.3
IGF-1R rev	TCCGGGTCTGTGATGTTGTAG		
INSR for	CCAACTGCTCAGTCATCGAA	164	XM_590552.5
INSR rev	GTTGGGGAACAAGTCCTTCA		
PRLR for	CATGGTGACCTGCATCCTC	172	NM_001039726.1
PRLR rev	ACCCTCATGCCTCTCACATC		
Transcription factors			
CEBPB for	GCACAGCGACGAGTACAAGA	152	NM_176788.1
CEBPB rev	GTTGCTCCACCTTCTTCTGG		
ELF5 for	ATACTGGACGAAGCGCCACGTC	134	NM_001024569.1
ELF5 rev	ACTCCTCCTGTGTCATGCCGCA		
JAK2 for	TCTGGTATCCACCCAACCATGTCT	201	XM_865133.2
JAK2 rev	AATCATGCCGCCACTGAGCAA		
RUNX2 for	ACCATGGTGGAGATCATCG	207	XM_002684501.1
RUNX2 rev	CCGGAGCTCAGCAGAATAA		
STAT5A for	GTGAAGCCACAGATCAAGCA	176	NM_001012673.1
STAT5A rev	TCGAATTCTCCATCCTGGTC		
YY1 for	GCTTGCCCTCATAAAGGCTGCACA	192	NM_001098081.1
YY1 rev	GCAGCCTTCGAACGTGCACTGA		
Glucose transporter			
SLC2A1 for	GTGCTCCTGGTTCTGTTCTTCA	84	NM_174602.2
SLC2A1 rev	GCCAGAAGCAATCTCATCGAA		
Translation factors			
AKT1 for	GATCACCGACTTCGGACTGT	202	NM_173986.2
AKT1 rev	CTTCTCGTGGTCCTGGTTGT		
EIF4EBP1 for	GAA CTC ACC TGT GAC CAA GA	157	NM_001077893.1
EIF4EBP1 rev	CTCAAACTGTGACTCTTCACC		
OAT for	ATACAGGAGTGGAGGCTGGA	150	NM_001034240.1
OAT rev	CAGTGGAGCTGGAGATAGCA		
RPS6KB1 for	GGCAGCCCACGAACACCTGT	96	NM_205816.1
RPS6KB1 rev	AGGCGTCTGCGGATTTGCCG		
References genes			
GAPD for	GTCTTCACTACCATGGAGAAGG	197	NM_001034034.1
GAPD rev	TCATGGATGACCTTGGCCAG		
H3F3A for	ACTCGCTACAAAAGCCGCTCG	232	NM_001014389.2
H3F3A rev	ACTTGCCTCCTGCAAAGCAC		
RPS9 for	CCTCGACCAAGAGCTGAAG	64	NM_001101152.1
RPS9 rev	CCTCCAGACCTCACGTTTGTTC		

Table 1 (Continued)

Gene*	Sequence (5′ → 3′)	Product size (bp)	GenBank accession no.
UBB for	GTC TTC ACT ACC ATG GAG AAG G	197	NM_174133.2
UBB rev	TCA TGG ATG ACC TTG GCC AG		

*CSN1S1, α_{S1}-casein; CSN1S2, α_{S2}-casein; CSN2, β-casein; CSN3, κ-casein; LALBA, α-lactalbumin; PAEP, progestagen-associated endometrial protein, known as β-lactoglobulin; KRT8, cytokeratin-8; IGF-1R, insulin-like growth factor 1 receptor; INSR, insulin receptor; PRLR, prolactin receptor; CEBPB, CCAAT/enhancer binding protein β; ELF5, E74-like factor 5; JAK2, Janus kinase 2; RUNX2, runt-related transcription factor 2; STAT5A, signal transducer and activator of transcription 5A; YY1, yin-yang-1; SLC2A1, solute carrier family 2, member 1; AKT1, v-akt murine thymoma viral oncogene homolog 1; EIF4EBP1, eukaryotic translation initiation factor 4E binding protein 1; OAT, ornithine δ-aminotransferase; RPS6KB1, ribosomal protein S6 kinase; GAPD, glyceraldehydes-3-phosphate dehydrogenase; H3F3A, H3 histone family 3A; RPS9, ribosomal protein 9; UBB, polyubiquitin.

curve fit'. The ΔCq values were calculated individually for each target gene as $\Delta Cq = Cq_{target\ gene} - meanCq_{reference\ genes}$ (Pfaffl, 2001). To avoid negative digits while allowing an estimation of a relative comparison between two time points, log2-transformed data are presented as LSM ± SEM subtracted from the arbitrary value 10 (10−ΔCq).

SAS (version 9.2 for Unix and Linux; SAS Institute, Cary, NC, USA) was applied for statistical analyses. All data were compared using the MIXED procedure with an autoregressive covariance structure, which (proved to) fit best according to the Akaike's information criterion (Littell et al., 1998). 'DIM' was regarded as a class variable and 'cow' as a repeated effect. If an overall significant effect of DIM was found, a subsequent Bonferroni's *post hoc* analysis was performed. A p-value of <0.05 was considered significant. Data are presented as LS-means ± standard error.

Results

Milk yield and composition

Morning milk and protein yields as well as milk composition during early and mid-lactation have been previously described in detail for the first 20 weeks of lactation (Sigl et al., 2012b). In short, in this study, all major milk traits comprising yield and composition were affected by DIM. The typical increase in milk yield was seen during early lactation up to 21.7 kg on day 57 pp with a subsequent decline up to the last day of sampling. Milk protein concentration also changed over the course of lactation, showing a marked nadir of 2.99 ± 0.06% on day 43 pp.

During early- and mid-lactation increase in NEB (day 26 pp vs. day 29 pp and day 141 pp vs. day 144 pp respectively; Table 2), morning milk yield decreased by roughly 2 kg (both $p < 0.05$; Fig. 1 left). Within 2 weeks after the first FR, milk yield increased significantly, whereas it remained on the same level after the second FR. Milk protein only decreased numerically during the first FR by 0.07% and during the second FR by 0.12% (both $p > 0.05$; Fig. 1 right). Protein yield decreased during early and mid-lactation FR by 76 and 96 g respectively (both $p < 0.05$; Fig. 1 left). Milk fat concentration varied strongly throughout the experimental period and was higher during the first FR as compared to the second FR ($p < 0.05$; Fig. 1 right). The somatic cell count varied highly during lactation between 50.6 ± 55.2 × 10^3/ml on day 57 pp and 294.0 ± 61.3 × 10^3/ml on day 155 pp, resulting in no statistical differences between sampling time points.

Table 2 Dry matter intake (DMI) and energy balance (EB) 3 days before, during and 3 days after feed restriction in early and mid-lactation (mean ± SD).

DIM	DMI, kg	EB, MJ NE_L
23–25	16.4 ± 2.4	−66 ± 20
26–28	10.5 ± 1.4	−102 ± 18
29–31	16.3 ± 2.5	−60 ± 24
138–140	19.8 ± 2.9	−7 ± 23
141–143	12.2 ± 2.5	−54 ± 16
144–147	19.7 ± 2.6	−3 ± 19

Epithelial cell marker expression

A statistical comparison of individual time points did not reveal differences in expression levels of the marker gene *KRT8*, although an overall influence of the sampling time point was evaluated ($p = 0.01$). The highest levels were seen on day 155 pp (7.25 ± 0.30). The lowest values were found after the second week of lactation (6.07 ± 0.27).

Milk protein genes

Transcript abundances of all six major milk protein genes were highly affected by the sampling time point ($p < 0.0001$) and decreased with increasing day of lactation (Fig. 2). During FR in early lactation, mRNA levels did not increase or only in tendency increased

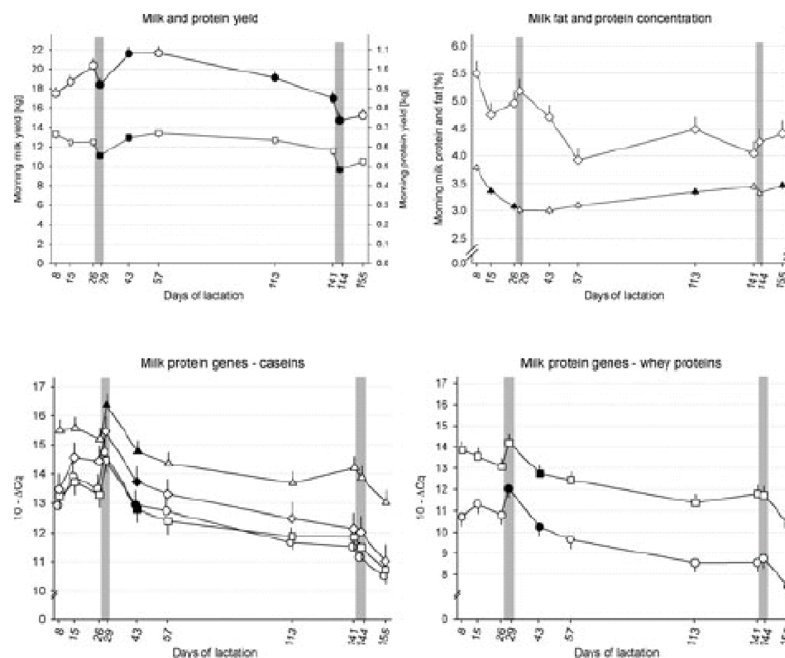

Fig. 1 Left: Morning milk yield (○) and protein yield (□); right: morning milk protein concentration (△) and milk fat concentration (◇) (right) during the first 155 days of lactation and after 3 days of restricted feeding during early (day 29 pp) and mid-lactation (day 144 pp). Grey bars represent sampling time point during FR. Values that differ significantly from the prior value are filled.

Fig. 2 Transcript abundance of casein genes (left: *CSN1S1* (○), *CSN1S2* (□), *CSN2* (◇) and *CSN3*(△)) and whey protein genes (right: LALBA (○) and PAEP (□)) during the first 155 days of lactation and after 3 days of restricted feeding during early (day 29 pp) and mid-lactation (day 144 pp). Grey bars represent sampling time points during FR. Values that differ significantly from the prior value are filled.

for *CSN1S1* (13.48 ± 0.49 vs. 14.74 ± 0.49; p = 0.11), *CSN1S2* (13.28 ± 0.44 vs. 14.47 ± 0.43; p = 0.08), *CSN2* (14.44 ± 0.51 vs. 15.45 ± 0.51; p = 0.9) and *PAEP* (13.08 ± 0.39 vs. 14.19 ± 0.39; p = 0.11; Fig. 2). Expression of *CSN3* (15.19 ± 0.37 vs. 16.38 ± 0.37; p = 0.007) and *LALBA* (10.79 ± 0.42 vs. 12.02 ± 0.42; p = 0.02) increased during early FR. An FR during mid-lactation did not influence the transcription of any major milk protein.

JAK/STAT pathway

Transcript levels of *JAK2* were affected by the sampling time (p = 0.04) and highest levels were seen during times of presumed positive energy balance (Fig. 3). *STAT5A* mRNA abundance was not influenced by DIM (p = 0.26; Fig. 3). Gene expression levels of *PRLR* were lowest of all measured genes, and variation between all time points was seen (p = 0.03; Fig. 3). During times of strong presumed and measured NEB, values were highest in tendency. Levels of *ELF5* mRNA were strongly affected by the sampling time (p < 0.0001). Abundances were highest during FR at day 29 pp (8.61 ± 0.33) and decreased until day 155 pp (5.98 ± 0.37; Fig. 3).

mTOR pathway

mRNA levels of *EIF4EBP1* were markedly affected by time and feed supply (p = 0.0008), showing the highest value at day 8 pp (5.37 ± 0.19) and a further peak during the first FR (5.19 ± 0.18; Fig. 4). Transcript abundances of *RPS6KB1* and *AKT1* remained stable throughout lactation and during FR (p = 0.07 and p = *0.12*; Fig. 4). Expression of *INSR* was strongly affected by the sampling time point (p = 0.001), peaked at day 26 pp (6.16 ± 0.18) and decreased as lactation progressed. Levels of transcript for *INSR* were lowest during FR in mid-lactation (4.96 ± 0.19; Fig. 4). Transcript abundances for *IGF-1R* were significantly affected by DIM (p = 0.02) with a peak at day 141 pp (6.13 ± 0.18; Fig. 4).

Protein expression exemplified for beta-casein transcription

mRNA levels of *CEBPB* were affected by time (p = 0.002), with an increase from day 8 to day 43 pp (5.83 ± 0.30–7.32 ± 0.29) and a decrease during FR in mid-lactation (6.88 ± 0.31; Fig. 5). *RUNX2* and *YY1* were constantly expressed during the first 155 days of lactation (p = *0.47* and p = *0.56*; Fig. 5).

AA transfer and glucose transporters

Transcript abundance of *SLC2A1* was affected by the feed supply (p = 0.03) as indicated by decreased levels during FR (Fig. 6). Levels of *OAT* mRNA remained at a constant level throughout the experimental period (p = *0.18*; Fig. 6).

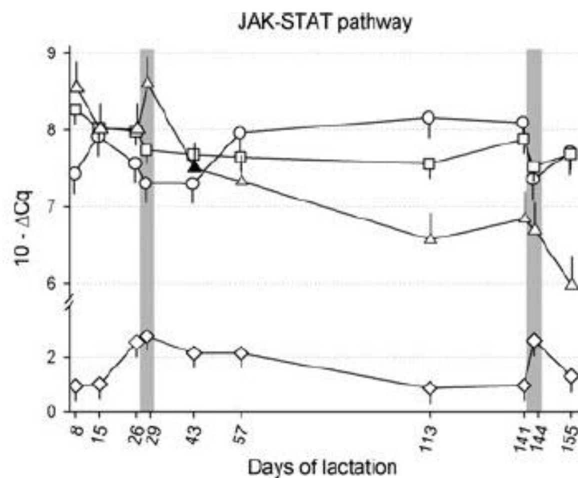

Fig. 3 Transcript abundance of *JAK2* (○), *STAT5A* (□), *PRLR* (◇) and *ELF5* (△) during the first 155 days of lactation and after 3 days of restricted feeding during early (day 29 pp) and mid-lactation (day 144 pp). Grey bars represent sampling time point during FR. Values that differ significantly from the prior value are filled.

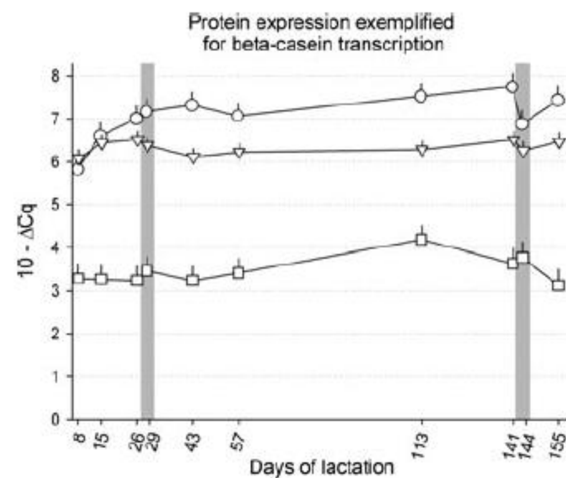

Fig. 5 Transcript abundance of *CEBPB* (○), *RUNX2* (□) and *YY1* (▽) during the first 155 days of lactation and after 3 days of restricted feeding during early (day 29 pp) and mid-lactation (day 144 pp). Grey bars represent sampling time point during FR. No value differs from the respective prior value.

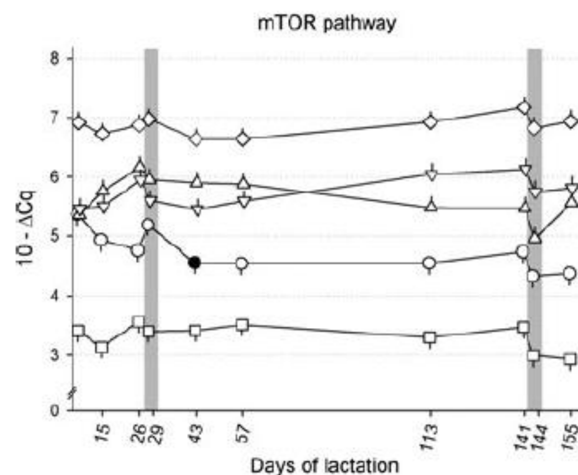

Fig. 4 Transcript abundance of *EIF4EBP1* (○), *RPS6KB1* (□), *AKT1* (◇), *INSR* (△) and *IGF-1R* (▽) during the first 155 days of lactation and after 3 days of restricted feeding during early (day 29 pp) and mid-lactation (day 144 pp). Grey bars represent sampling time point during FR. Values that differ significantly from the prior value are filled.

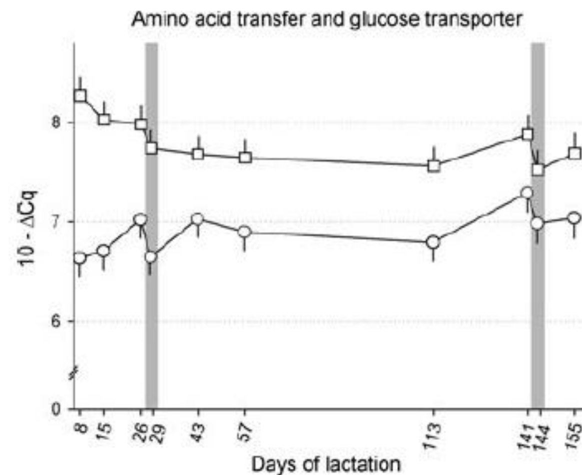

Fig. 6 Transcript abundance of *SLC2A1* (○) and *OAT* (□) during the first 155 days of lactation and after 3 days of restricted feeding during early (day 29 pp) and mid-lactation (day 144 pp). Grey bars represent sampling time point after FR. No value differs from the respective prior value.

Discussion

During milking, epithelial cells detach from the alveolar epithelium and discard the mammary gland within the milk. Despite the constant discharge of MEC, it has been established that the fraction of MEC of total milk cells represents 2% to 6.7% of total milk cells and approximately 2.1×10^6 cells can be purified from 1.8 l of milk using an indirect cell purification method (Sigl et al., 2012b). Alveolar cell purification from fresh milk using an immunomagnetic separation technique has been proved to circumvent the disadvantages of intricate mammary gland tissue biopsies or one-time collection after slaughtering for further studies on mRNA expression (Gomm et al., 1995; Boutinaud et al., 2008; Sigl et al., 2012b). However,

Krappmann et al. (2012) reported different expression levels of *CSN1*, *CSN3* and *GLUT1* in isolated MEC from fresh milk and in all cells extracted from tissue samples including MEC, fibroblasts, myoepithelial cells, immune cells, etc. after slaughtering. Yet, it has been demonstrated that *CSN1* and *CSN3* are expressed not only in MEC, but to a low extent also in blood mononuclear cells of goats or cows (Tokarska et al., 2001, 2009). *GLUT1* is believed to have an ubiquitous distribution in most cell types (Zhao and Keating, 2007), i. e., in cows' monocytes (O'Boyle et al., 2012). In MEC cytokeratin-8 derived from fresh milk and cultured for one passage and in MEC cytokeratin-8 extracted from mammary tissues, similar gene expression levels were measured (Sorg et al., 2012).

In this context, mammary keratins have been reported to be useful markers for epithelial cells (Boutinaud and Jammes, 2002; Sorg et al., 2012). Here, they were found in all extracted mRNA samples in roughly constant levels with the lowest amounts seen during early lactation and the highest values during mid-lactation.

Enhancing mammary milk protein synthesis is a highly desirable goal of dairy farmers and industry because of the associated positive health attributes as well as favourable dairy processing properties of that trait. In this study, gene expression profiles of all major proteins as well as of several important genes of central protein biosynthesis pathways were measured during the first half of lactation and two short-term FRs. During the first 155 day of lactation, dairy cows typically shift from a negative energy balance in early lactation to a positive energy balance within the first 40–80 DIM (de Vries and Veerkamp, 2000; Coffey et al., 2002). An NEB is associated with a decline in mammary milk protein biosynthesis, inter alia due to the lack of sufficient microbial substrate production in the rumen. Experimental perturbation of cows' energy status during early and mid-lactation was expected to cause short-term adaptation of gene expression patterns in MEC.

Milk protein expression

Gene expression profiles of all major milk proteins during the first 20 weeks of lactation have recently been published (Sigl et al., 2012b). A further enhancement in NEB during the 4th week of lactation resulted in highest gene expression values during the experimental period in all milk proteins. Particularly, expression of *CSN3* and *LALBA* rose markedly. This finding is in contrast to a decrease reported in mammary biopsies in goats after a 48-h feed restriction starting around day 48 pp (Ollier et al., 2007). Boutinaud et al. (2008) found no influence of restricted feeding on both genes after 7 days of restricted feeding (70% of allowance). However, taking the DIM or presented milk yield and energy intake of both studies into consideration, these animals were presumably in positive energy balance before nutrient limitation. Moreover, these results support our findings that the transcript abundance of the major proteins is not influenced during the short-term alteration of energy balance in mid-lactation. Transcription rates of all milk protein genes are dependent on the activation and synthesis of transcription factors, which in turn are controlled by the interaction of galactopoietic hormones and the nutrient supply.

JAK/STAT pathway

The luteotropic hormone prolactin is involved in MEC proliferation as well as differentiation during pregnancy and is essential for the initial secretion of milk into the alveolar lumen (Riley et al., 2010). It binds to the extracellular portion of *PRLR* and initiates events in the JAK/STAT signal transduction cascade (Darnell, 1997). Values of *PRLR* mRNA were constantly on a low level during the experimental period. This is in agreement with previous results presented by Bionaz and Loor (2011), who also reported no change in *PRLR* gene expression during the first 240 day of lactation. In general, plasma prolactin causes a down-regulation of its mammary receptor (Grattan et al., 2001; Bionaz and Loor, 2011). In ruminants, prolactin secretion decreases drastically shortly after parturition. As prolactin also induces *STAT5A* binding activity in mammary glands, it might explain the unaffected abundances of the primary transcription factor *STAT5A* during the experimental time frame. *STAT5A* is responsible for the signal transduction of prolactin in the mammary gland. On the other hand, several hormones, growth factors and cytokines can also activate *STAT5A* expression, and all milk protein genes contain at least one *STAT5A* binding site essential for basic milk protein expression (Rosen et al., 1999; Bionaz and Loor, 2011). Yet, in agreement with Bionaz and Loor (2011), variation in milk protein concentration during lactation and FR seems not to be strongly influenced by *STAT5A* signalling. In contrast to the abundance of *STAT5A*-mRNA, *JAK2* expression, which is triggered by prolactin as well as growth hormone binding to their receptors, was found to be highest towards the end of lactation and at times of presumed positive energy balance (Hayashi et al., 2009). JAK2 triggers a cascade of signalling events

that involve the insulin receptor substrate (IRS), followed by the phosphoinositide 3-kinase (PI 3-kinase) and protein kinase B (also known as *AKT1*; Harrington et al., 2005). These steps are important for downstream activation of the mTOR pathway (Hayashi and Proud, 2007). Therefore, *JAK2* activation is suggested to mediate the well-established positive linkage between growth hormone and milk protein yield.

Insulin has been shown to play a pivotal role in the coordinated induction of milk protein biosynthesis even though it does not stimulate glucose uptake in the mammary gland. The requirement of insulin may primarily be facilitated by the major milk protein transcription factor *ELF5* (Menzies et al., 2010). *ELF5* plays an important role in mammary gland development by regulating terminal differentiation of lobuloalveolar cells as it is associated with the prolactin-mediated mammary differentiation process (Harris et al., 2006; Choi et al., 2009). *ELF5* is also involved in the determination of cell fate and in the regulation of the stem/progenitor function of the mammary epithelium (Chakrabarti et al., 2012). With increased day of lactation, mRNA levels of *ELF5* declined continuously in the present study, which might be a result of a concomitant decline in mammary differentiation processes. These results were associated with a similar mRNA expression pattern of all six major milk protein genes during the first 21 weeks of lactation. Bionaz and Loor (2011) also pointed out the pivotal role of *ELF5* in bovine mammary protein synthesis. They explained that the decrease in *CSN3* expression at day 240 pp is a consequence of the decreased transcript levels of *STAT5B* and *ELF5*. However, the role of *ELF5* in bovine mammary gland warrants further investigations.

mTOR pathway

Recent studies in ruminants have highlighted a crucial role of the mTOR pathway in the regulation of milk protein synthesis (Toerien et al., 2010; Bionaz and Loor, 2011). Inter alia, insulin- and AA-induced stimulation of protein synthesis is at least partially mediated by mTOR as it catalyses the phosphorylation of *RPS6KB1* and nullifies the inhibiting effects on initiation of mRNA transcription of *EIF4EBP1* (Kimball and Jefferson, 2006). Due to the restricted availability of nutrients during FR, we expected changes in mRNA levels of key factors of mTOR signalling. Hayashi et al. (2009) found that the increased phosphorylation of *RPS6KB1* was correlated with increased milk protein syntheses. In contrast, the mRNA abundance of *RPS6KB1* was not affected by energy balance in this study. Nonetheless, differences in phosphorylation status during lactation could still result in greater rates of translational activity as it has been shown that mutations of the phosphorylation site (Thr^{389}) inhibit the blocking effect of rapamycin (Jefferies et al., 1997). Phosphorylation was not measured in this study, but this could warrant further investigations. Transcript encoding for the key regulatory component *EIF4EBP1* was strongly influenced by the feed supply. As it inhibits the initiation of mRNA translation, higher amounts during periods of shortage in the feed energy supply could contribute to the concurrent lower milk protein concentration during those times.

IGF-1 is an essential hormone that links nutrition with growth and fertility because blood concentrations are influenced by the nutritional status of the cow (Spicer et al., 1990). IGF-1 is a potent mammary growth as well as survival factor enhancing alveolar cell proliferation and inhibiting progression of involution (Modha et al., 2004). The action of IGF-1 through an IRS cascade is mediated by the *IGF-1R* in the mammary gland (McGrath et al., 1991; LeRoith et al., 1995). *IGF-1R* mRNA levels were low during the first 2 weeks of lactation and during FR, which underlines the correlation of the cow's energy state and IGF-1 secretion (Spicer et al., 1990). In comparison, transcript abundances of INSR decreased after an early peak at day 26 pp towards the end of the experimental period. Blood concentrations of insulin during lactation showed a comparable pattern to those of IGF-1 with a nadir shortly after parturition and a gradual increase towards mid-lactation. Nevertheless, Bionaz and Loor (2011) reported an increase in the insulin signalling cascade despite low plasma insulin concentrations and suggested an increase in insulin/IGF-1 sensitivity. However, the sharp increase in *AKT1* mRNA levels after parturition could not be confirmed by our results. Yet, the response of *INSR* expression to decreased insulin concentrations was more pronounced during the second FR in mid-lactation.

Protein expression exemplified for beta-casein transcription

CEBPs regulate several important genes involved in proliferation and differentiation. They play important functional roles in mammary development and lactation and are expressed during pregnancy and involution (Rosen et al., 1999). In the mammary gland, *CEBPB* is essential for milk protein β-casein expression as *CSN2* contains four *CEBPB* binding sites (Doppler et al., 1995). The transcript abundances of *CEBPB* increased during the first 3 weeks after parturition

and remained at a high level during the following 20 weeks of lactation.

To establish further potential factors mediating beta-casein expression, *RUNX2* and *YY1* were measured in MEC. It is known that *RUNX2* has a functional role in the regulation of gene expression in mammary epithelial cells (Inman and Shore, 2003). Besides the potential activators *STAT5A* and glucocorticoid receptor, *RUNX2* is said to be an essential factor required for β-casein transcription via the formation of a complex with an octamer element (Inman et al., 2005). It could recruit GR or interact with *STAT5A*, thus providing a link between prolactin signalling and *RUNX2* activity. *RUNX2* expression was not influenced by the stage of lactation or the nutrient supply in our study. This may again reflect the fact that prolactin has very little effect on the maintenance of lactation in cows. Also, expression of *YY1* did not differ during the experimental time period. The nuclear factor *YY1* is a multifunctional protein that can either activate or repress transcription, but it predominantly acts as a repressor of β-casein gene expression (Rosen et al., 1999). Earlier studies have reported that *YY1* is constitutively expressed in MEC with no response to galactopoietic hormones (Meier and Groner, 1994; Raught et al., 1994). Although CSN2 expression did not differ during the experimental time period (either), the role of *RUNX2* and *YY1* in milk protein gene expression seems to be of no or little importance.

Amino acid transfer and glucose transporter

The uptake of AAs from feed and their metabolic conversion are important preludes for milk protein synthesis. Deficits in non-essential AA can be partly overcome by synthesis of other precursors. Parts of the proline metabolism were selected for analysis as examples. Proline is required in rather high amounts for casein transcription, but is not available in sufficient concentration during peak lactation. It has been shown that ornithine, a readily available AA for mammary cells, can serve as a source for proline. The conversion of ornithine to proline requires the action of *OAT* (Basch et al., 1995). The unaffected mRNA levels of OAT might be a result of constitutive proline uptake in MEC. Arteriovenous difference analyses of AA uptake by the mammary gland were not performed during this study, and further investigations into essential AA could foster progress in this area.

SLC2A1 is predominantly responsible for the facilitative transport of glucose into mammary cells through a passive mechanism and plays a key role in maintaining glucose homoeostasis during lactation (Zhao et al., 1996; Bell and Bauman, 1997). Yet, the facilitative and sodium-dependent glucose transport systems are mediated to some extent by further energy-independent (GLUT) as well as sodium-dependent (SLC5A) transport systems, which were not determined in this study (Zhao and Keating, 2007). Glucose is the predominant energy substrate for lactose production. Changes in expression of glucose uptake are partly achieved by changes in *SLC2A1* mRNA expression. Komatsu et al. (2005) detected strong differences in the expression of *SLC2A1* between dry and lactating cows as *SLC2A1* mRNA was barely detectable in dry cows. They reported no variation between peak and late lactation. Mattmiller et al. (2011) measured a significant increase in *SLC2A1* abundance up to day 60 of lactation followed by a decrease towards the end of lactation. The expression pattern identified here differed from the current study in so far as no significant increase was measured shortly after parturition. During the first FR, *SLC2A1* abundances were low in relation to the highest values found shortly before the second FR. These findings coincide with the glucose concentration during those time periods (Sigl et al., 2012a). Expression of *SLC2A1* in MEC was also influenced in the FR study of Boutinaud et al., (2008).

In conclusion, pathway visualization indicated that the expression of investigated genes was quite stable during lactation, but revealed a few changes after restricted feeding. Milk protein biosynthesis is regulated at many levels within the bovine MEC. Our results suggest a particularly strong impact of *ELF5* on this signalling machinery. The decrease in *ELF5* mRNA expression throughout lactation might explain the concomitant decline in transcript abundances of all major milk proteins. Furthermore, an increase in mRNA levels for *ELF5* after FR in early lactation and a simultaneous increase in mRNA levels of all milk protein genes confirm the pivotal role of this transcription factor. Considering all other investigated pathways, additional studies on the phosphorylation status and on protein levels could contribute to the understanding of milk protein biosynthesis in the mammary gland. Further research is also needed on the energy and/or AA supply and concomitant milk protein synthesis at mammary tissue level as well as differences between local molecular regulation of cows yielding high milk protein and low milk protein.

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