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Effects of continuous milking during the dry period or once daily milking in the first four weeks of lactation on metabolism and productivity of dairy cows

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1 ABBREVIATIONS

a.m. = morning

AA = amino acid

ACADvl = acyl-coenzyme A dehydrogenase, very long chain

ACTA1 = actin alpha 1

ADF = acid detergent fiber

AI = artificial insemination

ANOVA = analysis of variance

ATP = adenosine triphosphate

BCS = body condition score

BHBA = β-hydroxybutyric acid

Bp = base pairs

bST = bovine somatotropin

BW = body weight

C = control

cDNA = complementary deoxyribonucleic acid

CF = crude fiber

CM = continuous milking

CP = crude protein

CPT 1A = carnitine palmitoyltransferase 1A

Cq = quantification cycle

CS = citrate synthase

CTSLb = cathepsin Lb

CV = coefficient(s) of variation

DIM = days in milk

DM = dry matter

DMI = dry matter intake

DNA = deoxyribonucleic acid

DP = dry period

ECM = energy-corrected milk

EDTA = ethylenediaminotetraacetate

EnoylCoA = enoylCoA hydratase

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

GfE = Gesellschaft für Ernährungsphysiologie

GH = growth hormone

Glut1 = glucose transporter 1

Glut2 = glucose transporter 2

Glut4 = glucose transporter 4

GnRH = gonadotropin-releasing hormone

GPAM = glycerol-3-phosphate acetyltransferase

Ig = immunoglobulin

IGF-1 = insulin-like growth factor 1

IR β = insulin receptor β

i.v. = intravenously

LDH = lactate dehydrogenase

LSM = least squares means

ME = metabolizable energy

mRNA = messenger ribonucleic acid

n = number of samples

N = Anzahl

NAD = nicotinamide adenine dinucleotide

NDF = neutral detergent fiber

NEFA = nonesterified fatty acids

NEL = net energy for lactation

NFC = non fiber carbohydrates

NS = nonsignificant

OD 260/280 = optical density 260/280

ODM = once daily milking

p.m. = afternoon

PEPCK = phosphoenolpyruvate carboxykinase

PMR = partial mixed ration

PGF2α = prostaglandin F2α

r = correlation coefficient

R^2 = coefficient of determination

REML = restricted maximum likelihood

RNA = ribonucleic acid

SAS = Statistical Analysis System

SCC = somatic cell count

SD = standard deviation

SE = standard error

SEM = standard error of means

TAT = tyrosin aminotransferase

Units and terms

celsius (with number)	°C	logarithm (base 10)	log10
day	d	micro	µ (prefix)
deciliter	dL	microliter	µL
gram	g	milli	m (prefix)
joule	J	milliliter	mL
kilo	k (prefix)	month(s)	mo
liter	L	week(s)	wk

2 ABSTRACT

The objective was to compare the effects of three management systems in high-yielding dairy cows on metabolic profile, reproduction, health parameters and milk production traits. Metabolic status was determined by reference to functional parameters in blood serum and mRNA expression of hepatic genes involved in major pathways of glucose, amino acid and lipid metabolism. Furthermore, mRNA expression of glucose transporters, fatty acid oxidation and energy metabolism related genes in myocytes of skeletal muscle should identify genes that were up- or down-regulated for poor or improved metabolic status in early lactation, when dissemination of glucose is impaired for the benefit of the mammary gland. The performance of the dairy cow is determined by individual adaptation to metabolic imbalance in early lactation and its subsequent effects on reproductive performance, health and productivity. We tested two strategies to improve the metabolic status of high yielding dairy cows in early lactation besides enhancing energy density of diet and nutrient intake: Omission of dry period or reducing milking frequency in early lactation.

For the present study, 36 multiparous Brown Swiss cows were randomly assigned to one of three treatment groups: cows of control (**C**) (n = 12) group were dried off 56 d before calving and milked twice daily throughout next lactation (305 d). Cows of the once daily milking (**ODM**) group were dried off 56 d before calving, milked once daily for the first four weeks of lactation and twice daily for the remaining lactation (n = 12). Cows of the continuously milked (**CM**) group were milked twice daily up to day of calving and also during the subsequent lactation (n = 12).

Serum glucose concentrations decreased below the critical value of 3 mmol/L between wk 1 and 4 exclusively in C-cows. Blood serum concentrations of NEFA and BHBA were highest in C-cows compared to ODM- and CM-cows in the first four weeks of lactation. Decrease of back fat thickness during early lactation and reduction of body condition score were markedly more pronounced in C-cows compared to ODM- and CM-cows. Neither fertility data nor incidence of production diseases differed significantly among treatment groups. The lack of differences was due to the animal number used for this trial which was too low to get a power of more than 0.8 that is required for statistical analysis of fertility data. Mean 305 d milk yield of C-cows (11,310 ± 601 kg ECM) was approximately 16 % higher compared to ODM-cows (9,531 ± 477 kg ECM) and CM-cows (9,447 ± 310 kg ECM), respectively. The additional milk yield of CM-cows from d -56 to parturition was 1,157 ± 83 kg ECM. The lactation curve of CM-cows compared to C-cows was characterized by a similar time of peak yield (wk 3), a reduced peak yield, and no obvious differences in

persistency. Mean percentage of milk protein of CM-cows was significantly higher (3.91 %) compared to C-cows (3.52 %). In contrast, once daily milking was accompanied by a reduced and significantly delayed peak yield (wk 8) compared to control group, whereas persistency was better and milk protein higher (3.79 %) than in C-cows.

Metabolic status of cows based on blood serum glucose, NEFA and BHBA concentrations was reflected by mRNA levels of CPT 1A, PEPCK, ACADvl, GPAM, CTSLb in liver, and EnoylCoA and LDH in skeletal muscle.

After calving, mRNA levels of CPT 1A were more down-regulated in CM-cows compared to precalving than in C- and ODM-cows, but were higher at wk 4 and 8 compared to C-cows. For CM-cows, higher mRNA levels of PEPCK were observed one week before expected calving and at wk 2 after calving. ACADvl was up-regulated in C- and ODM-cows with calving and remained on higher levels compared to precalving. These changes did not occur in CM-cows. Transcript abundance of GPAM was lower at calving compared to precalving in all groups, but was higher in CM- cows than in ODM-cows at wk 2 and higher in CM-cows compared to C-cows at wk 4 and 8. Gene expression associated with protein metabolism as well as CS were unaffected by metabolic status. In skeletal muscle, mRNA levels of glucose transporters Glut1 and Glut4 were not affected by omission of dry period or reduced milking frequency in early lactation. Gene expression related to fatty acid metabolism (ACADvl, Enoyl CoA) showed no variation over time or among groups, except that EnoylCoA was markedly up-regulated in C-cows at wk 6 compared to ODM- and CM-cows and remained on a higher level at wk 16 for this group. In cows of all groups, mRNA levels of LDH were up-regulated at wk 16 compared to wk 6 and compared to calving.

Significant negative correlations were found for all cows among NEFA blood serum concentration and mRNA level of LDH in muscle ($r = -0.41$) and positive correlations were found among daily milk yield and mRNA level of LDH in skeletal muscle ($r = 0.45$). Significant positive correlations were found only for C-cows among milk yield and hepatic PEPCK transcript abundance ($r = 0.47$) and among NEFA blood serum concentrations and CPT 1A and ACADvl transcript abundance ($r = 0.49$ and $r = 0.43$, respectively).

Our findings suggest that continuous milking or once daily milking during the first four weeks of lactation result in an improved metabolic status compared to traditionally managed cows that were given a standard 56 d dry period and twice daily milking. These cows experience more adaptive performance with the onset of lactation. However, continuous milking seems to keep the cow metabolically and physiologically adapted to lactation, once daily milking smoothes the transition from the non-lactating to the lactating state. This was indicated for

both treatments by moderate changes of transcript abundance of key enzymes related to glucose and lipid metabolism and functional parameters in blood serum.

3 ZUSAMMENFASSUNG

Ziel der Studie war es, die Effekte dreier unterschiedlicher Managementsysteme bei Hochleistungskühen auf metabolischen Status, Reproduktion, Gesundheit sowie Milchleistungsparameter vergleichend zu prüfen. Das metabolische Profil der Tiere wurde anhand funktionaler Parameter im Blutserum und anhand der mRNA-Expression wichtiger Enzyme des Glukose-, Aminosäure- und Fettstoffwechsels in der Leber beschrieben. Die mRNA Expression von Glukosetransportern und Genen, die in die Fettsäureoxidation und den Energiemetabolismus im Skelettmuskel involviert sind, sollten Gene identifizieren, die in Abhängigkeit vom metabolischen Status der Tiere in der Frühlaktation hoch- oder herunterreguliert werden, wenn nahezu die gesamte Glukose für die Milchsynthese im Euter benötigt wird. Die Leistung der Milchkuh wird determiniert von der individuellen Fähigkeit des Einzeltieres sich an metabolische Stresssituationen anzupassen und den sich daraus ergebenden Effekten auf Reproduktion, Gesundheit und Milchleistung. Wir überprüften zwei Strategien um den Stoffwechsel von Hochleistungskühen in der Frühlaktation zu entlasten, die darüber hinausgingen, die Energiedichte im Futter und die Futteraufnahme zu erhöhen: Das Weglassen der Trockenstehphase oder eine Reduktion der Melkhäufigkeit pro Tag in der Frühlaktation.

Für die vorliegende Studie wurden 36 Brown Swiss Kühe zufällig zu einer von drei Gruppen zugeordnet: Kühe der Gruppe „Kontrolle“ (**C**) (N = 12) wurden 56 Tage vor der erwarteten Abkalbung trockengestellt und nach der Abkalbung zweimal täglich gemolken, Kühe der Gruppe „einmal täglich Melken“ (**ODM**) (N = 12) wurden genauso 56 Tage vor der Kalbung trockengestellt, allerdings nach der Kalbung 4 Wochen lang nur einmal täglich, und zwar abends gemolken. Ab der fünften Laktationswoche wurden diese Tiere wieder zweimal täglich gemolken. Tiere der Gruppe „Durchmelken“ (**CM**) (N = 12) wurden nicht trockengestellt, sondern bis zur Kalbung und auch danach zweimal täglich gemolken.

Die Glukosekonzentration im Blutserum sank zwischen den Wochen 1 und 4 ausschließlich bei den Kontrolltieren unter den kritischen Wert von 3 mmol/L. Die Konzentrationen der freien nicht veresterten Fettsäuren (NEFA) und der β-Hydroxybuttersäure (BHBA) waren während der ersten vier Wochen bei den Kontrolltieren am höchsten verglichen mit den Kühen der Gruppe ODM und CM. Die Abnahme von Rückenfett und Körperkondition waren bei den Kontrolltieren stärker ausgeprägt als bei den ODM- und CM-Kühen. Bei der Fruchtbarkeit oder der Krankheitsinzidenz konnten keine Unterschiede zwischen den Gruppen gefunden werden. Die Ursache dafür ist die zu geringe Anzahl der Tiere für eine statistische power von mehr als 0,8. Diese ist erforderlich, um Fruchtbarkeitsdaten statistisch

auszuwerten. Die 305 Tage Leistung der Kontrolltiere war nahezu 16 % höher (11.319 ± 601 kg ECM) als die der Kühe der Gruppe ODM (9.531 ± 477 kg ECM) oder CM (9.447 ± 310 kg ECM). Die zusätzlich produzierte Milchmenge der CM-Kühe vom Tag -56 vor der Kalbung bis zum Tag der Abkalbung war 1.157 ± 83 kg ECM. Die Laktationskurve der CM-Kühe zeigte verglichen mit der der Kontrolltiere eine fast gleichzeitige (Woche 3), aber geringere Peakleistung und keine markanten Unterschiede bei der Persistenz. Der mittlere Milchproteingehalt der CM-Kühe (3,91 %) war höher als bei den Kontrolltieren (3,52 %). Ganz im Gegensatz dazu war die Laktationskurve der ODM-Kühe dadurch gekennzeichnet, dass die Peakleisung später (Woche 8) und geringer war als bei den Kontrolltieren und dass die Persistenz verbessert war. Der Milchproteingehalt (3,79 %) war ebenfalls höher als bei den Tieren der Kontrollgruppe.

Der metabolische Status der Kühe, der durch Konzentrationen von Glukose, NEFA oder BHBA charakterisiert wurde, spiegelte sich auch in den Expressionsmustern von CPT 1A, PEPCK, ACADvl, GPAM und CTSLb in der Leber und EnoylCoA und LDH im Skelettmuskel wider. Nach der Kalbung war die mRNA-Expression von CPT 1A bei den CM-Kühen mehr herabreguliert verglichen zu einer Woche vor der Kalbung als bei den C- und ODM-Kühen, aber in den Wochen 4 und 8 war sie höher als bei den C-Kühen. Bei den CM-Kühen wurde eine Woche vor und zwei Wochen nach der Kalbung eine erhöhte Expression von PEPCK in der Leber gefunden. ACADvl war bei den C- und den ODM-Kühen hochreguliert und blieb auch nach der Kalbung auf einem höheren Niveau als vor der Kalbung. Diese Änderungen traten bei den CM-Kühen nicht ein. Die Expression von GPAM war zur Kalbung geringer als vorher in allen Gruppen, aber war bei den CM-Kühen in der Woche 2 höher als bei den ODM-Kühen und in der Woche 4 und 8 höher als bei den Kontrolltieren. Die untersuchten Gene, die in den Proteinmetabolismus involviert sind, sowie die CS zeigten keine Regulation in Abhängigkeit vom metabolischen Status. Im Skelettmuskel war die Expression der Glukosetransporter Glut1 und Glut4 nicht beeinflusst vom Weglassen der Trockenstehphase oder einer reduzierten Melkhäufigkeit in der Frühlaktation. Gene des Fettstoffwechsels (ACADvl und EnoylCoA) blieben unverändert, außer dass EnoylCoA in der Woche 6 bei den C-Kühen deutlich höher exprimiert war als bei den ODM- und CM-Kühen und dass die Expression auch in der Woche 16 für die Kontrollkühe auf einem höheren Niveau blieb. Die mRNA-Expression von LDH war bei den Kühen aller Gruppen in der Woche 16 hochreguliert verglichen zur Woche 6 und zur Kalbung.

Die Konzentration der NEFA im Blutserum und der mRNA-Expression von LDH im Skelettmuskel waren bei allen Gruppen negativ korreliert (r = -0,41), hingegen waren Milchmenge und LDH positiv korreliert (r = 0,45). Für Kühe der Kontrollgruppe konnten signifikante Korrelationen gefunden werden zwischen der Milchmenge und der mRNA-Expression von PEPCK in der Leber (r = 0,47), sowie zwischen der NEFA Konzentration im Blutserum und jeweils der mRNA-Expression von CPT 1A und ACADvl in der Leber (r = 0,49 und r = 0,43).

Die Ergebnisse lassen den Schluss zu, dass Durchmelken oder einmal täglich Melken während der ersten vier Wochen der Laktation zu einem verbessertem metabolischen Status führt verglichen mit Kühen in traditionellen Managementsystemen mit einer 56 Tage dauernden Trockenstehphase und zweimal täglichem Melken. Diese Kühe machen eine ausgeprägtere Adaptation an die Anforderungen der Laktation durch. Die CM-Kühe hingegen schienen metabolisch und physiologisch an die Laktation angepasst zu bleiben, das einmal täglich Melken schien den abrupten Übergang vom nicht laktierenden in den laktierenden Zustand abzumildern. Das war bei beiden Managementverfahren an den moderateren Expressionsmustern von Schlüsselenzymen des Glukose- und Fettstoffwechsels, sowie funktionalen Parametern im Blutserum zu sehen.

4 INTRODUCTION

Modern dairy cows are genetically selected for high milk production (Söndergaard et al., 2002), but milk yield varies greatly among cows and across stage of lactation: individual cows have produced more than 14,000 kg of milk in a 365 d lactation, with a peak milk yield of over 70 kg/d (Allen et al., 2005). Higher milk outputs, however, increase the metabolic load of dairy cows, which may affect negatively health and welfare of these animals (Collard et al., 2000; Ingvartsen et al., 2003), particularly during transition from late gestation to early lactation. This timeframe is called as transition period and includes 3 weeks before and 3 weeks after calving (Drackley, 1999). The transition period remains a problematic area on many dairy farms, and metabolic disorders continue to occur at economically important rates on commercial dairy farms (Burhans et al., 2003). The nutrient demand of a transition cow increases from approximately 1 kg/d of glucose during late gestation to 2.5 kg/d during the first 3 weeks after calving (Reynolds et al., 2003). Most transition dairy cows enter a state of metabolic imbalance for 3 primary reasons: increased nutrient requirements at parturition, decreased dry matter intake (DMI) shortly before parturition, and lagging DMI compared with nutrient demands due to increasing milk production (Drackley, 1999). Due to this, transition period is characterized by mobilization of body fat, protein, and mineral stores to satisfy the requirements for milk production and maintenance. Some cows are able to overcome this period of metabolic imbalance well, whereas other do not, and these latter cows may develop metabolic and related diseases such as ketosis, retained placenta, mastitis, displaced abomasum, laminitis, or reproduction problems (Goff and Horst, 1997). In most cases, these disorders are followed by reduced milk yield or in case of sustaining a high level of milk production by a postpartum decrease of milk protein percentage. Due to this, a nadir of milk protein percentage may be the result of a high milk yield and exacerbated metabolic imbalance using amino acids and other substrates for gluconeogenesis. For example, given a daily milk yield of 50 kg, a decrease of milk protein percentage from 3.6 % to 3.2 % means a loss of 0.2 kg milk protein per day. Thus, high protein percentage in early lactation requires an improved metabolic situation in early lactation. There is a tremendous demand of the dairy industry for milk with elevated milk protein content and low fat content. Moreover, milk and milk products play an important role in a balanced and healthy human diet predominantly due to the high quality and quantity of protein. In Germany, the main focus of the consumer is to get protein with a high biological value while avoiding ingesting fat of animal's origin. Given the current pricing system for milk and based on the energy supply that is necessary for

generating fat or protein, milk protein is paid more than five-fold higher than milk fat, but needs nearly half of energy for biosynthesis (Kirchgessner, 1987).

In the system of metabolic adaptation to lactation, the physiological transitions involved are reflected by changes of several parameters such as levels of glucose decrease, concentrations of BHBA and NEFA increase, concomitantly with related changes of endocrine systems. The circulating metabolites NEFA and BHBA are commonly used indices as they reflect metabolic status or ketosis in transition animals. Although some elevation of these metabolites is normal as these animals balance nutrient intake and nutrient demands in early lactation, excessive elevation of NEFA or BHBA can indicate poor adaptation to the ongoing lactation (Herdt, 2000). This complex system of adaptation occurs gradually and can differ considerably among cows (Jorritsma et al., 2003). The fact that some cows are able to deal with this metabolic challenge more successfully than others under similar management conditions implies, that metabolic adaptation may have a genetic impact. The liver plays a key role in the metabolic adaptation, through coordination and interconversion of nutrients to support pregnancy and lactation (Reynolds, 2003). This is illustrated by Loor et al. (2005), who found that mRNA expression of several hepatic genes related to lipid metabolism were changed during the transition period.

Searching for strategies to reduce metabolic imbalance in early lactation put optimizing of feeding strategies in focus by enhancing energy density of diet. Another option to reduce metabolic status in early lactation is reducing milking frequency as an important factor influencing milk yield in high-producing dairy cows (Patton et al., 2006; Rémond et al., 2004). The metabolic stress associated with high-yielding animals in early lactation may be reduced by adopting once-a-day milking, taking into consideration that this milking strategy is associated with decreased milk production (Davis et al., 1999). In earlier studies, this milking strategy focused only on the improved productivity of large or fragmented farms, as well as facilitate overcoming labor shortages, and enhanced lifestyle of the producer because of fewer work constraints.

Another tool to reduce the risk for metabolic imbalance during early lactation is to omit the dry period (DP) (Andersen et al., 2005; Rémond and Bonnefoy, 1997). It is generally accepted that a DP of 50 to 60 d is required to maximize milk production in the subsequent lactation (for recent reviews, see Bachman and Schairer, 2003; Annen et al., 2004; Grummer and Rastani, 2004). There has recently been substantial interest in decreasing the duration of

the DP as it affects DMI during early lactation. Cows that avoid severe decreases in DMI pre- and postpartum have improved metabolic status (Grummer, 1995). Recently, it has been demonstrated that omitting the DP results in dramatic alterations in metabolic situation, shown by metabolic profiles of continuously milked cows (Rastani et al., 2005). For these cows, plasma concentrations of glucose and insulin were higher and plasma concentrations of NEFA and BHBA were lower than in control cows (with a conventional 56 d DP) during the first 5 weeks of early lactation (Andersen et al., 2005). However, continuously milking compromised colostrum quality (Caja et al., 2006; Grummer and Rastani, 2004) and caused a 12-25 % decrease in milk yield during the subsequent lactation (Rémond et al., 1992; Rémond and Bonnefoy, 1997; Swanson, 1965). Dramatic production increases and improved persistency of lactation in today`s dairy cow provide new opportunities for omission of the DP: additional days of lactation maximize income generated per cow per lactation and decrease the number of replacement animals needed to keep a dairy at desired cow number capacity. Further, such high yields of > 30 kg/d at milk stasis result in extreme changes in metabolic and physiological state complicated with dramatic diet changes to add essentially a second transition period to the lactation cycle.

To date, continuous milking has not been compared with once daily milking in early lactation as a way to easier cope with the metabolic challenge of high-yielding dairy cows. Most of previous studies on short or omitted DP were retrospective and based on production records that included observational data without any randomized assignment of cows to different DP length.

Several other studies focusing on major and minor compounds of milk that are correlated to the metabolic status of the individual animals were linked to the study presented here.
In the first study published recently (Sigl et al., 2010), it was hypothesized that a reduction of milk fat percentage may improve the metabolic resilience of high yielding dairy cows in early lactation. Thus, it was the objective to investigate whether milk composition and metabolic key parameters were affected by adding rumen-protected CLA to the diet of dairy cows in the first 4 weeks of lactation.
As the biochemical milk profile has been related to health status of the individual cow and to imbalances during early lactation (Duffield et al., 2009), characterizing metabolic status in early lactation by non-invasive methods may be an option to select that cows easily adapt to metabolic stress (Klein et al., 2010).

5 AIM OF THE STUDY

Continuous milking or milking once daily for the first four weeks of lactation in comparison with a traditional management system including a dry period and twice daily milking were used to provoke poor or improved metabolic status during early lactation. Metabolic status is commonly reflected by alterations in concentrations of functional metabolic key parameters and results in different production levels and milk composition. Related to altered milking regimes the study was designed to meet the following requirements or to give a profound, scientific reply to some well known issues of the high yielding dairy cow:

- There are just a few studies available about once daily milking or continuously milking, but there are no experiments that conducted these two management regimes within the same herd and under the same housing and feeding conditions in comparison with a traditional management system of 56 d dry period and twice daily milking throughout lactation. Therefore, cows of different treatment groups should experience the same environmental system to ensure that poor or improved metabolic status is only triggered by the milking regimen.
- The approach to attenuate metabolic disturbances by altered milking regimes and the identification of factors that cause or that follow such a reduced metabolic load of dairy cows should be in main focus of the study.
- Against to other previous experiments, the study should be designed for a run over the entire 305 d lactation period.
- Effects of these different milking regimes on peak yield, time point of peak yield and persistency could be compared.
- Actually, there is a limited body of information about the effects of these two milking regimes on other affected parameters besides milk yield. Therefore, a close-mashed, intense sampling scheme was designed to obtain further data from blood and liver or muscle tissue samples based on established metabolic key parameters as well as potentially interesting new genes that were not previously suspected to be influenced by poor or improved metabolic status.

The hypothesis to be tested was that metabolic profiles and as a consequence parameters of productivity were different among cows that were either milked continuously or once daily for the first four weeks of lactation compared to those managed with a traditional 56 d dry period and twice daily milking after calving.

6 MATERIAL AND METHODS

The study started in December 2006 and ended in January 2009. A total of 45 multiparous Brown Swiss dairy cows were randomly assigned to one of three treatment groups: cows of the control group(**C**; n = 16) were dried off 56 d before expected date of calving and milked twice daily after calving. Cows of the second group (once daily milking, **ODM**; n = 16) were dried off 56 days before expected day of calving, but these cows were milked only once day for the first 4 weeks of lactation. Cows of the third group (continuously milking group, **CM**; n = 13) were milked continuously twice daily without a DP and troughout the subsequent 305 d lactation.

Figure 1 shows the timeframe of the whole study including all the samples starting for each cow four weeks before expected day of calving and ending at the individual 305 day of lactation.

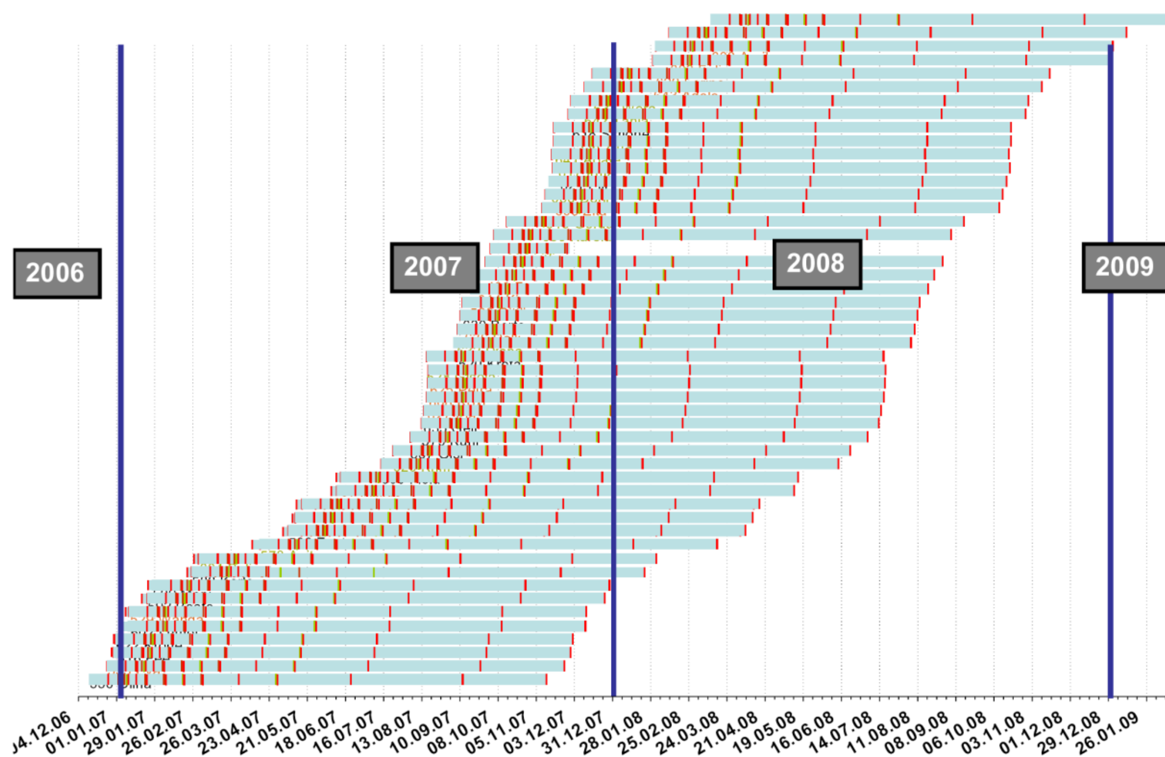

Figure 1. Timeframe of the study: Each line represents the timeframe of a 305 d lactation including the marked lines, that represent samples (blood serum and plasma, liver or muscle tissue samples) obtained from each animal.

Nine cows were removed from the study; the reasons for animals leaving the study were not associated to any of treatments and are presented in the following table (Table 1). There was no evidence of any effect of treatment on typical indices of cow health.

Previously, an additional group was planned, fed with fat supplement as concentrates containing CLA. But due to limited cow number, this experiment was done with a subset of heifers and is published by now (Sigl et al., 2010).

Table 1. Summary of the number of cows in each group, cows removed from each group, and reasons for removal

		Cows left trial before 305 DIM[1]		
Treatment[2]	Number of cows n for statistical analysis[3]	Cows removed	DIM	Reasons for removal
C	12	4	4, 29, 194, 297	milk fever (n = 1) euth, injured (n = 1) euth, curtailed tendons (n = 1) slaughtered , abortion (n = 1) slaughtered
ODM	12	4	1, 48, 137, 159	died suddenly (n = 1), injured (n = 1) euth, mastitis (n = 1) slaughtered, lameness (n = 1) slaughtered
CM	12	1	106	injured (n = 1) euth

[1] DIM = days in milk.

[2] C = 56 d dry period and twice daily milking after parturition (n = 12), ODM = 56 d dry period and once daily milking for the first 28 d of lactation (n = 12), CM = 0 d dry period and continuously milking twice a day throughout lactation (n = 12).

[3] Only those cows were included in statistical analysis that finished the 305-d lactation.

Percentage of culled cows (20 % according to 9 culled cows out of a total of 45 cows during a timeframe of 305 days) is even below the average culling rate in Germany. According to the point of time of occurrence of disease-related culling, it can be ruled out that the results of the study were contorted by the cows rejected from the study. Cows were randomly assigned to ODM, CM or C. Rejected cows were not excluded from the study due to be maladaptation to the management system, but only because of health problems or disorders. So, the decision to exclude any of the cows out of the study was the severity of illness, which was not related to experimental treatment.

Exclusively cows with recordings over the total 305 d experiment period 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 Bavarian State animal welfare committee (Munich, Germany).

6.1 Housing and feeding

All cows were kept in a cubicle housing system fitted with rubber coated slatted floors and bedded with wood shavings. Cows were milked in a 2 x 2 tandem milking parlour (GEA WestfaliaSurge GmbH, Boenen, Germany), either once (1545 h) or twice daily (0415 and 1545 h). For drying off (C-cows and ODM-cows only), milking was done once daily (1545 h) for 3 d, and then an intramammary antibiotic treatment containing 1 g cloxacillin (Orbenin®, Pfizer, Berlin, Germany) was given 56 d before expected calving. Thereafter, C-cows and ODM-cows were transferred to a separate stable and fed the DP ration. The following table shows the ingredients and chemical composition of diets.

Table 2. Chemical composition and ingredients of diet

	LD[1]	DPD[1]
Ingredient, %		
Corn silage	60	43
Grass silage	23	25
Hay	4	32
Feed pellets[2]	12	-
Mineral mix[3]	1	-
Chemical composition, %		
DM	45.2	52.0
CP	12.2	12.9
CF	18.6	19.2
NFC	26.4	9.92
Ether extract	3.16	3.16
NE_L (Mcal/kg)[4]	1.55	1.36

[1] Lactation Diet (LD) was fed to all cows, dry period diet (DPD) was fed only to cows having a 56 d dry period (control and once daily milking cows) but not to continuously milking cows.

[2] Composition: corn gluten 18.4 %, turnips molasses chips 13.8 %, wheat 10.0 %, triticale 10.0 %, rape cake 10.0 %, maize 8.8 %, malt germ 6.0 %, grain distillation residual (ProtiGrain) 5 %, rape extraction grist 5 %, rumen protected rape extraction grist 5 %, palm corn cake 3.3 %, soy extraction grist 2.8 %, sodium bicarbonate 1.0 %, calcium bicarbonate 0.99 %, plant oil (palm coconut) 0.40 % (Raiffeisen Kraftfutterwerke Süd GmbH, Würzburg Germany).

[3] Ingredients: calcium 14 %, sodium 10.0 %, phosphorous 5.0 %, magnesium 5.0 % (Josera, Kleinheubach, Germany).

[4] Estimates determined from tabulated values of feeds (GfE, 2007) and according to the following formula: NEL (Mcal/kg) = $0.6*[1+0.004*(q-57)]*ME$ (Mcal/kg), with q = ME/GE, here q = 88.9.

Approximately one week before expected calving, all cows were moved to a single calving pen bedded with barley straw. After calving, cows were moved to the lactating herd and fed the lactation diet. The CM-cows were not separated from the herd during the last 2 mo of pregnancy, as they were milked throughout the entire pregnancy-lactation cycle. All lactating cows were fed the partly mixed ration (**PMR**) formulated to meet nutrient requirements, as indicated by GfE (GfE, 2007) (Table 2), and had *ad libitum* access to fresh water.
In addition, hay was fed immediately after milking (0600 h). The PMR, formulated on the basis of a milk yield of 22 kg/d, was delivered once daily (0700 h) and intended to provide *ad libitum* intake (> 5 % residual feed). Cows producing more than 22 kg/d were fed additional concentrates (0.5 kg concentrate per kg milk; maximal 9 kg/d, Raiffeisen Kraftfutterwerke Süd, Würzburg, Germany) in feeding stations.

6.2 Milk yield recording, milk samples, and analysis of milk composition

Milk yield was measured twice daily with electronic milk meters (Metatron P21, GEA WestfaliaSurge, Boenen, Germany) and stored electronically (DairyPlan C21, GEA WestfaliaSurge, Boenen, Germany). Daily milk yield was standardized to ECM (4.0 % fat and 3.4 % protein content) according to (Fischer et al., 2002) by the following formula:

$$\text{ECM [kg/d]} = [(0.38 * \text{fat \%} + 0.21 * \text{protein \%} + 1.05)/3.28] * \text{milk yield [kg/d]}$$

Analysis of milk components (protein, fat, lactose, urea, and SCC) was done on samples collected at the afternoon milking. Due to almost similar milking intervals between morning and afternoon milking, measurements in afternoon samples were reliable estimations of the contents in daily milk in agreement with Lee and Wardrop (1984). Samples were collected during milking (about 1 L). The amount of milk separated into the sample pot was controlled by milk flow rate and total amount of milk to get a proportional subsample (Metatron P21, GEA WestfaliaSurge, Boenen, Germany). A 50 mL aliquot was taken and stored at 4°C for a maximum of 10 d with a preservative (acidiol) until analysis.
Milk protein, milk fat, lactose and urea were analyzed by infrared-spectrophotometry (MilkoScan^{FT}6000), and SCC was determined with a fluorescence-optical counting system (Fossomatic^{TM}FC) in the laboratories of Milchprüfring Bayern e.V. (Wolnzach, Germany). Milk composition of CM-cows was analyzed twice weekly until calving. After parturition, milk composition of all cows was determined daily for the first week, twice weekly to 56 days in milk (DIM), once weekly from 57 to 100 DIM, and then biweekly to 305 DIM.

Skim milk samples were quantitatively analyzed for progesterone by an enzyme immunoassay (Prakash et al., 1988) twice weekly from parturition to confirmed pregnancy (Inter- and intrassay coefficient of variation (CV) < 11.0 %). If skim milk progesterone concentration exceeded 2.5 ng/mL for at least 1 wk after a nadir (< 0.5 ng/mL), a corpus luteum was assumed to be present, indicating a preceding ovulation (Schopper et al., 1989).

6.3 Blood collection, and analysis of serum metabolites

Blood samples from all cows were collected by jugular venipuncture at wk -4, -2, and -1 before expected calving, within 24 h postpartum (wk 0), and at wk 1, 2, 4, 6, 8, 12, 16, 24, 36, and 44. Samples were collected into 7 mL evacuated EDTA-coated tubes and 7 mL evacuated serum-gel tubes (Vacuette®, Greiner Bio-One, Kremsmünster, Austria) at 0700 (after milking). Plasma was separated immediately after milking by centrifugation (2,000 x *g*, 4 °C, 15 min) and within 1 h, blood serum, was separated also by centrifugation (2,000 x *g*, 4 °C, 15 min). Three aliquots (1.5 mL) of both serum and plasma were stored at -20 °C. Serum concentrations of glucose, NEFA, BHBA, and total bilirubin (TB) were determined with an automated clinical chemistry analyzer (ABX Pentra 400, Horiba, Montpellier, France); precision of 20 measurements of one sample was expressed as the respective relative CV. Glucose concentrations were analyzed by using hexokinase method (Hoffmann La-Roche, Basel, Switzerland, CV 2.3 %), NEFA concentrations were measured using colorimetric enzymatic reactions (CV 6.2 %), BHBA was determined using a spectrophotometric enzymatic analysis (Sigma-Aldrich Diagnostics, Munich, Germany, CV 7.1 %), and TB was determined with the Jandrassik/Grof reaction (Jandrassik and Grof, 1938). Calibration and quality controls were done daily. The threshold values are based on Macrae et al. (2006).

6.4 Back fat thickness and body condition score

Subcutaneous adipose tissue (Back fat thickness) was assessed with ultasonography (Sonovet 2000, Universal Ultrasound, NY, USA) near the pelvic region (Schroeder and Staufenbiel, 2006). The BCS was determined by the same person biweekly from wk -8 to the end of the study (wk 44), using a scale from 1 to 5 (1 = emaciated, 5 = obese), in increments of 0.25 (Edmonson et al., 1989).

6.5 Reproduction and health check

A transrectal examination was done approximately 40 d after calving. During lactation, cows were monitored for estrus by milking personnel at milking time and by experienced herdmen.

After detection of estrus, cows were inspected by herd veterinarians; In the absence of any apparent reproductive dysfunction, cows were inseminated utilizing the a.m./p.m. rule (Graves et al., 1997). In case of anestrus, appropriate treatments, including the use of prostaglandin F2α or GnRH, were done to enable timed artificial insemination or for therapy of dysfunction. Pregnancy confirmation tests were performed 60 d after insemination by rectal palpation. Reproductive records maintained included days open, services per conception, first-service conception, days to first service, and pregnancy rate. Pregnancy diagnosis (transrectal palpation) was done 60 d after the last insemination and pregnancy rate was defined as the proportion of cows in each group diagnosed pregnant.
Technicians visited the herd every day and recorded health status, and recordings were verified by the herd verterinarian. Retained fetal membranes, periparturient paresis, primary ketosis, mastitis, and lameness occurred during the study.

6.6 Liver and muscle tissue collection, RNA extraction, and quantitative real-time reverse transcription-PCR

Muscle tissue samples of musculus semitendinosus (approximately 600 mg) were removed from the animals by an open muscle biopsy procedure at three times of lactation: within 24 hours after parturition, at wk 6 and 16 of lactation. Cows received a local subcutaneous anesthesia (7 mL, Procasel®, Selectavet, Weyarn, Germany) and caudal epidural anesthesia (5 mL Procainhydrochlorid (2 %), 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°C until analysis of mRNA levels.

Liver biopsies were obtained from all cows at wk -1 relative to calving, within 24 hours after calving and at wk 2, 4, and 8 of lactation by blind percutaneous needle biopsy (Bard®Magnum^{TM}, Covington, USA). A field of 15 x 15 cm² was shaved, washed and disinfected with 70 % ethanol and iodine solution. Local anesthetic (5 mL Procasel ®, Selectavet, Weyarn, Germany) was used to desensitize skin and underlying body wall. 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 (Buckley et al., 1986) and was just large enough to admit the trocar. Liver tissue (60-100 mg) was directly given into cryo-cups, frozen in liquid nitrogen and stored at -80°C until RNA extraction.
Total ribonucleic acid (RNA) was isolated from 50 mg tissue samples according to the manufacturer’s instructions of peqGOLD TriFast^{TM}. RNA was quantified by

spectrophotometry (Nanodrop, Eppendorf, Hamburg) obtaining an OD 260/280 ratio of 1.7 to 2.0 for all samples and diluted in 50 µL RNase-free water. Degradation of the RNA was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) in connection with the RNA 6000 Nano Assay.

Constant amounts of 1 µg RNA were reverse transcribed respectively to cDNA using the following RT 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, Mannheim, Germany).
The reverse transcription reaction was carried out according to the manufacturer in a 60-µL reaction volume in 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.

For the LightCycler reactions a master mix of the following reaction components was prepared to the indicated end concentration: 6.4 µL water, 1.2 µL $MgCl_2$ (4 µM), 0.2 µL Forward Primer (0.4 µM), 0.2 µL Reverse Primer (0.4 µM) and 1.0 µL LightCycler DNA Master SYBR Green I. A volume of 9 µL of LightCycler master mix was filled in the LightCycler glass capillaries and 25 ng reverse transcribed total RNA in 1 mL was added as PCR template. The capillaries were closed, centrifuged in a microcentrifuge, and placed in the LightCycler rotor (Roche, Mannheim, Germany). A conventional LightCycler amplification cycle contains three segments: In the 1st segment DNA is denaturated at 95°C. In the 2nd annealing segment the primer annealing takes place and the chosen temperature should be as high as possible to improve specificity. Within the following 3rd elongation segment at 72 °C, the elongation time should be adapted to the length of the desired product, which is limited by Taq Polymerase processing rate (1000 bp/min elongation time). The following LightCycler protocol was used for real-time PCR: *denaturation program* [95°C for 30 s], a 3 segment *amplification and quantification program* repeated 40 times [95°C for 3 s; 60°C for 10 s; 72 °C for 10 s]; *melting curve program* [60°C to 95°C with a heating rate of 0.1°C/s] and a final *cooling program* down to 40°C.
The display mode and the fluorimeter gains of channel 1 were set to 5.

Table 3. Primer information, annealing temperature, and reference

Primer[1]	Primer sequence 5´- 3´	Tissue	Function	Annealing temperature	Accesion no
PEPCK for	TACGAGGCCTTCAACTGGCGT	liver	gluconeogenesis	60°	XM_583200
PEPCK rev	AGATCCAAGGCGCCTTCCTTA				
CPT 1A for	CCATACTCACATAATTGGTAGCC	liver	β-oxidation	54°	BF_039285
CPT 1A rev	GCAACTAGTGAAGCCTCTTATGAA				
GPAM for	TCTGACTGAAGATGGGGATG	liver	glycerolipid-biosynthesis	54°	AF_469047
GPAM rev	ATGGGGAATTTGCCGCTTAT				
GLUT1 for	GTGCTCCTGGTTCTGTTCTTCA	muscle	glucose transport	61°	NM_174602
GLUT1 rev	GCCAGAAGCAATCTCATCGAA				
GLUT4 for	GGACCGCGAATAGAAGAAAGA	muscle	glucose transport	61°	NM_174604
GLUT4 rev	CAACTTCATCATCGGCATGG				
ACADvl for	CGTACATGGTGAGTGCCAAC	muscle/ liver	β-oxidation	60°	NM_174494
ACADvl rev	GTCATTTGTCCCCTCGAAGA				
EnoylCoA for	GCTGCTGTCAATGGCTATGC	muscle	β-oxidation	60°	NM_001025206
EnoylCoA rev	ACCAGTGAGGACCATCTCCA				
GAPDH for	GTCTTCACTACCATGGAGAAGG	liver	glycolysis, reference gene	60°	NM_001034034
GADPH rev	TCATGGATGACCTTGGCCAG				
Histone H3 for	ACTTGCCTCCTGCAAAGCAC	muscle/ liver	ordering DNA, reference gene	54°	NM_001014389
Histone H3 rev	ACTTGCTACAAAAGCCGCTC				
Ubiquitin for	AGATCCAGGATAAGGAAGGCAT	muscle	post-translational modification, reference gene	60°	Z18245
Ubiquitin rev	GCTCCACCTCCAGGGTGAT				
LDH for	GTGGCTTGGAAGATAAGTGG	muscle	pyruvat metabolism	62°	NM_005566
LDH rev	ACTAGAGTCACCATGCTCC				
ACTA1 for	TATTGTGCTCGACTCCGGCGA	muscle	muscle protein cell motility	60°	NM_174225
ACTA1 rev	GTCACGAAGGAGTAGCCACG				
IRß for	TCCTCAAGGAGCTGGAGGAGT	muscle	insulin receptor β	60°	M_37211
IRß rev	TAGCGTCCTCGGCAACAGG				
Glut2 for	GGACCTTGGTTTTGGCTGTC	liver	glucose transport	60°	XM_614140
Glut2 rev	CACAGACAGGGACCAGAACA				
CS for	TGGACATGATGTATGGTGG	liver	citric acid cycle	60°	BC_114138
CS rev	AGCCAAGATACCTGTTCCTC				
CTSLb for	CAC TGG TGC TCT TGA AGG ACA	liver	protein metabolism (degradation)	60°	NM_174032
CTSLb rev	TAA GAT TCC TCT GAG TCC AGG C				
TAT for	ACC CTT GTG GGT CAG TGT TC	liver	AA degradation	60°	NM_001034590
TAT rev	ACA GGA TGG GGA CTT TGC TG				

[1] PEPCK = phosphoenolpyruvate carboxykinase; CPT 1A = carnitine palmitoyltransferase 1A; GPAM = glycerol-3-phosphate acetyltransferase; Glut1 = glucose transporter 1; Glut4 = glucose transporter 4; ACADvl = acyl-coenzyme A dehydrogenase very long chain; EnoylCoA = EnoylCoA hydratase; GAPDH = Glyceraldehyde 3-phosphate dehydrogenase; LDH = Lactate dehydrogenase; ACTA1 = actin alpha 1; IRβ = insulin receptor β; Glut2 = glucose transporter 2; CS = citrate synthase; CTSLb = Cathepsin Lb; TAT = Tyrosin aminotransferase.

Target genes muscle

The enzymes selected and measured involved in lipid metabolism of muscle were acyl-coenzyme A dehydrogenase very long chain (**ACADvl**) and EnoylCoA hydratase (**EnoylCoA**), both involved in β-oxidation of fatty acids. Lactate dehydrogenase (**LDH**) is part of energy metabolism. It converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply and it performs the reverse reaction during the cori cycle in the liver. Glucose transporters **GLUT1** and **GLUT4** are involved in glucose transport: The insulin independent glucose transporter **GLUT1** is predominantly located in the muscle cell plasma membrane, and accounts for the basal glucose supply of the myocyte. The insulin-regulated glucose transporter **GLUT4** recycles between the muscle cell plasma membrane and an intracellular tubulovesicular pool, where it is associated with cytoplasmic vesicles. Actin alpha 1 (**ACTA1**) is a protein that is involved in cell motility, structure and integrity. It is a major constituent of the contractile apparatus. The insulin receptor β (**IRβ**) is a transmembrane receptor that is activated by insulin. Insulin receptor β substrates binding and phosphorylation eventually leads to an increase in the high affinity glucose transporter (**GLUT4**) molecules on the outer membrane of insulin-responsive tissues, including muscle cells and adipose tissues, and therefore to an increase in the uptake of glucose from blood into these tissues.

Target genes liver

Most important for hepatic adaptation is phosphoenolpyruvate carboxykinase (**PEPCK**), which is described as ratelimiting enzyme for hepatic gluconeogenesis (Greenfield et al., 2000). Hepatic oxidation of long chain-fatty acids occurs in mitochondria and peroxisomes (Drackley et al., 2001). Mitochondrial fatty acid oxidation involves 4 key steps: 1) uptake and activation of fatty acids to fatty acylCoA, 2) translocation of fatty acyl-CoA ino the motchondria, 3) β-oxidation of fatty acyl-CoA, and 4) ketogenesis. Carnitine palmitoyltransferase 1A (**CPT 1A**), an integral protein located on the outer mitochondrial membrane, catalyzes the formation of fatty acyl-carnitine from fatty acyl-CoA and carnitine and is believed to be a key regulatory step in metabolism of long-chain fatty acids (McGarry and Brown, 1997). Glycerol-3-phosphate acetyltransferase (**GPAM**) catalyzes the initial and committing step in glycerolipid biosynthesis and is predicted to play a pivotal role in the regulation of cellular triacylglycerol and phospholipid levels. Before glycerol can enter the pathway of glycolysis or gluconeogenesis (depending on physiological conditions), it must be converted to their intermediate glyceraldehyde 3-phosphate, which is mediated by GPAM.

Acyl-coenzyme A dehydrogenase, very long chain (**ACADvl**) catalyzes the initial step of each cycle of fatty acid β-oxidation. The protein encoded by Cathepsin Lb (**CTSLb**) is lysosomal cysteine proteinase that plays a major role in intracellular protein catabolism. Tyrosin-amino-transferase (**TAT**) is involved in the frist step of AA degradation. Glucose transporter 2 (**GLUT2**) is a transmembrane carrier protein that enables passive glucose movement across cell membranes. It is the principal transporter for transfer of glucose between liver and blood. The enzyme citrate synthase (**CS**) stands as a pace-making enzyme in the first step of the citric acid cycle. Oxalacetate will be regenerated after the completion of one round of the citric acid cycle.

Intron-spanning primers were designed (Table 3) using Primer3 online-software and synthesized by Metabion International AG (Martinsried, Germany) to amplify cDNA from the above mentioned genes except for Glut1 and Glut4, which were according to Komatsu et al., (2005).

7 STATISTICAL ANALYSIS

7.1 Power analysis

Discrepancies in results of different studies on interaction of DP length and parity apparently arise from management schemes (Gulay et al., 2003; Rastani et al., 2005), genetic potential of milk yield (Rastani et al., 2005), experimental method (between-cow model or within-cow and half-udder model) and statistical power. Because of high variation for milk yield in early lactation, it is important to evaluate the statistical power of these types of experiments before they are conducted. For experiments with a low number of animals, the likelihood of a type II error is increased. A power calculation indicated that the required number of animals for a power value of 0.8 and a minimum detectable difference of means with $P < 0.05$ was 11 cows for each group for blood serum parameters, milk yield and milk composition. To detect differences in frequency of health disorders and reproduction data among treatments, a minimum of 38 cows for each treatment group was required.

7.2 Statistical analysis of functional parameters in blood and performance data

End points measured repeatedly (daily milk yield, milk composition) were pooled to weekly means before statistical analysis. For BCS and back fat, an average value for every 4 wk from wk -8 to wk 40 was used.

For any metabolic key parameters in blood, as for milk yield, percentage milk protein, milk fat content, and milk protein and fat yield, as well as hepatic and muscular gene expression, treatment effects and differences among groups were determined using the REML in the Mixed Model procedure in SAS (SAS Institute, 2002). The model contained fixed effects of treatment and week and random effects of cow within treatment. For the repeated measurements, the model also contained weeks relative to calving and the interaction between treatment and weeks relative to calving. The effects of time, group, and time x group interactions were tested.

The following model was used by defining covariance structure as described above:

$$\mathbf{Y_{ijk} = \mu + treatment_i + cow_j(treatment)_{ik} + week_l + (treatment\ week)_{ikl} + \varepsilon_{ijkl}}$$

Y =	dependent variable
μ =	the overall mean
treatment =	fixed effect of treatment ik (i = ± dry period, k = milking frequency)
week =	fixed effect of week l postpartum (l = -8, -7, -6, …, 44)
cow (treatment)=	random effect of cow within treatment
interaction	treatment x 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. There are basically two aspects of the correlation. First, two measures on the same animal are correlated simply because they share common contributions from the animal. This is due to variation between animals. Second, measures on the same animal close in time are often more highly correlated than measures far apart in time. This is covariation within animals. Usually, when using PROC MIXED, the variation between animals is specified by the RANDOM statement, and covariation within animals is specified by the REPEATED statement.

The following paragraph shows the SAS program used for data analysis for each parameter:

```
options noxwait noxsync;
x 'C:\name and location of folder\name of excel file.xls';
filename a dde    'excel|name of sheet!z2s1:zxsy';
data _null_;
      x=sleep(5);
      run;
      data name of library (replace=yes);
      infile a notab dlm='09'x dsd missover;
      informat group$1.;
      informat cow$3.;
      informat week$2.;
      informat variable x 5.2;
      informat variable y 5.2;
      informat variable z 5.2;
      INPUT group cow week variable x variable y variable z;
      Format group$1.;
      Format cow$3.;
      Format week$2.;
      format variable x 5.2;
      format variable y 5.2;
      format variable z 5.2;
RUN;
filename d dde 'excel|system';
data _null_;
      file d;
      put '[schliessen()]';
      run;
quit;


ods html;
ods rtf file="d:\name and location of folder\name of word file.rtf" ;
proc mixed data=C;
class week_num group cow;
Model variable x= week_num group week_num*group;
repeated/type=ar(1) subject=cow(group);
lsmeans week_num group week_num*group/pdiff=all;
ods html close;
Ods rtf close;
run;


proc format ;
value group_num 1='control'
                  2='once daily milking'
                  3='continuously milking';
value week_num 0='parturition';
                        run;
options nodate pageno=1 linesize=80 pagesize=60;
ods rtf file="d:\name and location of folder\name of word file.rtf";


proc tabulate data=C;
class week_num group_num;
var variable x;
table week_num, all='name of table'*(variable 1)*group_num*(mean std
stderr)/rts=20 condense;
format group_num group_num. week_num week_num.;
run;
ods rtf close;
run;
```

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.

Incidence of health disorders and the proportion of blood samples from wk 1 to 4 with low glucose, high NEFA and high BHBA were tested for differences with PROC ANOVA, using Dunnetts one-tailed t-test to locate differences.

Results are reported as least square means ± standard error of means. Means were considered to differ significantly in case $P < 0.05$.

7.3 Statistical analysis of mRNA abundance in liver and muscle tissue

For valide comparisons between functional parameters of metabolism and productivity on the one side and mRNA levels of genes encoding for specific metabolic pathways on the other side, data for milk yield and fat yield presented here originate from day of sampling and were not pooled weekly means. So, corresponding daily milk yield and daily fat yield as well as NEFA, BHBA and glucose concentrations were available at each biopsy time point.

Data of mRNA were analyzed using the second derivate maximum method described in the LightCycler Relative Quantification Software, obtaining a crossing-point (Cq) value for each gene on each animal at each time point. These values were translated to normalized expression quantities (ΔCq) using two reference genes for each tissue in a form of normalization index (glyceraldehyde-3- phosphate dehydrogenase (GAPDH) and histone H3 in liver tissue samples, and histone H3 and ubiquitin in muscle tissue samples (Table 3 for primer sequences). Δ quantitative Cycle (Cq) values were calculated as:

$\mathbf{\Delta Cq = Cq_{target\ gene} - meanCq_{reference\ genes}}$ (Pfaffl, 2001)

For graphical representation of regulated genes in **liver tissue**, a relative expression index (ΔΔCq-values) was calculated according to:

$\Delta\Delta Cq = \Delta Cq_{\text{timepoint (calving to wk 8)}} - \text{mean}\Delta Cq_{\text{(wk -1)}}$ to detect regulations within groups between times or

$\Delta\Delta Cq = \Delta Cq_{\text{treatment group}} - \text{mean}\Delta Cq_{\text{control group}}$ to detect regulations within times between groups.

For graphical representation of regulated genes in **muscle tissue**, a relative expression index (ΔΔCq-values) was calculated according to:

$\Delta\Delta Cq = \Delta Cq_{\text{time point (calving or wk 6)}} - \text{mean}\Delta Cq_{\text{wk 16}}$ to detect regulations within groups between times.

In order to avoid negative numbers in the table that shows mRNA abundance while allowing an estimation of a relative comparison between two genes, data are presented as means ± SEM subtracted from the arbitrary value 5 for liver samples and 10 for muscle samples (ΔCq). Thus, a high ΔCq resembles high transcript abundance (Livak and Schmittgen, 2001). An increase of one ΔCq represents a two-fold increase of mRNA transcripts.
Correlation and linear regression coefficients used to describe relationships between gene expression and plasma metabolites and parameters of productivity were estimated using the CORR procedure. If data were normal distributed, Pearson correlation coefficients are shown, if data were not normal distributed, spearman rank-order correlation coefficients are shown in the tables.

8 RESULTS AND DISCUSSION

8.1 Animals and performance

In most studies in dairy science previous lactation milk yield is often used as covariate. In our study a clear relationship between parity and previous lactation milk yield was found for all treatment groups. Previous lactation milk yield and parity were similar among groups (Table 4).

Table 4. Previous lactation milk yield and number of parity for each group

	Treatment group[1]		
	C	ODM	CM
Previous energy corrected 305-d milk yield [kg][2]	8,826 ± 377	8,725 ± 360	8,664 ± 336
Parity[3]	3.5 ± 1.6	3.8 ± 1.8	3.3 ± 2.1

[1] C = 56 d dry period and twice daily milking after parturition (n = 12), ODM = 56 d dry period and once daily milking for the first 28 d of lactation (n = 12), CM = 0 d dry period and continuously milking twice a day throughout lactation (n = 12).

[2] Calculation of previous ECM yield was based on data of LKV Bayern 2006.

[3] Parity ranged for all groups from 2 to 7 lactations.

Milk yield

Total lactational ECM yield (305 d) was highest for C-cows (11,311 ± 601 kg ECM). Compared to C-cows, total ECM was 15.7 % and 16.5 % lower for ODM-cows (9,531 ± 477 kg ECM) and CM-cows (9,446 ± 310 kg ECM), respectively. The milk yield in the present experiment was significantly higher than in previously reported studies concerning continuous lactation in cows not treated with bST (Buckley et al., 1986; Rémond and Bonnefoy, 1997; Swanson, 1965) or cows milked once a day (Andersen et al., 2005; Rémond et al., 2004). Moreover, the milk yield was similar to recent experiments in which cows were milked once daily (Loiselle et al., 2009) or even higher compared with cows milked continuously in a Danish experiment from 2004 (Andersen et al., 2005). Therefore, the present experiment contributes new information to the impact of continuous lactation throughout gestation or once daily milking in high-yielding dairy cows on both, milk production as well as metabolic adaptation to the following lactation.

CM$^+$. The abbreviation of CM with the appendix "+" means that for any comparisons of the continuously milked cows with control or once daily milked cows the last 8 weeks of gestation were included. These extra days of milking are accompanied by additional produced milk during this time, while C- and ODM-cows were dried off. However the reduced milk yield in the subsequent lactation is related to a lack of sufficient time for the mammary gland to involute and redifferentiate, but this milk loss could quite entirely recovered by the additional days of milking in late gestation. In this case, the most obvious potential benefit from continuously milking would be increased income from milk. This will occur if extra income from milk obtained by extending the previous lactation is greater than lost income if less milk is produced in the subsequent lactation. The total milk yield of CM-cows from d -56 to parturition was 1,157 ± 83 kg ECM. Daily ECM yield was 21.2 ± 1.5 kg. Daily ECM yield declined by 57.3 % from wk -8 to wk -1 relative to calving. During the last week before calving average daily milk yield of CM-cows was 14.4 ± 2.1 kg ECM.

CM. In our study, the 305-d lactation ECM yield was 17 % lower in CM- compared to C-cows. The reduced milk yield for CM-cows was reported elsewhere (Farries and Hoheisel, 1989; Rémond et al., 1992; Swanson, 1965) and may be due to secretory activity per unit of mammary tissue and the physiological factors that affect the cells during lactogenesis, but not due to number of mammary epithelial cells (Annen et al., 2004). Involution, proliferation and differentiation of mammary parenchyma during dry period (DP) represent decisive preconditions for a high subsequent lactation yield (Capuco et al., 1997) independently of the genetic merit of the cow (Andersen et al., 2005; Smith et al., 1966). The lack of detectable differences between CM and ODM in 305 d milk yield supports the hypothesis that improved energy status of animals may overcome any differences in secretory function as lactation progresses. It is likely that omitting the DP do not provide optimum time for mammary cell turnover, leading to decreased milk yield during the next lactation (Annen et al., 2007).

ODM. Cows of this group were milked only once a day during the first four weeks of lactation. The obvious reduction of total lactation ECM yield for ODM-cows compared to C-cows by 16 % was primarily due to the reduced yield during the first four wks of lactation, with a smaller carry-over effect after resumption of twice daily milking during the following four weeks. ODM-cows had the lowest daily ECM yield (31.3 ± 1.1 kg ECM/d, $P < 0.001$) compared to C-cows (43.3 ± 1.8 kg ECM/d) or CM-cows (35.0 ± 1.1 kg ECM/d, $P = 0.005$) during this time. This reduction of milk yield may be caused by functional changes in the

mammary gland: mammary cell number and their metabolic activity, tight junction permeability, regulation of alveolar cell activity by feedback inhibitor of lactation, and apoptosis stimulating factors (Stelwagen, 2001). Our approach to implement milking once a day with cows of high production level was to improve the metabolic situation of these cows after calving. In contrast, there are some economic environments, especially low cost-low return extensive production systems like in New Zealand, where farmers have found merit in reducing milking frequency to once daily. The switch to once daily milking may be driven by lifestyle preferences or include a substantial increase in labour productivity and better utilization of milking parlours (Davis et al., 1999). Only one recent study is available that compared the effects of once daily milking in comparison with three times daily milking on the energy status of dairy cows in early lactation (Patton et al., 2006). In that study, however, the production level was much lower compared with that of the high yielding dairy cows used in our trial. Milk yield for ODM-cows increased from the start of twice-daily milking at wk 5 ($P < 0.001$), but remained approximately 8.1 kg/d ECM (18 %) lower than for C-cows ($P = 0.01$) till wk 8.

Milk composition

In this study evening samples were used to predict the daily value of milk composition for fat % and protein %. Due to this, the following experiment was conducted to evaluate the method of taking milk samples at evening milkings:

Twelve cows (each four cows in early, mid or late lactation) were milked twice daily on five subsequent days. At each milking, a representative milk sample was taken and analyzed for fat, protein, lactose and SCC. Each individual milking interval and milk yield was recorded. Results indicated that the variance of milk composition was influenced more by the animal itself than by milking intervals or time of milking (milking intervals were 720 ± 56 min). We calculated the correlation between the estimated value (exclusevily based on afternoon milking) and the actual value (based on morning and afternoon milking). The coefficient of correlation between the estimated and the actual value were 0.99 for protein and 0.92 for fat percentage. The mean of the absolute deviation of evening samples from the actual value was 0.01 units for fat and 0.05 units for protein. The relative deviation of the estimated from the actual value was 7.7 % for fat and 1.6 % for protein percentage. So the deviation of observed and predicted protein or fat percentages was negligible, as both were predicted well from unadjusted afternoon samples. According to our milking interval we had no bias using

afternoon samples to predict daily values of cows milked twice daily in comparison with cows milked once daily. This is in agreement with Lee and Wardrop (1984).

Milk composition before calving

CM. Milk of CM-cows before calving –an extra time of milking, while ODM- and C-cows were dried off- was enriched with fat and protein. Interestingly, the daily protein yield remained constant (0.71 ± 0.32 kg/d), while the daily fat yield decreased during this time (1.02 ± 0.40 kg/d to 0.64 ± 0.31 kg/d, $P < 0.01$). Protein percentage was more than 7.2 % last week before calving perhaps caused by an accumulation of immunoglobulins in the mammary gland. However, majority of literature suggests that colostral immunoglobulin and protein content are reduced in CM-cows (Rémond and Bonnefoy, 1997). Enhanced protein and immunoglobulin concentrations in colostrum are believed to be the result of an accumulation of secretion in the udder prior to calving for cows with a 56 d DP in late gestation (Wheelock et al., 1965). Continuously milked cows may have reduced colostrum quality due to the lack of a secretion-accumulation period. Interestingly, cows that were given a DP length of 1 to 10 d produced colostrum with immunoglobulin concentrations that are 60 to 70 % of colostrum from cows given a 60 d DP (Rémond and Bonnefoy, 1997). Overall, during the last 8 weeks of gestation CM-cows produced a total amount of 42 kg milk protein.

Milk composition during lactation

For the first 4 weeks after calving, milk protein concentration was higher in both ODM-cows (3.97 ± 1.07 %, $P = 0.041$) and CM-cows (4.06 ± 0.63 %, $P = 0.009$) compared to C-cows (3.65 ± 0.12 %). The increased milk protein content in early lactation indicates improved availability of energy due to a reduced milk yield (Rémond et al., 1997) as milk protein content is considered as indicator for energy supply in a herd monitoring system. However, reduced milk volume for ODM-cows resulted in lower daily protein yield for these cows (0.83 ± 0.22 kg/d) compared to C-cows (1.13 ± 0.3 kg/d, $P = 0.007$) and compared to CM-cows (1.15 ± 0.14 kg/d, $P = 0.007$). CM-cows had a higher weekly milk protein concentration for the first 20 wks of lactation compared to C-cows (3.85 ± 0.07 % vs. 3.34 ± 0.07 %, $P = 0.005$). During the 305 d lactation, CM-cows had the highest milk protein content (3.89 ± 0.28 %) in comparison to C-cows (3.52 ± 0.3 %) and ODM-cows (3.79 ± 0.41 %, Table 5). Beside milk yield, milk protein content is a matter of growing economic interest compared to all other milk components by the processing dairy industry. It has to be emphasized that CM as well as ODM are useful tools to produce milk with higher milk

protein content and a higher price compared to traditional management of dairy cows. Also Patton et al. (2006) demonstrated a higher milk protein concentration (3.55 %) and milk fat concentration (4.70 %) during the first 28 d of lactation for cows milked once daily compared to cows milked thrice daily. However, no changes in milk protein concentration were found in short-term studies (ODM for 1 wk) (Guinard-Flament et al., 2007; Loiselle et al., 2009).

In our study, ODM was associated with the highest milk fat concentration during the first four weeks of lactation. Due to the lower milk yield, ODM-cows produced only three quarters of the daily fat yield produced by C-cows. The high milk fat concentrations may be caused by an enhanced lipomobilization, as long chain fatty acids are precursors for milk fat synthesis.In addition to the high milk protein content throughout lactation, CM-cows produced the highest lactational milk protein yield from d 56 before parturition to the end of lactation. Total lactational protein yield was 367 ± 14 kg for CM cows, including the extra days of milking, while C-cows had a protein yield of 342 ± 15 kg and ODM-cows a protein yield of 311 ± 16 kg in the following 305 d lactation.

Table 5. Parameters of productivity for the 305 d lactation[1]

	Treatment group[2]			
Item[3]	C	ODM	CM	CM+
ECM yield [kg/d]	38.2 ± 0.60	32.4 ± 0.40	**30.5 ± 0.50**	**29.4 ± 1.20**
Protein %	3.52 ± 0.30	3.79 ± 0.40	**3.91 ± 0.30**	**4.04 ± 0.30**
Fat %	5.50 ± 0.20	5.15 ± 0.10	**4.70 ± 0.10**	5.33 ± 0.30
Lactose %	4.66 ± 0.08	4,65 ± 0.05	4.66 ± 0.05	4.70 ± 0.04
Protein kg /d	1.08 ± 0.05	0.99 ± 0.05	1.10 ± 0.04	1.03 ± 0.05
Fat kg/d	1.87 ± 0.08	1.42 ± 0.08	**1.38 ± 0.08**	**1.26 ± 0.08**
Lactose kg/d	1.48 ± 0.05	1.28 ± 0.08	1.27 ± 0.08	1.20 ± 0.07

[1] For calculation of values in column CM+, the extra days of milking (56 days before calving) were included.

[2] C = 56 d dry period and twice daily milking after parturition, ODM = 56 d dry period and once daily milking for the first 28 d of lactation, CM = 0 d dry period and continuously milking twice a day throughout lactation.

[3] Values in boldface are different from values of C-cows ($P < 0.05$).

Persistency

ECM yield peaked for C-cows at 35 ± 5 DIM (53.6 ± 3.4 kg ECM). The time period of almost constant yield (defined as the number of days with a daily milk yield of > 90 % of peak yield) persisted 70 ± 13 d up to 105 ± 15 DIM resulting in an average daily ECM of about 49.6 ± 3.12 kg during this time frame. For ODM-cows, milk yield peaked 3 weeks later than

for C-cows at 56 ± 4 DIM (43.4 ± 1.6 kg). Milk yield persisted for 64 ± 13 days, i.e. up to 120 ± 14 DIM. During this time peak yield declined for about 0.12 kg ECM/day. In CM-cows, daily milk yield peaked at 54 ± 8 DIM (45.2 ± 1.6 kg ECM) and persisted 66 ± 10 d up to 120 ± 16 DIM resulting in a daily ECM yield of 41.8 ± 1.5 kg.

SCC

SCC differed only at wk 1 between cows of group C (5.34 ± 0.4 $\log_{10}$SCC) and ODM (5.47 ± 0.4 $\log_{10}$SCC, P = 0.042). No differences were found for SCC among treatment groups at any other time points.

CM. A typical pattern of SCC during a traditional lactation cycle is characterized by high concentrations at freshening, a nadir at peak milk and midlactation, and concentrations gradually rising in late lactation. Rémond and Bonnefoy (1997) observed a tendency for elevated SCC in milk from cows given a shortened or omitted DP. This increase was not accompanied by an increase in clinical mastitis cases. One could speculate that continuous milking of cows could reduce clinical mammary infections by eliminating new infections associated with cessation of milking and the DP. In our study, SCC of CM-cows did not differ from that of C-cows either in early lactation nor during the entire lactation. There were two cows with mastitis at day -7 and -1 relative to calving, which may caused by the very low milk yield for these cows (< 2 kg/d) followed by a lack of intramammary pressure, longer stripping times and as a consequence a higher risk for mastitis for these cows which are known to be immunosuppressed immediately before and after calving.

ODM. A typical concern in respect to ODM is udder health as udder distension in ODM-cows may lead to a higher probability of inflammatory responses. One study indicated a doubled SCC in comparison to twice daily milking when cows were milked once daily for a longer time (more than 4 weeks, Lacy-Hulbert et al., 2005). However, no differences were obvious in the incidence of mastitis for ODM-cows compared to C-cows or CM-cows in our study which corresponded with others (Clark et al., 2006). Rémond et al. (2004) found no differences in mean SCC between once and twice daily milking despite a sharp increase noted for ODM-cows during the last third of lactation. Loiselle et al. (2009) found neither differences for SCC nor for chemotaxis, phagocytosis, or oxidative burst activity during ODM in the first week postpartum compared to twice daily milking or thereafter up to wk 14 of lactation.

Lactose

The level of water secretion into milk largely determines the fat and protein content of milk. The rate of water secretion is mostly determined by the rate of lactose synthesis, because lactose is the major factor responsible for the osmolality of milk. Lactose percentage was similar among groups for the 305 d lactation (4.66 ± 0.08 % for C- and CM-cows, and 4.65 ± 0.05 % for ODM-cows).

CM. The lactose content has been shown to increase during the last 2 mo of gestation in a similar, but inverse, temporal pattern as observed with fat and protein (Rémond et al., 1997). For CM-cows, lactose percentage rised up to more than 5.0 ± 0.05 % at wk -4 and -3 prepartum and decreased to normal value of about 4.7 ± 0.2 % at wk -1. Postpartum lactose content in milk from CM glands or cows decreased in some experiments (Rémond et al., 1992, 1997) and was unaffected by length of DP in others (Wheelock et al., 1965; Smith et al., 1967). In our study, lactose content was 4.1 ± 0.13 % 4.05 ± 0.1 % 4.5 ± 0.12 %, in the first week postpartum for C-, ODM-, and CM-cows, respectively. Lactose is the major osmoregulator of milk, and a decrease in lactose production from CM-cows or glands would explain reduced milk yield. However, not all experiments with decreased milk yield following an omitted DP demonstrate reductions in lactose content. Reduced milk yield without reduced lactose synthesis would suggest fewer cells in the secretory state in CM glands. However, Andersen et al. (2005) did not find any differences for lactose concentration in milk of CM-cows compared to those having a 56 d DP in the following early lactation.

ODM. In two studies which have been conducted in cows over the course of an entire lactation (Claesson et al., 1959; Holmes et al., 1992), concentration of lactose decreased, in common with our study. Lactose concentration was 4.4 ± 0.08 % during the first 4 weeks of lactation for ODM-cows, compared to 4.7 ± 0.12 % of C-cows. Loiselle et al. (2009) found no differences in lactose percentage among cows milked either once daily or twice daily during the first week of lactation. Similar results for lactose concentration of cows milked once daily compared to those milked twice daily on a standard diet were observed earlier by Patton et al. (2006). In our study, significant differences observed for daily lactose yield during the treatment period were largely due to milk volume differences.

Urea

Milk urea nitrogen is a normal NPN component in milk. Urea is a major end-product of nitrogen metabolism in dairy cows. It is synthesized primarily in the liver and transported in blood to the kidney to be excreted in urine. From the blood, its concentration equilibrates rapidly with other body fluids, including milk (Gustafsson and Palmquist, 1993). Urea originates mainly from excess ammonia released from dietary protein degradation in the rumen or from deamination of amino acids in excess of requirements. Milk urea nitrogen has been used as a noninvasive measurement to monitor the animal's protein status and the efficiency of nitrogen utilization (Eicher et al., 1999). In our study, milk urea was similar among groups during the entire lactation (239 ± 11 mg/dL, 205 ± 12 mg/dL, and 211 ± 7 mg/dL for C-, ODM-, and CM-cows, respectively) and did not significantly change in late gestation for CM-cows or during the first 4 weeks of lactation.

Animal welfare

CM. There were no indicators for us, that CM-cows experienced discomfort because of two times daily milking even in late pregnancy, when rest and lying times were longer compared to the non-pregnant state.

ODM. From the perspective of the cow, once daily milking has several advantages. ODM-cows produce less milk (Davis et al., 1999) and, therefore, may have reduced risk of metabolic disorders (Rauw et al., 1998). In New Zealand production systems once daily milking may be an option for utilizing pasture further from the milking parlour without forcing animals to walk distances beyond the current average of 1.9 km 1-way (Tucker et al., 2007). In contrast to these findings, there are concerns that ODM-cows may experience discomfort associated with udder distension and inflammatory response. In our study, however, we did not observe in ODM-cows any indicators revealing severe discomfort, which is in accordance to others (Davis et al., 1999; Rémond et al., 2004; Tucker et al., 2007). One of the 12 cows mooed and lowed during the first few days of ODM-treatment before and during afternoon milking, but after 3 days the animal accepted once daily milking without any problems. There were two leaking cows in this group, one of them because of teat damage. In general, ODM did not pose problems in terms of routine management of cows.

8.2 Metabolic status of cows

Recent studies have shown that numerous milk metabolites were correlated to the metabolic status of cows in early and late lactation. A total of 44 different milk metabolites that are related to energy metabolism were identified by nuclear magnetic resonance and mass spectrometry (Klein et al., 2010). In this study, the aim was to select milk constituents to monitor metabolic status of cows that cope well with metabolic stress in early lactation. This sampling method is less invasive than taking blood samples by jugular venipuncture. Despite of this, in our own study metabolic status of cows was identified by concentrations of functional parameters in blood serum such as glucose, BHBA and NEFA. Most of studies focusing on metabolic profile of dairy cows in early lactation analyze blood plasma samples. In this study serum samples were used for analyzing concentrations of functional parameters in blood. As shown by Dettmer et al. (2010), concentrations of central metabolites were similar among these two sample types.

The following table (Table 6) shows the main remarks of the results of blood analysis. Furthermore, considering blood samples collected in wk 1, 2 and 4 of lactation proportion of samples was assessed in which established alarm levels of metabolic key parameters in herd medicine were exceeded. In group C, a higher percentage of cows exhibited hypoglycemia compared to ODM- and CM-cows (47 % vs. 20 % vs. 14 %, respectively, $P < 0.05$). Moreover, the proportion of C-cows with excessive lipomobilization and ketonemia was higher compared to those of the other treatment groups

Table 6. Percentage of blood samples (36 samples for each group) collected in wk 1, 2 and 4 of lactation exceeding established alarm levels of metabolic key parameters in herd medicine of all treatment groups

		Treatment group[1]			P-value[2]		
Parameter	Threshold value[3]	C	ODM	CM	C vs. ODM	C vs. CM	CM vs. ODM
Glucose	≤ 3.0 mmol/L	47.1	20.0	13.9	< 0.001	< 0.001	0.81
BHBA	≥ 1.4 mmol/L	29.4	2.9	0	< 0.001	< 0.001	0.62
NEFA	≥ 0.7 mmol/L	41.2	17.1	8.3	0.012	< 0.001	0.90

[1] C = 56 d dry period and twice daily milking after parturition, ODM = 56 d dry period and once daily milking for the first 28 d of lactation, CM = 0 d dry period and continuously milking twice a day throughout lactation.

[2] Means are different for $P < 0.05$.

[3] Threshold values are based on our own experience and data of Macrae et al. (2006).

Glucose, NEFA and BHBA concentrations in blood serum were analyzed for monitoring metabolic status of experimental cows.

Glucose

Glucose is a key molecule in ruminant metabolism, e.g. in lactogenesis (Guinard-Flament et al., 2006). The glucose need of a transition cow increases from approximately 1 kg/d of glucose during late gestation to 2.5 kg/d during the first 3 weeks after calving (Reynolds, 2003). Blood glucose concentration reflects the balance between glucose input and output. Hypoglycemia is prevented under physiologic conditions by the effects of different hormones including glucagon, catecholamines, glucocorticoids and the GH-IGF-1-system. In our study, 47 % of serum samples of C-cows collected between wk 1 and wk 4 revealed hypoglycemia (< 3.0 mmol/L; Table 6). In contrast, only 14 % of samples of CM-cows and 20 % of samples of ODM-cows collected from wk 1 to 4 revealed hypoglycaemia. The sustained physiological serum glucose levels in early lactation for CM-cows indicate a more stable metabolic status together with lower blood serum BHBA and NEFA concentrations. Similar results were reported by Rastani et al. (2007) during the first week of lactation and by Andersen et al. (2005) and Madsen et al. (2008) during the first 5 weeks of lactation for CM-cows compared to C-cows milked twice daily after a preceding 56 d DP, respectively. As glucose is the primary metabolic fuel and is absolutely required for vital organ function, fetal growth, and

milk production (LeBlanc et al., 2005), it is a sensitive measure of metabolic status although is subject to tight homeostatic regulation.

NEFA

Stored energy from fat is mobilized as NEFA, some of which is taken up by the liver. In the liver, NEFA are oxidized or reesterified into triglycerides that are either exported as very low density lipoproteins or stored in the liver. Oxidation of NEFA and other substrates provides ATP needed for gluconeogenesis and is necessary for stimulated gluconeogenesis from lactate or pyruvate, at least in part by provision of the activator of pyruvate carboxylase (PC). During the periparturient period, high rates of NEFA enter the liver and sometimes exceed the liver's capacity to secrete triglycerides as very low density lipoproteins, resulting in an accumulation of triglycerides (Drackley et al., 2001b). Increased amounts of NEFA removed by the liver along with CPT 1A activity regulate ketogenesis and thus, BHBA production (Hegardt, 1999). Herdt (2000) proposed that the NEFA concentration during the first week postpartum is an indicator for adaptive performance.

BHBA

In many nonruminant species, hepatic conversion of NEFA to ketone bodies is considered a strategy to spare glucose during times of deficit. Although oxidative use of glucose generally is lower in dairy cows than in nonruminat animals, similar adaptive processes may occur in dairy cows during the transition period that further decrease oxidation of glucose. Therefore, increased ketogenesis during the transition period may be an additional strategy to compensate for insufficient intake of glucose precursors. BHBA concentrations were higher after calving than precalving because of the high energy demands associated with the onset of lactation (Vazquez-Anon et al., 1994). BHBA concentrations rose sharply up to approximately 10 DIM and slowly decrease thereafter. Walsh et al. (2007) suggested a concentration threshold of serum BHBA of ≥ 1.0 mmol/L in the first week postpartum or ≥ 1.4 mmol/L in the second week postpartum as a diagnostic measure of subclinical ketosis. Similarly, Duffield et al. (2009) defined hyperketonemia above a threshold level of 1.4 mmol/L of BHBA resulting both in increased disease risk during the first two weeks postpartum and in substantial loss of milk yield in early lactation.

The case definition of subclinical ketosis in dairy cattle is an excess level of circulating ketone bodies in the absence of the clinical signs of ketosis (Andersson, 1988). Beta-hydroxybutyrate

is the predominant circulating ketone body in ruminants, but there is a strong correlation between the whole blood concentrations of BHBA and acetoacetate (Kauppinen, 1983). Conceivably, subclinical ketosis may start at levels of BHBA greater than 1.0 mmol/L; however, the decision to set an appropriate subclinical threshold using serum or plasma BHBA appears to be somewhat arbitrary. Kelly (1977) suggested that 1.0 mmol/L be used to separate cows with low and high BHBA concentrations. Nielen et al. (1994) selected a subclinical ketosis threshold of 1.2 mmol/L of BHBA based on the data appearing to be skewed around that value.

Ketone utilization by cells is a normal part of dairy cow metabolism. In fact, individual capacity to handle elevated ketones may vary from cow to cow. Despite this limitation, elevations in ketone body concentrations are likely reasonable surrogate indicators of impaired energy metabolism.

Analysis of other metabolites in milk for characterization of metabolic status in early lactation

Measurement of selected milk constituents to monitor the udder health or the metabolic status of cows has attracted much attention in dairy research. The biochemical milk profile has been related to the health status of the cow and, in particular, to imbalances during early lactation (Duffield et al., 2009). Ketone bodies such as acetone, acetoacetate, and BHBA in milk are biomarkers for subclinical ketotic conditions (Geishauser et al., 2000; Enjalbert et al., 2001). On the basis of the close interaction between blood circulation and milk secretion, changes in additional milk metabolites that have yet to be defined can be assumed to reflect the metabolic and health conditions of the cow and the mammary gland. For the study presented here, only major milk components were analyzed, but the study of Klein et al. (2010) showed that there are clear interrelationships between concentrations of specific milk metabolites that act as biomarkers for energy metabolism (acetone and BHBA, α-aminobutyric acid and Glycin) and metabolic status of cows. Metabolic status of cows is rather influenced by the individual ability of the dairy cow to cope with the metabolic stress than production level (Klein et al., 2010).

Before parturition

Before parturition, there were no differences in serum concentration of glucose, NEFA, BHBA, and TB, among treatment groups except during last week before calving. At this time, serum NEFA concentrations were higher for ODM-cows compared to CM-cows

(467 ± 88 µmol/L vs. 216 ± 79 µmol/L, P = 0.025). Despite of the lactating status of CM-cows in late pregnancy, we found moderate concentrations of NEFA and BHBA in blood serum. This may be due to the lower milk yield of these cows of on average 21.2 ± 1.5 kg ECM/d that did not require mobilization of body reserves to meet energy demand. Continuous milking seems to keep the cow metabolically adapted to lactation.

Day of calving

Serum glucose concentration did not differ among groups at day of calving. C-cows and ODM-cows had higher blood NEFA concentrations compared to CM-cows. C-cows had similar blood BHBA concentrations compared to ODM-cows, but higher blood BHBA concentrations compared to CM-cows. TB was highest for ODM-cows compared to C-cows.

CM. The lack of a NEFA and BHBA peak concentration shortly after parturition in this study suggests, that cows in this group did not experience enhanced lipolysis of adipose tissue at the onset of lactation and that the parturition stress was not marked.

ODM. At calving, ODM-cows had blood NEFA concentrations more than twice as high as CM-cows. At this time, ODM-cows are practically in same metabolic situation as C-cows. However, already one week after calving ODM-cows had higher blood glucose concentrations and lower NEFA and BHBA blood concentrations compared with C-cows.

Wk 1 to wk 4 after calving

Blood glucose concentration was lowest for C-cows for the first 2 weeks of lactation (2.62 ± 0.82 mmol/L at wk 1 and 2.72 ± 0.81 mmol/L at wk 2) compared to CM-cows (3.29 ± 0.27 mmol/L at wk 1, P = 0.006 and 3.40 ± 0.30 mmol/L at wk 2, $P < 0.001$) and ODM-cows (3.16 ± 0.5 mmol/L at wk 1, P = 0.032, and 3.29 ± 0.32 mmol/L at wk 2, $P < 0.005$). During the first 4 weeks of lactation, average blood glucose concentration was lowest for C-cows compared to ODM-cows and CM-cows. During the first 4 weeks of lactation, blood serum NEFA concentrations were 44 % lower for ODM-cows and 66 % lower for CM-cows compared to C-cows. Like NEFA, blood BHBA concentrations for C-cows were nearly twice as high as for ODM-cows, and nearly thrice as high as for CM-cows.

CM. Blood serum BHBA concentrations were higher in C-cows compared to CM-cows, because in case of large amounts of NEFA entering the hepatocytes, ketogenesis represents an

alternative metabolic pathway in hepatic mitochondria (Heitmann and Fernandez, 1986). Differences in serum concentration of glucose and BHBA suggest an improved metabolic status for CM-cows in early lactation.

ODM. In our study, reduced milking frequency in early lactation led to a decrease in metabolic imbalances as reflected in findings similar to those described previously (Patton et al., 2006). Accordingly, an increase in milking frequency in early lactation has been reported to accentuate metabolic imbalances (Patton et al., 2006). As expected, NEFA and BHBA blood serum concentrations increased after calving. The concentrations of NEFA and BHBA provide an indication of fat mobilization, and NEFA is often used as an indicator of NEB. The NEFA and BHBA profiles differed between ODM-cows and maintained lower even after the switch to twice daily milking was made in the fifth week of lactation. The blood concentrations of metabolic key parameters of ODM-cows did not differ during the first four weeks of lactation from those of CM-cows, but were lower compared to C-cows. In particular, the drop of serum glucose concentration at wk 2 as observed for C-cows was not observed in ODM-cows. This result is consistent with the positive effects observed on energy status and metabolite profiles. The glucose concentration in cows milked once daily remained higher even after the treatment period. Ketone bodies are known to impair gluconeogenesis (Goff and Horst, 1997). Therefore, these cows have greater glucose levels, probably because of their lower milk production, lower fat mobilization, and increased gluconeogenesis.

Wk 5 to wk 8 and throughout the remainder of lactation

From wk 5 to wk 8 glucose concentration did not differ between groups. NEFA and TB were higher for C-cows than for ODM-cows and CM-cows. Blood serum BHBA concentrations were higher in C-cows than in CM-cows.

After the treatment period, there were no marked differences among groups for glucose, NEFA or BHBA concentration at any time up to 305 DIM.

Due to the close interaction between blood circulation and milk secretion, changes in concentration of milk metabolites may reflect changes in concentrations of the corresponding blood metabolites and, therefore, serve as markers of metabolic and health status in dairy cows (Klein et al., 2010). Additionally, milk reflects the systemic situation of an approximately 12 h period in twice daily milked cows in comparison to the considerable

circadian variation measured for generally accepted key parameters in blood. Future research should focus on how milk can be used as a readily available biofluid to provide a noninvasive source for metabolic analyses.

8.3 Back fat thickness and body condition score

Back fat thickness was not different among treatments at any time point. It declined from month -2 before to month 2 after calving by 24 % for C-cows, by 19 % for ODM-cows and by 25 % for CM-cows. After calving, back fat thickness declined from month 1 to 4 by approximately 40 % for C-cows (21.4 ± 2.3 to 13.1 ± 1.9 mm; $P < 0.001$), and by 17 % for ODM-cows (23.7 ± 3.3 to 19.6 ±3.9 mm; $P = 0.06$), which was reflected by high blood serum NEFA concentrations in early lactation, especially for C-cows in early lactation.

Before calving, BCS did not differ among groups. BCS was lower in the first month of lactation in C-cows compared to ODM- and CM-cows. Decrease of BCS between the first and the third month of lactation was more pronounced in C-cows. A smaller reduction in BCS was found for ODM- and CM-cows. BCS increased from the first to the tenth month of lactation. BCS increased by 24 % from the third to the tenth month of lactation, whereas for ODM-cows increase was only 15 % during the same timeframe. These postcalving BCS changes demonstrated that CM-cows experienced less negative energy balance than cows with a preceding DP, presumably because of greater feed intake. The DMI of CM-cows was found to be similar (Andersen et al., 2005) or even higher (Rastani et al., 2005) compared with 56 d dry cows. Similar to these results, reduction in BCS loss after parturition by omitting or giving a short DP is reported by others (Farries and Hoheisel, 1989; Gulay et al., 2003; Swanson, 1965). ODM-cows lost less body reserves during early lactation as reported elsewhere (Rémond et al., 2002).

8.4 Reproduction

Reproductive efficiency in high-yielding dairy cows has decreased in recent decades (Royal et al., 2000; Butler, 2003; Evans et al., 2006). Intensive genetic selection for increased milk production has led to remarkable improvements in milk yield per cow, but has also been associated with a worldwide decline in dairy cow fertility. It has been demonstrated that a negative correlation between genetic merit for milk yield and reproductive performance exists (van Arendonk et al., 1989; Pryce et al., 1997). The severity and duration of metabolic imbalance experienced in early lactation affects the postpartum interval to first ovulation and

has a detrimental effect on subsequent likelihood of conception (Butler and Smith, 1989). A delay in the onset of ovulatory ovarian activity limits the number of estrous cycles before breeding, reducing the likelihood of conception and increasing the number of days open (Butler, 2003).

Due to the failure of power, no differences were observed for any reproductive parameters among treatments. Neither milking frequency during early lactation nor management during DP had any effects on intervals to conception, services per conception or overall pregnancy rate ($P > 0.05$, Table 7). Patton et al. (2006) found that the interval to first ovulation was shorter for ODM-cows than for cows milked three times daily for the first four weeks of lactation. Beam and Butler (1999) reported that cows with a shorter interval to energy balance nadir and a more positive energy balance during the first 3 weeks of lactation had a reduced interval to first ovulation. Furthermore, indicators of energy status, e.g. time of energy balance nadir, body weight loss, body condition score, and body condition score loss, have been related to reproductive performance. In our study, the majority (87 %) of cows had resumed cyclicity by 40 d, and conception rate and final pregnancy rate did not differ due to treatment, despite the earlier onset of cyclicity for the ODM- and CM-cows measured by progesterone. Grummer (2007) found that shortening or eliminating the DP may be a more successful approach to improving reproductive efficiency than diet manipulation.

Table 7. Parameters of reproduction (means ± SEM) of all treatment groups

	Treatment group[1]			P-value[2]		
	C	ODM	CM	C vs. ODM	C vs. CM	CM vs. ODM
Dry period length	69 ± 4	66 ± 2	-			
Open days	64 ± 6	58 ± 4	58 ± 5	0.47	0.49	0.96
Calving to conception interval	112 ± 18	123 ± 20	89 ± 13	0.66	0.34	0.16
Services per conception	3.0 ± 0.8	2.7 ± 0.5	2.0 ± 0.3	0.74	0.23	0.38
Pregnancy rate at first insemination	0.33	0.16	0.5	0.49	0.22	0.62
DIM at drying off[3]	342 ± 26	303 ± 7	314 ± 13	0.11	0.22	0.62
Calving interval	395 ± 21	390 ± 13	380 ± 13	0.81	0.50	0.68

[1] C = 56 d dry period and twice daily milking after parturition (n = 12), ODM = 56 d dry period and once daily milking for the first 28 d of lactation (n = 12), CM = 0 d dry period and continuously milking twice a day throughout lactation (n = 12).

[2] Means are different for $P < 0.05$.

[3] DIM = days in milk when C-cows, ODM-cows and CM-cows were dried off 56 d before expected date of calving. This calving was the onset of the lactation that followed our study.

The onset of luteal activity as measured by milk progesterone (Table 8) was similar among treatment groups. Intraassay CV for the EIA is 5.3 % and Interassay CV is 10.58 % according to Prakash et al. (1988).

Table 8. Occurrence of the first ovulation (percent of cows) measured by progesterone concentration in skim milk samples of all experimental groups in subject to three timeframes (till 28, 56 and 100 DIM)

	Treatment group[1]			P-value[2]		
	C	ODM	CM	C vs. ODM	C vs. CM	CM vs. ODM
1 to 28 DIM[3]	8.3	16.7	33.3	0.45	0.11	0.29
29 to 56 DIM	58.3	50.0	41.7	0.80	0.90	0.41
56 to 100 DIM	33.3	33.3	25.0	0.92	0.82	0.91

[1] C = 56 d dry period and twice daily milking after parturition (n = 12), ODM = 56 d dry period and once daily milking for the first 28 d of lactation (n = 12), CM = 0 d dry period and continuously milking twice a day throughout lactation (n = 12).

[2] Means are different for $P < 0.05$.

[3] DIM = days in milk.

The use of exogenous PGF2α was started in case of anestrus first 6 weeks after calving. Most noticeably may be the ratio of cows with first ovulation during the first 28 days of lactation.

Aspects of health

We failed to detect differences in the incidence of health events among the three groups. The following table (Table 9) shows occurrence of health disorders during study.

Table 9. Occurence of diseases (number of events) in the treatment groups during study.

	Treatment group[1]			*P*-value[2]
Diagnosis	C	ODM	CM	group
Mastitis[3]	5 (3, 15, 109, 248, 263)	2 (61, 118)	3 (-7, -5, 60)	0.47.
Retained placenta	2 (1)	5 (1)	1 (1)	0.61
Hypocalcemia	0	1 (1)	0	0.56
Ketosis	3 (13, 31, 32)	0	0	0.18
Lameness	2 (33, 53)	2 (86,88)	0	0.21

[1] C = 56 d dry period and twice daily milking after parturition (n = 12), ODM = 56 d dry period and once daily milking for the first 28 d of lactation (n = 12), CM = 0 d dry period and continuously milking twice a day throughout lactation (n = 12).

[2] Means are different for $P < 0.05$.

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

Economical aspects

CM. Because many milk pricing plans and quota systems pay the dairy producer not only for the quantity of milk produced, but also for the composition of that milk, the effects of CM on milk compositon are important. Further, changing milk compositon could have implications at the consumer level and poses issues regarding salable milk. Changes in colostrum quality following omission of the DP are also important due to the importance of good quality colostrum on calf morbidity and mortality. Additional financial benefit could accrue because omitting DP, if proven to be equally effective, would allow for changes in nutritional management that could reduce the amount of stress and the incidence of disorders that dairy cows experience postpartum as they transition into a copious lactating status.

ODM. A reduced peak yield is in practice associated with a reduced lactational yield. Since the last decade in German production systems, the focus was more and more on healthy and metabolic solid cows, with a milk yield that does not exorbitantly exceeds the nutritional requirements. Due to this a slightly reduced peak yield (compared to a peak yield of about 60 kg/d and more) may be favorable for farmers. Peak yield occuring not in the second but in the fifth or sixth week of lactation when DMI is increased markedly would be the second tool

for an economical milk production system with healthy and durable dairy cows. A delayed time of peak yield may be economically more favorable for the farmer if, on the one hand, costs can be saved due to a reduced incidence of health disorders in early lactation, and, secondly, the reduced milk yield in early lactation is compensated by a higher milk yield in late lactation due to a better persistency. Results of the study presented here reveal evidence that once daily milking in early lactation seems to be a tool to achieve this objective.

8.5 Relative quantification of mRNA levels of candidate genes

Reference genes used in liver or muscle samples

The two genes tested to be used as a reference in the relative expression calculations were GAPDH and Histone for liver tissue samples, and Histone and Ubiquitin in muscle tissue samples. In each tissue, both had consistent level of expression across all time points; therefore these genes were used as reference for calculating relative expression.

Expression of GLUT4 in musculus semitendinosus

Under hyperglycemic conditions, insulin is secreted from the pancreas and increases the translocation of cytoplasmatic Glut4 vesicles towards and into the plasma membrane, thus stimulating the transport of glucose into the cells (Kahn, 1996 in Dühlmeier, 2007). Glut4 was identified as the main glucose transporter in fat and muscle cells (James et al., 1989). Insulin triggers the movement of the sugar transporter that is found in these cells from an intracellular store to the plasma membrane (Bryant et al., 2002).

In our study, Glut4 mRNA levels in skeletal muscle did not differ among groups, but changed over time in all groups. Transcript abundance was higher at wk 6 compared to calving (1.69 fold) and similar to calving at wk 16 (Table 10). This is in contrast to findings of Komatsu et al. (2005), who did not detect changes of mRNA level in muscle tissue among the various stages of lactation, e.g. peak, late and non lactating state. One reason for this discrepance may be the fact that in the study of Komatsu et al. (2005) the whole pregnancy lactation cycle was included, while in our study the first 16 weeks of lactation were in focus. The most metabolic adaptation to lactation occurs within the first third of lactation. While Komatsu et al. (2005) compared peak- and late lactating cows, samples in our study were obtained at time points accompanied by daily milk yield more than 33 kg ECM/d. Peak lactating cows of Komatsu et al. (2005) had a daily milk yield of 26.6 kg/d. It is considered that insulin resistance increases during lactation (Rose et al., 1997) and an insulin deficiency with regard to glucose utilization has been reported in lactating goats (Debras et al., 1989).

Insulin-resistance in peripheral tissue may be caused by inhibition of Glut4 translocation to the cytomembrane, not by inhibition of Glut4 expression or Glut4 content in general (Komatsu et al., 2005). In humans suffering from insulin-independent diabetes mellitus, muscle cell Glut4 contents are like those of healthy persons, but the ability of insulin to provoke Glut4 translocation into the myocyte plasma membrane is interrupted (Zierath et al., 1998). In our study, mRNA levels of Glut4 were increased at wk 16 compared to calving, for ODM-and C-cows, but not for CM-cows. A possible explanation for these findings is that the activity of insulin did not increase the Glut4 translocation in myocytes in CM-cows, but in C- and ODM-cows. For this reason, the bovine insulin-independent glucose transport via Glut1 may compensate for the smaller insulin-induced Glut4 translocation.

Furthermore, in our study muscle tissue samples were obtained from musculus semitendinosus which was metabolically characterized to be a glycolytic muscle by Dühlmeier et al (2005). Glut4 contents were 3.0 times lower in musculus semitendinosus (glycolytic) than in musculus masseter and diaphragm (oxidative) of bovine skeletal muscle. They found higher Glut1 contents and lower Glut4 contents in glycolytic muscles. However, we detected higher mRNA levels of Glut4 than of Glut1 in (bovine glycolytic) skeletal muscle, but these findings were exclusively based on transcript abundance of these genes related to glucose transport quantified by qRT-PCR. Although physiological mechanisms cannot be completely explained through RNA abundance, protein expression data have been extensively demonstrated to be largely concordant with gene transcription profiling (Mootha et al., 2003). Higher Glut1 and a lower Glut4 level in glycolytic muscles of lactating cows could be an effective mechanism for maintaining a basic glucose supply, which prevents insulin-sensitive excessive glucose transport into these muscle cells, thus facilitating a preferential insulin-independent glucose supply for the mammary gland. Insulin-dependent glucose transport is mediated through changes in compartmentalization of Glut4 within cells and Glut4 gene expression. The fact that Glut4 is not expressed in bovine mammary epithelial cells supports a lack of insulin-responsive glucose uptake by mammary tissue (Zhao and Keating, 2007). Dühlmeier et al. (2007) found that Glut1 may be of greater importance in the whole body glucose utilization in herbivores with forestomachs than in monogastric omnivores. It would be worthwhile to test the hypothesis of such an interaction between lactation and glycolytic muscle in further experiments on cows.

Expression of Glut1 in musculus semitendinosus

Insulin independent glucose transporter Glut1 is expressed in many tissues, including brain, kidney, and mammary gland (Burant et al., 1991). It is generally assumed that Glut1 accounts for basal muscle glucose demands (Dühlmeier et al., 2005), as it is predominantly located in the muscle cell plasma membrane. Komatsu et al. (2005) could not detect Glut1 protein or mRNA in muscle of lactating or non-lactating cows, but in the mammary gland. There they could detect no difference between Glut1 protein and mRNA abundance at peak and late lactation. In dry cows, Glut1 protein and mRNA were barely detectable in the mammary gland (Komatsu et al., 2005). However, qRT-PCR analysis showed that mRNA levels of bovine Glut1 did not change from calving up to wk 16 of lactation in skeletal muscle in our own study (Table 10). It is well known, that Glut1 mRNA in mammary gland increases from at least 5-fold to several hundred-fold from late pregnancy to early lactation and peaks during lactation (Zhao et al., 2007). However, Glut1 mRNA levels in skeletal muscle tissue remained unchanged at wk 6 and 16 of lactation, and was unchanged by DP management or milking once daily in early lactation. In our study, transcript abundance of Glut1 seemed to be lower than expression level of Glut4. That's in contrast to findings of Dühlmeier et al. (2005), who found higher levels of Glut1 content than of Glut4 content in bovine glycolytic muscles. Data from the study of Dühlmeier et al. (2005) strongly indicate that Glut1 is the predominant glucose transporter of bovine glycolytic skeletal muscle. However, Dühlmeier et al. (2005) indicated that fluctuations of plasma insulin and glucose levels seem to require a glucose transport pathway partly independent from an insulin-stimulated uptake to maintain glucose levels in large locomotor muscles of dairy cows. In ruminants, the insulin-independent glucose utilization is of greater importance than in monogastric omnivores, and it may compensate at least in part of the impaired in vivo insulin sensitivity in adult ruminants (Debras et al., 1989). Most important of our findings is that transcript abundance of Glut1 was unchanged by improved metabolic status triggered by milking regimes in late gestation and early lactation.

Expression of LDH in musculus semitendinosus

Pyruvate is a central intermediate for energy metabolism, and partitioning of pyruvate metabolism can greatly affect energy supply to the cell. Instead of accumulating inside the muscle cells, lactate produced by anaerobic fermentation is taken up by the liver. If muscle activity has stopped, the glucose is used to replenish the supplies of glycogen through glycogenesis. So LDH is part of the energy metabolism of cells.

No previous studies known by the authors have investigated the expression of LDH related to pyruvate metabolism in bovine skeletal muscle. In our study, transcript abundance of the gene encoding LDH was quantified. With the onset of lactation, mRNA levels of LDH in muscle were down-regulated compared with later times of lactation (wk 16), especially for C- and ODM cows. But we could not detect differences for transcript abundance among treatments (Table 10). So, transcripts of LDH constantly rose from calving up to wk 16 in control and treatment groups (3.6 fold for control animals and 2.7 fold for ODM and 2.6 fold for CM-cows, Figure 2). Higher transcript abundance at later times of lactation (wk 16) compared to earlier times (wk 6) or compared to calving may suggest, that after the first 100 days of lactation the metabolic state of peripheral tissue like skeletal muscles is more stable than at earlier times.

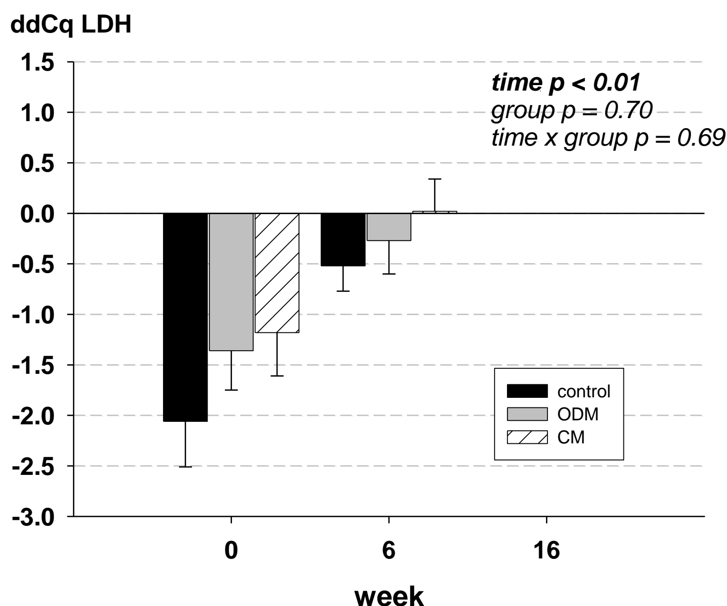

Figure 2. Relative expression of LDH in muscle of cows with different metabolic status triggered by dry period management and milking frequency in early lactation. Metabolic situation was determined by metabolic parameters in blood serum and by production traits. Cows of control group (control ■) were dried off 56 d before expected day of calving and milked twice daily after parturition. Cows of the second group were also dried off 56 d before expected calving, but these cows were milked only once daily for the first 28 days of lactation (ODM ▭). Cows of the third group were not dried off in late gestation, but were milked continuously (CM ▨). Data are expressed as the fold change compared with the expression at wk 16 of lactation..

Expression of ACADvl and EnoylCoA in musculus semitendinosus

ACADvl and EnoylCoA are two enzymes involved in catalyzing the first step of the β-oxidation of long chain fatty acids. We found effects of group on EnyolCoA mRNA levels and effects of time on mRNA levels of ACADvl, but fatty acid metabolism in muscle was not markedly influenced by treatments, as shown by similar and unchanged mRNA levels of these two enzymes (Table 10). However, C- and ODM-cows exhibited an increased transcript abundance of EnoylCoA compared to CM-cows at time of calving (1.8 and 1.6 fold for C and ODM-cows, respectively). At wk 6, expression of EnoylCoA was highest for C-cows compared to ODM and CM-cows suggesting that these cows appropriately adjust their metabolism to support increased nutrient requirements of early lactation. At wk 16, transcript abundance in muscle of C-cows was higher compared to CM-cows, but not to ODM-cows. These differences may be caused by the improved metabolic status and the lower milk yield of these cows, which did not need the utilization of body fat as energy fuel in the range as C-cows did.

Expression of IRβ in musculus semitendinosus

The insulin receptor (IRβ) is a transmembrane receptor that is activated by insulin. Transcript abundance of Insulin receptor beta showed marked variation among groups and across time (Table 10). Around calving, mRNA levels were higher in ODM- and CM-cows compared to C-cows. Surprisingly the higher levels of mRNA encoding for the IRβ in ODM-cows at calving were not expected whereas for CM-cows higher levels of IRß might have caused an improved metabolically situation as determined by a higher blood glucose concentration. These cows easily adapt to lactation perhaps by an efficient translocation of glucose into cells by the insulin dependent glucose transporter Glut4. For all groups, transcript abundance of IRβ decreased from calving to wk 6 (1.8 fold) and 16 (2.6 fold) (Figure 3). However, mRNA levels of Glut4 in muscle were down-regulated at wk 6 and up-regulated at wk 16 of lactation. That result emphasizes the widely accepted doctrine that in ruminants early lactation is characterized by distinctive metabolic changes including the development of insulin resistance in skeletal muscles and adipose tissue (Bell and Bauman, 1997) to maintain mammary gland lactose production. In contrast, Dühlmeier et al (2007) suggested that this insulin resistance is rather due to a decreased Glut4 expression than a downregulation of insulin receptors as suggested in our study. Please remember that concurrently transcript of Glut4 mRNA abundance did not decrease from calving to wk 16. Sano et al. (1991) found that during late gestation and early lactation, lowered responsiveness and sensitivity of extra

hepatic tissues to insulin facilitate partitioning of nutrients for the rapidly growing fetus and the mammary gland. Significant changes of mRNA levels of IRβ suggest that ODM and CM may improve the basal glucose supply in musculus semitendinosus. However, the question is, if the major site of differences concerning the insulin sensitivity occurs with the insulin receptor or with GLUT4 that recycles between membrane structures and an intracellular tubulovesicular pool.

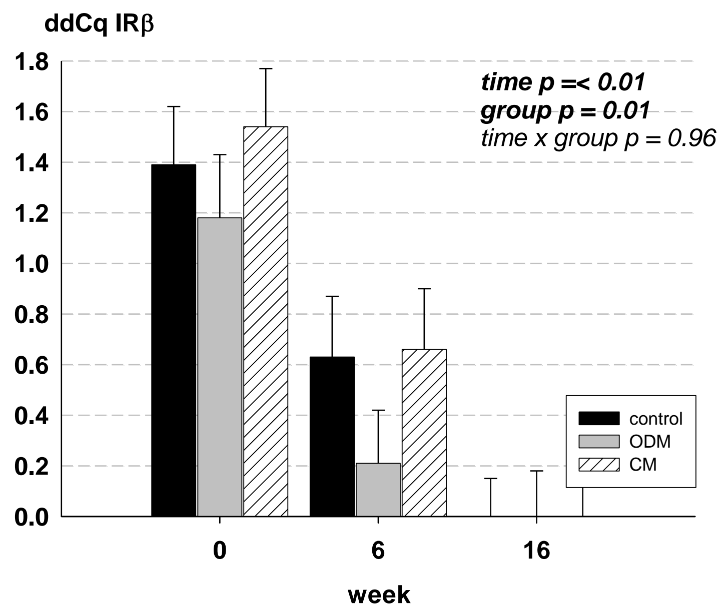

Figure 3. Relative expression of IRβ in muscle of cows with different metabolic status triggered by dry period management and milking frequency in early lactation. Metabolic situation was determined by metabolic parameters in blood serum and by production traits. Cows of control group (control ■) were dried off 56 d before expected day of calving and milked twice daily after parturition. Cows of the second group were also dried off 56 d before expected calving, but these cows were milked only once daily for the first 28 days of lactation (ODM ▭). Cows of the third group were not dried off in late gestation, but were milked continuously (CM ▨). Data are expressed as the fold change compared with the expression at wk 16 of lactation.

Expression of ACTA1 in musculus semitendinosus

Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells. Transcript abundance of Acta1 did not differ among groups but was increased at wk 16 compared to day of calving for all groups (Table 10). The increase of mRNA levels of muscle protein expression was not unexpected despite of the observed mobilization of body reserves during the first three months of lactation. At wk 16, cows of all groups replenish body reserves as time of peak of milk yield lags behind. The higher transcript abundance at wk 16 compared to day of calving was significant for all groups (3.8 fold for C-cows, 2.5 fold for ODM- and 3.3 fold for CM-cows,

Figure 4) and showed that catabol status was altered to anabol status. However, these results might only be valid for the musculus semitendinosus as muscle types respond differently to metabolic status, as has been shown earlier by biochemical approaches (Hoquette, 2000).

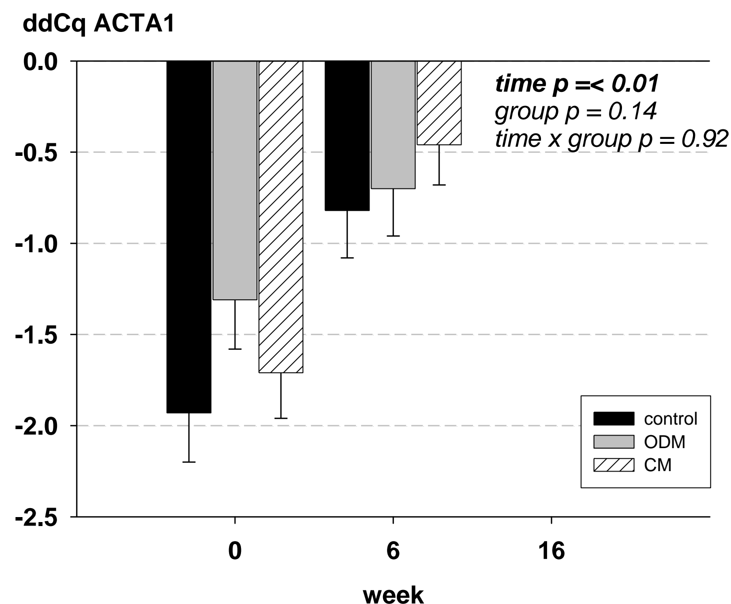

Figure 4. Relative expression of ACTA1 in muscle of cows with different metabolic status triggered by dry period management and milking frequency in early lactation. Metabolic situation was determined by metabolic parameters in blood serum and by production traits. Cows of control group (control ■) were dried off 56 d before expected day of calving and milked twice daily after parturition. Cows of the second group were also dried off 56 d before expected calving, but these cows were milked only once daily for the first 28 days of lactation (ODM ▭). Cows of the third group were not dried off in late gestation, but were milked continuously (CM ▨). Data are expressed as the fold change compared with the expression at wk 16 of lactation

Expression of CPT 1A in liver

Uptake of NEFA into hepatic mitochondria, where oxidation occurs, is regulated by CPT 1A. Activity of CPT 1A and sensitivity of CPT 1A to malonyl-CoA and methylmaloyl-CoA inhibition during different physiological and pathological states have been investigated by Dann and Drackley (2005). Aiello et al. (1984) showed that CPT 1A activity in dairy cows was higher at d 30 than at d 60, 90, or 180 of lactation. The elevated activity of CPT 1A in early lactation was associated with higher rates of gluconeogenesis and ketogenesis, possibly due to a greater negative energy balance in early lactation. Similar to Aiello et al. (1984), Dann et al. (2006) showed that CPT 1A activity peaked at 1 DIM and decreased at 21 and 65 DIM. Mizutani et al. (1999) compared CPT 1A activity of cows in early (0 to 110 DIM), mid (111 to 220 DIM), and late (220 DIM) lactation and found no difference among stages of lactation. Energy status of the cows at the various stages was not reported by Mizutani et al.

(1999); energy balance among groups may have been similar and therefore no difference in CPT 1A activity would be expected. That's in contrast to our findings where mRNA levels of CPT 1A were higher for CM-cows than for C- and ODM-cows especially before calving (Table 11). That's may be a result of an improved metabolic status in early lactation for these cows. Figure 5 shows the expression levels for each group relative to one week before calving. We found that transcript abundance was down-regulated for CM-, but not for C- and ODM-cows. Oxidation of NEFA and other substrates provides ATP needed for gluconeogenesis. For CM-cows, lower serum NEFA levels combined with reduced mRNA levels of CPT 1A relative to calving compared with C-cows and ODM-cows suggest that there was no need to use body fat as energy fuel in CM-cows. The influx of NEFA from the adipose tissue mobilization 1d after parturition was not in a range that up-regulates the carnitine palmitoyltransferase system, likely be due to the absence of elevated NEFA concentration around calving.

Loor et al (2005) observed increased mRNA expression of CPT 1A 1d after parturition compared to prepartum. That's in accordance to results we found for C-cows for the first 8 weeks of lactation and for ODM-cows for the first 4 weeks of lactation. Surprisingly, ODM-cows had the lowest energy corrected milk yield during this time compared with the other groups due to once daily milking. CPT 1A mRNA levels increased for ODM-cows in this group two weeks after calving.

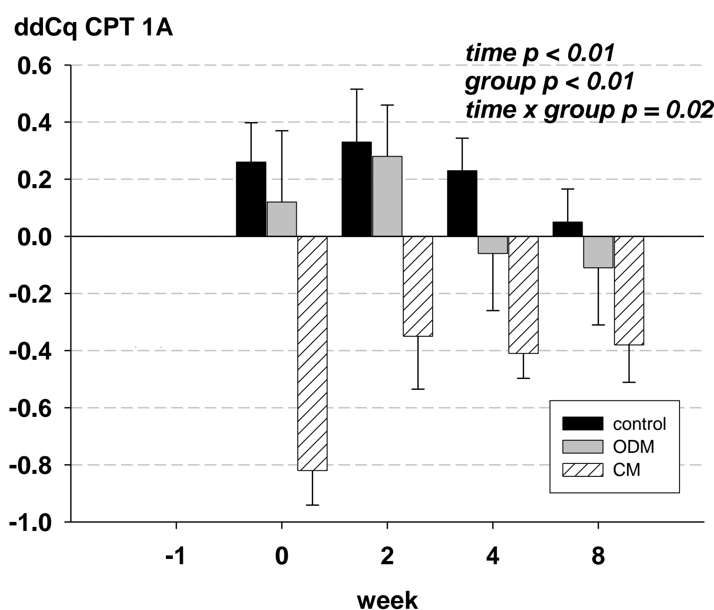

Figure 5: Relative expression of CPT 1A in muscle of cows with different metabolic status triggered by dry period management and milking frequency in early lactation. Metabolic situation was determined by metabolic parameters in blood serum and by production traits. Cows of control group (control ■) were dried off 56 d before expected day of calving and milked twice daily after parturition. Cows of the second group were also dried off 56 d before expected calving, but these cows were milked only once daily for the first 28 days of lactation (ODM ▭). Cows of the third group were not dried off in late gestation, but were milked continuously (CM ▨). Data are expressed as the fold change compared with the expression at wk -1 relative to calving.

Expression of PEPCK in liver

It is the major criterion of glucose metabolism in ruminants that the majority of dietary carbohydrate is fermented to volatile fatty acids in the rumen. Thus, gluconeogenesis represents a major metabolic activity required to maintain glucose homeostasis and is the primary source of glucose for maintenance and productive processes. In our study, levels of PEPCK mRNA were elevated after calving compared to precalving more pronounced in CM-cows. For these cows, we quantified markedly higher levels one week before calving and at wk 2 after calving compared to C- and ODM-cows. At other time points, there were no significant differences with respect to milking regimen (Table 11). Similar findings in the literature suggest differences in gluconeogenic enzyme regulation of PC and PEPCK in the transition cow (Greenfield et al., 2000). We found the strongest and rapidly increase of PEPCK transcript abundance for CM-cows around calving, which may suggest that these cows were able to cover demands for gluconeogenesis. We found constantly rising mRNA levels of PEPCK up to wk 8 for C- and ODM-cows, whereas for CM-cows already at wk 4 mRNA levels of PEPCK turned to levels lower than one week precalving. Greenfield et al. (2000) observed an increased abundance of PEPCK mRNA following the transition period,

but it increased only slowly and only by about 50 % in samples at day 28 postpartum compared with values compared to day -28 prepartum. In our study, mRNA expression was highest for CM-cows before calving and at wk 2 postpartum compared to C- and ODM-cows. Especially C- and ODM-cows showed lower transcript abundance of PEPCK after calving with significant lower levels at wk 2. This time was associated with the highest daily milk yield for C-cows (Schlamberger et al., 2010). This observation could imply an inhibition of PEPCK mRNA expression in hypoglycemic and ketotic cows, possibly by insulin (Hanson and Reshef, 1997). The increasing PEPCK mRNA level with time after calving may represent the incremental glucose demand for milk production (Greenfield et al., 2000). For C-cows, transcript abundance rosed 1.65 fold, but 2.0 fold for ODM and 2.94 fold for CM-cows at wk 2 compared to time of calving. For these latest cows, the most obvious up-regulation of gluconeogenesis regulating enzyme occurred and may be followed by an enhanced gluconeogenesis and therefore an improved metabolic status. Gene expression of PEPCK may be affected by propionate as well as lactate and amino acid supply and was therefore not different at all times among groups, but changed significantly over time.

Expression of GPAM in liver

In our study, GPAM mRNA levels were down-regulated with the onset of lactation in all groups, but up-regulated earlier during succeeding lactation in CM-cows than in C-cows. This is in accordance to Loor et al. (2005) who detected differential expression of glycerol-3-phosphate acyltransferase at specific times during the DP and through peak lactation. Concerted down-regulation of GPAM around parturition channels NEFA toward fatty acid oxidation. Differences among groups are obvious at wk 8 of lactation, when CM- and ODM-cows have already higher mRNA levels compared to precalving (wk-1), although mRNA levels of GPAM were still reduced compared to wk -1. Glycerol released from adipose tissue during lipid mobilization also represents recycling of glucose, but this recycling occurs over the course of a lactation cycle, not on minute-to-minute basis as for lactate (Drackley et al., 2001a). For that reason, we found significant changes over time for GPAM mRNA levels in hepatic tissue, especially the higher mRNA levels of this enzyme at wk 8 compared to day of calving showed that glycerol may be an important gluconeogenic precursor as the cow adapts to lactation. During extensive mobilization of adipose triglyceride of approximately 3.2 kg/d, glycerol may provide maximally 15 to 20 % of the glucose demand at 4 d postpartum (Bell et al., 1995). Accordingly dietary circumstances and particulary the metabolic status in early lactation that decrease or increase the amount of adipose tissue mobilized will increase or

decrease the contribution of glycerol to gluconeogenesis during early lactation. Furthermore up-regulation of GPAM is a necessary response in mice to accommodate the greater influx of NEFA into liver, i.e. there is an imbalance between fatty acid oxidation and lipid synthesis. The improved metabolic status in CM-cows is reflected by higher mRNA levels of these enzymes in early lactation compared to C- and ODM-cows (Table 11).

Expression of ACADvl in liver

Transcript abundance of ACADvl rose from one week precalving to time of calving 2.04 fold and from one week precalving to wk 2 of lactation 0.78 fold for C-cows and 2.6 fold and 0.75 fold, respectively, for ODM-cows ($P < 0.01$, Figure 6). For CM-cows, transcript abundance returned to levels similar to precalving at wk 2. Higher mRNA levels of GPAM channels NEFA toward fatty acid oxidation as described above. This was accompanied by increased mRNA abundance of ACADvl in hepatocytes for cows of all groups with the onset of lactation (Table 11). Milking cows continuously did not really change transcript abundance of this enzyme of fatty acid oxidation in liver. Blood serum NEFA concentration differed at wk 2 and wk 4 between groups after calving and was in a critical range only at calving for C-cows and ODM-cows and at wk 2 only for C-cows. Serum NEFA concentration is followed by an increased β-oxidation which was reflected by expression of genes encoding for this metabolic pathway.

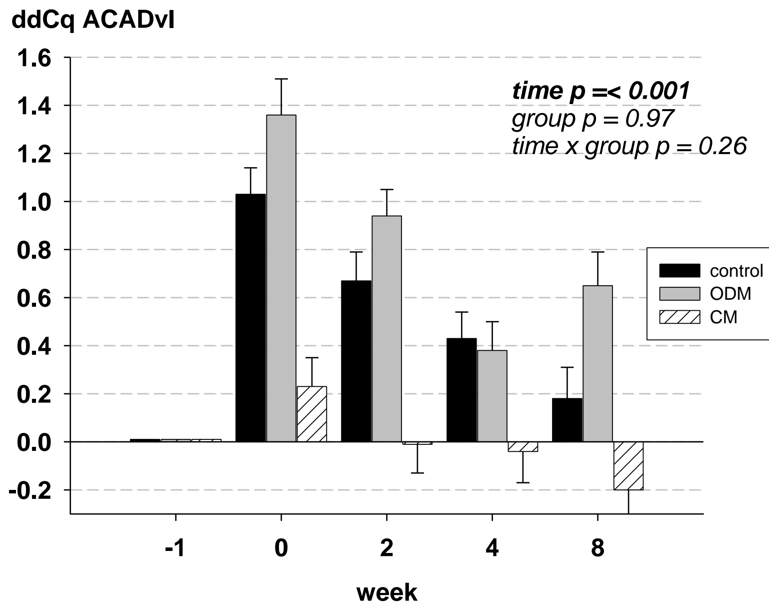

Figure 6. Relative expression of ACADvl in muscle of cows with different metabolic status triggered by dry period management and milking frequency in early lactation. Metabolic situation was determined by metabolic parameters in blood serum and by production traits. Cows of control group (control ■) were dried off 56 d before expected day of calving and milked twice daily after parturition. Cows of the second group were also dried off 56 d before expected calving, but these cows were milked only once daily for the first 28 days of lactation (ODM ▭). Cows of the third group were not dried off in late gestation, but were milked continuously (CM ▨). Data are expressed as the fold change compared with the expression at wk -1 relative to calving.

Expression of CS in liver

The CS catalyzes the condensation of acetyl-CoA and oxalacetate to form citrate, which represents the first step in the citric acid cycle (Voet and Voet, 2004). Transcript abundance revealed no variation for CS subjected either to time during lactation or to group. Surprisingly neither C-cows neither ODM- or CM-cows did show any changes in mRNA encoding for CS (Table 11), suggesting that an increase in the activity of the citric acid cycle in response to an increase in acetyl-CoA did not occur as shown by higher levels of BHBA for C-cows at wk 2 and 4 after calving compared to ODM- and CM-cows. We suggest that the lack of difference of mRNA expression of CS among groups and over time in our study showed that for this enzyme no functional regulation was necessary to adapt to metabolic stress. Perhaps other mechanisms work to adapt to requirements of lactation.

Expression of TAT in liver

The gene tyrosin-amino-transferase catalyzes the decomposition of L-tyrosine. At the end of this reaction where two deoxygenases are necessary, fumarate and acetoacetate were liberated, which can enter into the citric acid cycle. Despite the observed adaptations to

lactation for genes of gluconeogenesis or β-oxidation, we did not find marked changes in transcript abundance of TAT in liver for all groups (Table 11). Noticeably is the elevated transcript abundance of TAT for ODM- compared C-cows (2.23 fold) at wk 4. Perhaps effects of once daily milking were stronger after these four weeks of lactation resulting in the lowest daily milk yield compared to C- and CM-cows. In general, breakdown of protein did not occur even in C-cows where daily milk yield exceeded the nutrient input.
In the present study we measured the abundance of mRNA sequences that encode for major proteolytic systems. In lactating sows, a greater mRNA abundance for key components of the ubiquitin-mediated, ATP-dependent pathway was associated with increased muscle protein catabolism (Clowes et al., 2005, Chibisa et al., 2008).

Expression of CTSLb in liver

The protein encoded by Cathepsin Lb (CTSLb) is lysosomal cysteine proteinase that plays a major role in intracellular protein catabolism. Transcript abundance of CTSLb did not change over time, but mRNA levels were different among groups, showing higher levels for ODM-cows compared to C- and CM-cows before calving and at wk 2 and 4 after calving (Table 11). The elevated transcript abundance precalving for ODM-cows is doubtful as both ODM- and C-cows were dried off in late gestation, so there is no reason available why this gene encoding intracellular protein metabolism was more intense prepartum in ODM-cows compared to C- and CM-cows. Most obvious are higher mRNA levels of CTSLb at wk 2 (1.7 fold for ODM- and 2 fold for CM-cows) and wk 4 after calving (1.8 fold for ODM- and 1.6 for CM-cows, respectively) compared to C-cows.

Expression of GLUT2 in liver

Glut2 is one of the facilitative glucose transporters. It mediates the uptake and release of glucose by hepatocytes based on a high-capacity low affinity transport and release of absorbed glucose across the basolateral surface of epithelial cells of the kidney and small intestine. It is thought to be involved in the regulation of insulin secretion from β-cells. In liver samples of the study presented here, we could not detect any changes of Glut2 expression among groups and across time (Table 11). During the first 8 weeks of lactation we found a tendency for a lower transcript abundance compared to precalving. Glucose supply to hepatocytes is an important condition for functionality of these cells especially gluconeogenesis. More important is the release of this hepatic glucose into the bloodstream. Lower serum glucose concentrations of C-cows especially at wk 2 of lactation seem to be

more the result of a dramatic supply of glucose for lactose synthesis in the mammary gland than an impaired availability of glucose perhaps due to restricted hepatic glucose release induced by downregulation of GLUT2. The unchanged mRNA levels of GLUT2 provide sufficient glucose supply to hepatocytes even in early lactation.

Table 10. mRNA abundance[1] (log_2) of Glut1, Glut4, LDH, ACADvl, EnoylCoA, IRβ and ACTA1 in muscle of dairy cows managed with different milking regimen in early lactation or milked continuously

			Group[2]				ANOVA	
Variable	Week[3]	C	ODM	CM	SEM	Milking regimen	time	Milking regimen x time
Glut1	0	2.39	2.59	2.77	0.19	0.1078	0.06	0.1355
	6	2.15	1.71	2.94	0.18			
	16	1.70	2.19	2.12	0.19			
Glut4	0	6.70	6.44	6.21	0.17	0.0742	0.0103	0.8556
	6	7.23	7.44	6.94	0.16			
	16	7.04	6.82	6.22	0.18			
LDH	0	9.08*	9.22*	8.99*	0.16	0.70	<0.01	0.6933
	6	10.41	10.30	10.39	0.15			
	16	10.92	10.67	10.35	0.16			
ACADvl	0	5.72	5.99	5.60	0.14	0.38	0.05	0.70
	6	6.04	5.76	5.81	0.14			
	16	5.68	5.41	5.16	0.14			
EnoylCoA	0	3.81[a]	3.66[a]	2.96[b]	0.14	<0.01	0.84	0.58
	6	4.12[a]	3.34[b]	3.10[b]	0.14			
	16	3.99[a]	3.47[ab]	2.81[b]	0.15			
IRβ	0	3.78*[a]	4.99*[b]	4.53*[ab]	0.23	0.01	<0.01	0.96
	6	3.02	4.02	3.65	0.23			
	16	2.39[a]	3.81[b]	2.99[ab]	0.24			
ACTA1	0	11.56*	11.05*	11.37*	0.27	0.14	<0.01	0.92
	6	12.67	11.66	12.62	0.26			
	16	13.49	12.36	13.08	0.27			

[1] Data are presented as means ± SEM subtracted from the arbitrary value 10 (10 - ΔCq). Thus, a high ΔCq resembles high transcript abundance (Livak and Schmittgen, 2001). An increase of one ΔCq represents a two-fold increase of mRNA transcripts.

[2] Groups are C = 56 d dry period and twice daily milking after parturition (n = 12), ODM = 56 d dry period and once daily milking for the first 28 d of lactation (n = 12), CM = 0 d dry period and continuously milking twice a day throughout lactation (n = 12).

[3] Weeks are relative to calving.

[a, b] Means within a row with different superscripts differ ($P < 0.05$). Means with * indicate a difference within one group compared to wk 16 ($P < 0.05$).

Table 11. mRNA abundance[1] (log $_2$) of CPT 1A, GPAM, PEPCK, TAT, Glut2, CS, CTSLb and ACADvl in liver of dairy cows managed with different milking regimen in early lactation or milked continuously

		Group[2]				ANOVA		
Variable	Week[3]	C	ODM	CM	SE	Milking regimen	time	Milking regimen x time
CPT 1A	-1	4.34[a]	4.44[a]	5.36[b]	0.09	0.002	<0.001	0.0147
	0	4.56	4.35	4.49*	0.09			
	2	4.66	4.81	5.00	0.09			
	4	4.56[ab]	4.38[b]	4.86[a]*	0.10			
	8	4.37[a]	4.20[a]	4.89[b]*	0.10			
GPAM	-1	5.59[a]	5.18[b]	5.75[a]	0.08	<0.001	<0.001	0.54
	0	4.98[a]*	4.52[b]*	5.19[a]*	0.08			
	2	5.46[ab]	5.14[a]	5.53[b]	0.08			
	4	5.21[a]*	5.29[ab]	5.65[b]	0.07			
	8	5.33[ab]	5.17[a]	5.64[b]	0.08			
PEPCK	-1	5.78[a]	5.71[a]	7.79[b]	0.47	0.092	0.001	0.57
	0	5.06	4.39	6.48	0.45			
	2	5.79[a]	5.39[a]	8.04[b]	0.45			
	4	5.95	5.72	7.73	0.45			
	8	6.01	5.90	6.17	0.46			
TAT	-1	7.48	7.33	6.93	0.22	0.67	0.85	0.08
	0	7.44	7.82	7.08	0.21			
	2	7.82	7.26	7.19	0.20			
	4	6.88[a]	8.04[b]	7.29[ab]	0.21			
	8	7.41	7.32	8.08*	0.21			
Glut2	-1	5.34	5.89	5.70	0.22	0.12	0.05	0.74
	0	5.41	5.30	5.41	0.21			
	2	5.18	5.61	6.14	0.21			
	4	5.22	5.86	5.95	0.20			
	8	4.53	4.77*	5.50	0.21			
CS	-1	3.11	2.64	2.88	0.17	0.52	0.64	0.31
	0	2.94[ab]	2.52[a]	3.40[b]	0.16			
	2	2.79	3.14	2.89	0.16			
	4	2.68	2.55	3.11	0.16			
	8	2.53	2.80	2.53	0.17			
CTSLb	-1	6.04[a]	7.07[b]	6.93[b]	0.17	<0.001	0.26	0.89
	0	6.57	7.09	6.74	0.16			
	2	6.56[a]	7.32[b]	7.56[b]	0.16			

	4	6.46[a]	7.32[b]	7.19[ab]	0.16			
	8	6.59	7.17	7.27	0.16			
ACADvl	-1	5.66	5.40	6.09	0.15	0.97	<0.001	0.26
	0	6.69*	6.76*	6.32	0.15			
	2	6.33*	6.34*	6.08	0.14			
	4	6.09	5.78	6.05	0.14			
	8	5.84	6.05	5.89	0.15			

[1] Data are presented as means ± SEM subtracted from the arbitrary value 5 (5 - ΔCq). Thus, a high ΔCq resembles high transcript abundance (Livak and Schmittgen, 2001). An increase of one ΔCq represents a two-fold increase of mRNA transcripts.

[2] Groups are C = 56 d dry period and twice daily milking after parturition (n = 12), ODM = 56 d dry period and once daily milking for the first 28 d of lactation (n = 12), CM = 0 d dry period and continuously milking twice a day throughout lactation (n = 12).

[3] Weeks are relative to calving.

[a, b] Means within a row with different superscripts differ ($P < 0.05$). Means with * indicate a difference within one group to wk -1 precalving ($P < 0.05$).

8.6 Metabolic status of cows: functional parameters and mRNA levels of key enzymes

Metabolic regulation in complex organisms relies partly on transcriptional control as a long-term mechanism affecting the level of expression of several key enzymes (Desvergne et al., 2006). Furthermore, a cellular gene network can be defined as a collection of DNA segments that interact with a regulator such as a transcription factor or nuclear receptor, but also with each other through their RNA and protein products and with other molecules in the cell (Wittkopp, 2007).

It must be acknowledged that changes in gene expression do not necessarily indicate differences in protein abundance caused by post-translational regulation or enzyme activity. Gene expression data provide only a snapshot of information regarding the quantity of a given transcript in a cell, but its assessment strengthens the functional parameters measured in this study.
In the following chapter, possible relationships between functional parameters of blood serum and transcript abundance of genes coding for regulating enzymes in liver and muscle should be determined. In general, it should be clarified if adaptations to lactation were reflected by changes in mRNA abundance of genes related to central metabolic pathways. Most important, adaptation to lactation was quite different among groups, that were either milked in late pregnancy or milked once daily in early lactation. So, we associated mRNA levels of hepatic genes and genes of muscle with functional parameters like glucose-, BHBA- and NEFA concentration in blood serum.

Detailed information about improved metabolic situation postpartum triggered by different milking regimes is described above (Schlamberger et al., 2010). In short, C-cows having a 56 d DP and two times daily milking after calving showed the highest percentage of hypoglycemic blood glucose levels (47 %) compared to ODM-cows (20 %) and CM-cows (14 %, $P < 0.001$) from wk 1 to wk 4. C-cows peaked at much higher level (43.7 ± 2.6 kg ECM/d) at wk 4, CM-cows at wk 4 (38.2 ± 2.5 kg ECM/d), and ODM-cows at wk 8 (37.3 ± 2.2 kg ECM/d). As a consequence, C-cows experienced marked stress with the onset of lactation accompanied by enhanced lipolysis of adipose tissue and ketogenesis as proven by higher levels of BHBA and NEFA in blood serum compared to ODM- and CM-cows.

The enhanced lipolysis was also reflected by the highest daily milk fat yield for these cows (2.24 ± 0.14 kg/d) at wk 4, compared to ODM-cows (1.64 ± 0.14 kg/d) and CM-cows (1.81 ± 0.15 kg/d) at wk 4 and wk 2, respectively (Figure 7).

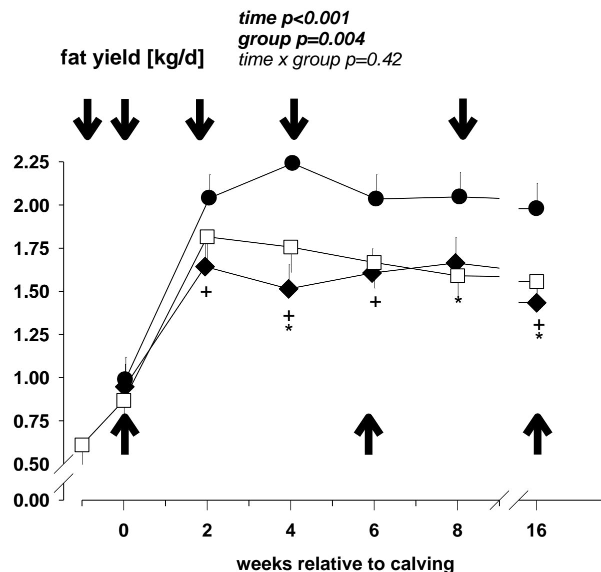

Figure 7. Daily fat yield (kg/d) from calving up to wk 16 of lactation for Brown Swiss dairy cows assigned to one of three management strategies: 56 d dry period (DP) and twice daily milking after parturition (controls, C, ●), 56 d DP and once daily milking (ODM) for the first 28 days of lactation (♦), and zero days dry with continuously milking (CM) (□) twice a day throughout of lactation. Data presented here originate from day of sampling and are no pooled weekly means. So, at each biopsy time point corresponding daily fat yield was available. Arrows on the top shows biopsy time points of liver biospy, arrows on the bottom show time points of muscle biopsies. Asterisks (*) indicate differences between C and CM; plus signs (+) indicate differences between C and ODM ($P < 0.05$); circle signs (°) indicate differences between ODM and CM. Values are LSMeans ± SEM.

The higher milk fat yield of C-cows might have been caused by a higher infiltration of long chain fatty acids stored in the body from blood into milk fat (Bauman et al., 2006) and also Pullen et al. (1989) found a positive correlation between milk fat content and plasma NEFA. C-cows experienced the largest drop in serum glucose at wk 2 and wk 4 (Figure 8) which might have been a consequence of a high mammary uptake of glucose for lactose synthesis, followed by an increased availability of fat used as energy fuel.

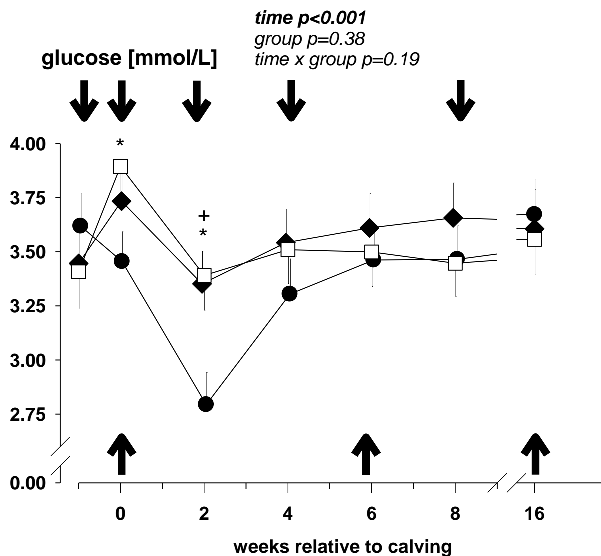

Figure 8. Blood serum glucose concentration from calving up to wk 16 of lactation for Brown Swiss dairy cows assigned to one of three management strategies: 56 d dry period (DP) and twice daily milking after parturition (controls, C, ●), 56 d DP and once daily milking (ODM) for the first 28 days of lactation (♦), and zero days dry with continuously milking (CM) (□) twice a day throughout of lactation. Arrows on the top shows biopsy time points of liver biospy, arrows on the bottom show time points of muscle biopsies. Asterisks (*) indicate differences between C and CM; plus signs (+) indicate differences between C and ODM ($P < 0.05$); circle signs (°) indicate differences between ODM and CM. Values are LSMeans ± SEM.

The decreased glucose concentrations postpartum are probably related to low DMI, and the concomitant reduction in propionate absorption. This reduced blood serum glucose concentration was followed by higher NEFA and BHBA concentrations in blood serum of these cows (Schlamberger et al., 2010). The high mammary uptake of glucose soon after parturition especially for C-cows with peak of daily milk yield at wk 2 of lactation (Schlamberger et al., 2010) resulted in low serum concentrations of glucose. Higher blood serum concentrations postpartum in CM- cows compared to C-cows were rather the result of an enhanced gluconeogenesis immediately postpartum than of a reduced demand of glucose for lactose synthesis in the mammary gland. CM-cows had a daily milk yield similar to C-cows at wk 2 but with blood serum BHBA and glucose concentrations out of critical range.

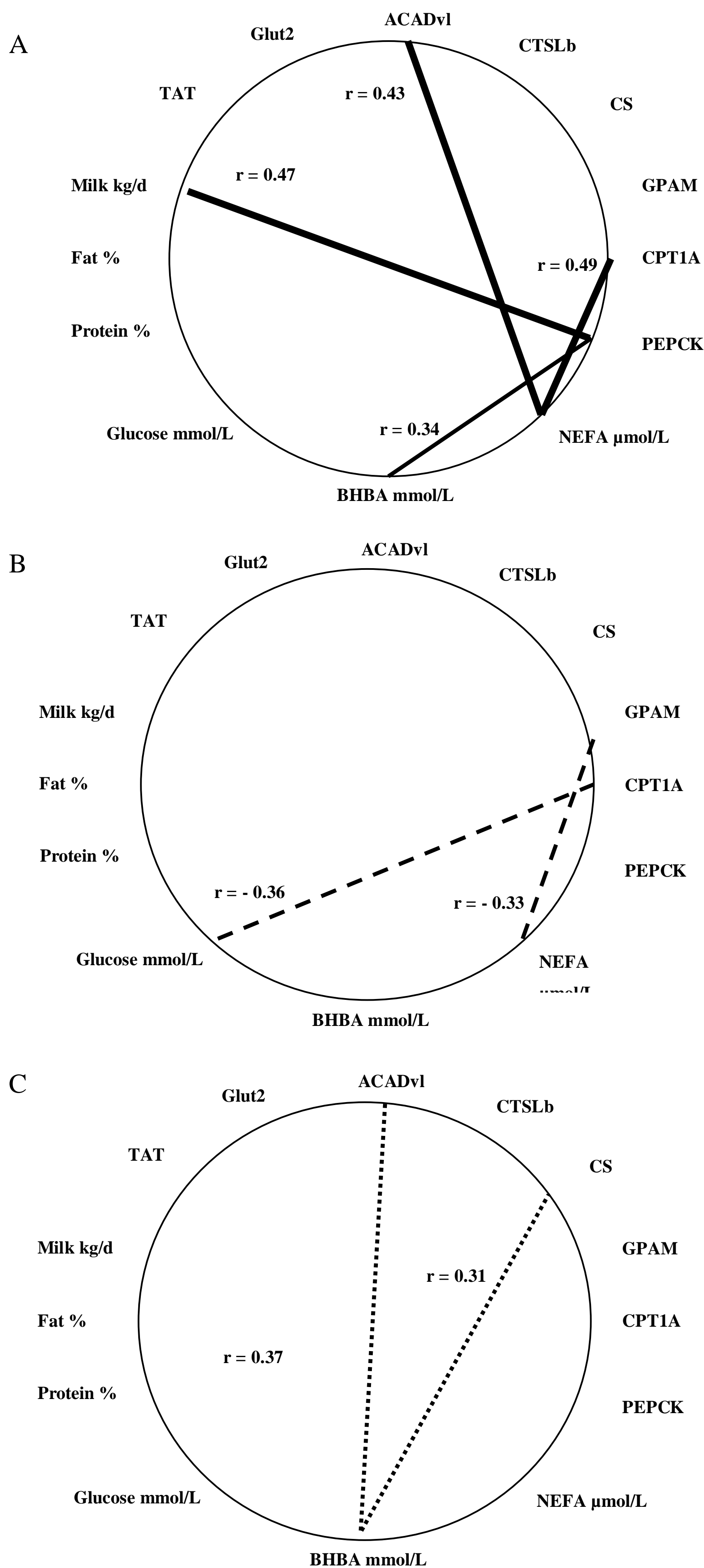

Figure 9. Pearson and Spearman rank order coefficients of correlation among concentrations of blood serum parameters and production traits and transcript abundance of target genes in liver of C-cows (A, solid lines), ODM-cows (B, dashed lines), and CM-cows (C, dotted lines). Lines in boldface mean $P < 0.01$, normal lines mean $P < 0.05$.

Gluconeogenesis indicated by transcript abundance of PEPCK seemed to start earlier in CM- than in C-cows. Metabolism of CM-cows seemed to be ready for the onset of lactation as shown by higher transcript abundance of PEPCK before calving, at calving and thereafter compared to C-cows (Table 11). For C-cows, a significant positive correlation was found between milk yield and PEPCK mRNA expression. Gene expression of PEPCK may be affected by propionate as well as lactate and amino acid supply and was therefore different among groups. Normally, a shift in these gluconeogenic substrates is well-known. The strong relationship between milk yield and PEPCK mRNA expression for C-cows shows that adaptation to lactation occurs according to increasing milk yield. Modulation of this regulating enzyme seems really essential.

Also mRNA levels of CPT 1A before calving were higher for CM- cows compared with those of ODM- and C-cows. We suggest, that twice daily milking in late gestation up to day of calving may influence well ahead of parturition hepatic adaptation to the onset of parturition. Perhaps other factors besides the hormonal environment characteristic of the periparturient period regulate hepatic adaptations to lactation. Most important is that hepatic gene expression changes are more pronounced in C- and ODM-cows than in CM-cows. That is also reflected by downstream activation of genes with key functions in fatty acid oxidation (CPT 1A, ACADvl, EnoylCoA) that were also influenced by the milking regimen. Transcript abundance of ACADvl in hepatocytes showed a marked increase at time of calving and wk 2 of lactation followed by a marked decrease up to wk 8 of lactation for C-and ODM- but not for CM-cows. Up-regulation of ACADvl with calving was more pronounced in C- and ODM- than in CM-cows, suggesting that regulation of metabolic adaptation was moderate in CM-cows compared with C-cows. Up-regulation of ACADvl abundance around calving compared to precalving was also found by Loor et al (2005). In our study, serum concentrations of BHBA and NEFA were correlated positive with transcript abundance of ACADvl in liver, but this correlation was really marked for NEFA blood serum concentration and mRNA levels of ACADvl for C-cows ($r = 0.43$, $P = 0.007$, Figure 9).

We could not detect any changes of transcript abundance of the GLUT 2 transporter, suggesting that hepatic glucose release remained unaffected by treatments and might not, therefore, be regulated by metabolic status of cows (Drackley, 2001a). For enzymes of protein catabolism we could not detect any differences among groups prepartum in liver. The onset of lactation is associated with increased AA uptake by the mammary gland and liver (Verbeke et al., 1972). The increased protein supply either originate through increases in DMI and muscle protein degradation, as muscle stores large amounts of AA and release them for

gluconeogenesis and milk protein synthesis. Most important is that all the adaptations to lactation seem to occur in CM-cows before calving perhaps over a long timeframe, while adaptions especially for C-cows seem to occur within a short timeframe, at worst within two weeks, as milk yield peaked at this early time point of lactation.

One week before calving, levels of blood serum NEFA and BHBA concentrations did not differ among groups, but transcript abundance of CPT 1A, GPAM and PEPCK in liver differed among groups. Higher levels of mRNA abundance for CM-cows compared to C and ODM-cows were found in enzymes of liver (CPT 1A, PEPCK, GPAM). Metabolism of these cows seemed to be more adapted to lactation compared to C-cows and ODM-cows. Serum concentrations of NEFA correlated positive with mRNA levels of hepatic CPT 1A ($r = 0.49$, Figure 9), but correlation was significant only for C-cows, suggesting that these cows showed a greater adaptive performance than others. The correlation between NEFA and CPT 1A mRNA level was also observed by (Loor et al., 2005).

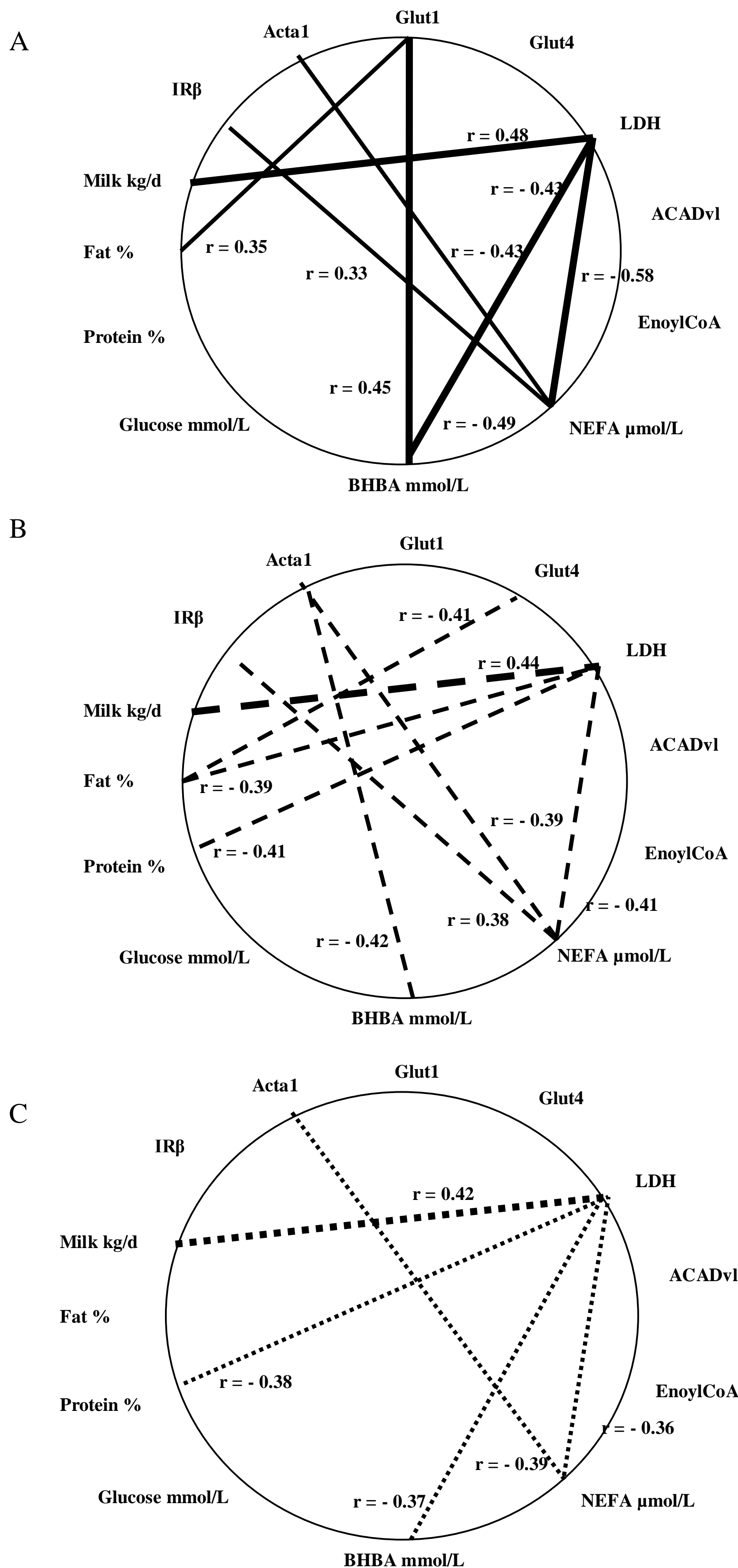

Figure 10. Pearson and Spearman rank order coefficients of correlation among concentrations of blood serum parameters and production traits and transcript abundance of target genes in skeletal muscle of C-cows (A, solid lines), ODM-cows (B, dashed lines), and CM-cows (C, dotted lines). Lines in boldface mean $P < 0.01$, normal lines mean $P < 0.05$.

In skeletal muscle, EnoylCoA transcript abundance was significant lower in CM-cows than in C-cows at time of calving and wk 16 (Table 10). At wk 6 transcript abundance of EnoylCoA were lower for both, CM- and ODM-cows compared to C-cows. These C-cows showed a stronger lipolysis marked by higher mRNA levels of this regulatory step of the β-oxidation. Levels of ACTA1 were upregulated at wk 6 and 16 of lactation for cows of all groups, suggesting that metabolism returns more and more from catabol to anabol status. Blood serum concentrations of NEFA were negative correlated with ACTA1 transcript abundance for all groups ($r = -0.37$ $P < 0.05$, Schlamberger et al., 2010, in review). We could not detect any relationships between transcript abundance of insulin receptor β or glucose transporter Glut1 and Glut4 to functional parameters in blood serum. Perhaps changes in transcript abundance of several key enzymes are more pronounced in liver than in skeletal muscle of dairy cows. Despite of this, we found changes in the expression of IRβ in skeletal muscle showing decreasing levels from calving to wk 16 for all groups, interestingly more pronounced for ODM- and CM-cows. Serum concentrations of BHBA were correlated with mRNA abundance of LDH in muscle. These correlations were negative for all groups (Schlamberger et al., 2010, in review), but most obvious for C-cows, with a significant correlation ($r = -0.49$, $P < 0.01$, Figure 10). LDH transcript abundance was found to be positive correlated with daily milk yield for all groups ($r = 0.45$), and was negative correlated with blood serum NEFA concentrations ($r = -0.41$). It was suggested that energy metabolism in muscle was impaired with increasing BHBA concentrations in blood serum. In addition with increasing BHBA and NEFA, mRNA levels of ACTA1 decreased with the plainest correlation for C-cows ($r = -0.43$, $P < 0.05$ for NEFA) and ODM-cows ($r = -0.42$ for BHBA, $P < 0.01$, Figure 10). It remains an open question why this gene involved in energy metabolism of cells seems to play a central rule in metabolic adaptations to early lactation.

A main finding from the calculated correlations is that the correlations between gene expression of hepatic genes and blood serum metabolites and parameters of productivity varied between treatments. Correlations were more pronounced in C-cows than in ODM- and CM-cows (Figure 9 and Figure 10). This could suggest that metabolic regulation of cows of each group was different. The metabolic imbalance of C-cows compared to ODM- and CM-cows was reflected by changes in relative mRNA expression of specific genes in liver and muscle, measured at different time points. Furthermore, more correlations were observed for C-cows, which may indicate a higher metabolic acitivity in the liver and skeletal muscle. In contrast to these cows, the metabolic changes in early lactation affected ODM- and CM-cows

only moderately. This was also concluded from the results of the concentrations of blood metabolites for these cows (Schlamberger et al., 2010).

9 CONCLUSIONS AND OUTLOOK

In the present study, the importance of the periparturient management of the dairy cow based on the milking regimen has been shown. Eliminating the dry period and milking once daily for the first four weeks of lactation were studied for the first time within the same herd and under the same feeding and housing conditions. Before this work, there was a paucity of data to determine the effects of eliminating the dry period or reducing milking frequency in early lactation on metabolic profile, mRNA levels of key enzymes and productivity.

Metabolism

It is well accepted that the dairy cow experiences multiple physiological states throughout a lactation cycle, including extended periods of poor or improved metabolic status. During the lactation cycle, the nutrient requirement varies several-fold and nearly half of the cow`s fat may be lost and regained during a lactation. These wide ranging physiological states throughout lactation provide us the opportunity to alter traditional management systems to smooth changeovers of physiological regulation. With this study, information already exists to substantially **reduce metabolic stress in early lactation by altering milking regimen**. Both management arrangements presented here resulted in an improved metabolic status, which was further evidenced by increased blood serum concentrations of glucose and reduced concentrations of blood serum NEFA and BHBA for the first four weeks after calving.

Gene expression

A fundamental response to nutrient supply is a change in signals that regulate metabolic pathways such as hormones or the direct and indirect actions of key nutrients and metabolites to alter metabolism by changes in gene expression. In our study, relative quantification of gene expression in liver and muscle was done using reference genes for normalization. It has been shown, that functional metabolic changes triggered by different milking regimen are reflected by changes in expression of genes involved in central metabolic pathways in liver and skeletal muscle. **Traditionally managed dairy cows** with a standard 56 d dry period and at least twice daily milking after calving **required a more pronounced adaptation at the onset of lactation**, reflected by marked changes of transcript abundance of regulating factors of glucose and lipid metabolism compared to ODM- or CM-cows.

Milk yield, health and milk composition

The more favorable metabolic status of CM- and ODM-cows obviously arised from a more favorable relationship between nutrient provision and nutrient output in milk due to a lower capacity for milk production. **Milk yield was reduced for both milking regimen, but not in a dramatic range**, which was likely to result in healthier cows with improved reproductive performance, especially for CM-cows. The lactation curve for ODM-cows peaked later and proceeded flatter. The optimal duration of ODM in high-yielding dairy cows potentially combined with increased milking frequency for a limited timeframe remains an issue for further investigations.

Whereas milk fat content is known to be the most sensitive component of milk to dietary manipulation, the study presented here revealed an option to elevate milk protein content by alteration of standards to manage the high yielding dairy cow. **Milk protein percentage was markedly elevated** in continuously milked cows (+ 0.5 %) and to a lesser extent also in ODM-cows (+ 0.3 %). To the dimension that milk pricing is linked to milk components especially milk protein, producers may become motivated to alter standard dairy production systems as a means to modify milk composition for maximum economic return.

Transfer of knowledge

In what extent these management systems can transferred into dairy production systems remains an open question, but there are **many basic approaches arising from results of the present study**. Ultimately, the decision to eliminate the dry period or reducing milking frequency becomes an issue of economics. Profitability of these presented management systems will depend on milk yield and composition during the extra days of milking and the subsequent lactation, colostrums quality and calf survival, incidence of metabolic disorders and disease, reproductive performance, herd level effects, and management factors such as labor and housing costs. The ability to translate emerging knowledge into on-farm application and actual prevention of problems requires understanding of the farm as an integrated system, a major component of which is educating and motivating farmers to implement well designed practices. Understanding and accomplishing this final major step of managing the dairy cow in future is both an advance and an ongoing challenge.

Methods of sampling and analyzing

Physiological processes are governed by several genes acting in concert rather than by only one or a few individual genes. Due to this, genomics can be applied to characterize the

metabolic status of an individual cow by relative quantification of target genes involved in major metabolic pathways in liver. So, attention is directed to specific biological pathways regulated by rate-limiting enzymes or key genes. More recently, genomic technologies (array technology, proteomics and metabolomics) have enabled the analysis of thousands of genes or proteins or metabolites in a single sample. Metabolomics aims at quantifying and characterizing all metabolites present within cells, biofluids or tissues under a given set of conditions. Nuclear magnetic resonance (NMR), mass spectrometry (MS), or gas or liquid chromatography (GC, LC) with detections limits in the micromolar range are well-established analytical methods. To record metabolic patterns of an individual cow by repeatedly determination of hepatic glycogen and triglyceride content, validation of tissue specific **assays based on µg-sized tissue samples are necessary**. Furthermore, the metabolic patterns of samples could be compared and classified using multivariate statistical tools. Most important, all these analytical methods mentioned above presuppose a representative blood or tissue sample originating from the individual animal. Hence, taking into account animal welfare, small-sized tissue samples are required, likely taken by non- or slightly invasive methods, which may be another area of future research.

Fields of future research

Today, the important contributions of milk and dairy products in meeting our dietary requirements for energy, **high quality protein**, and several key minerals and vitamins are unequivocal. In the last quarter century, nutritional quality has become increasingly important in food choices because of consumer awareness of the link between diet and health. The greatest opportunities on the horizon for manipulating milk composition will be directed at using milk for delivery of nutraceuticals to enhance **human health** and to combat clinical diseases such as obesity, lactose intolerance, or osteoporosis. But this may be an option of future research. In addition to this study, further basic research must also take place **to investigate factors in the mammary epithelial cells** where conversion of circulating nutrients into milk components occur.

The current thesis reveals a major advance for scientists and veterinarians, producers and breeders how to meet some problems of modern dairy production systems by modification of traditional management standards. Importantly, additional animal trials are needed that assign cows randomly to specific treatments related to dry period length or milking frequency as they were evaluated in the present study, to determine the optimal production system for the modern dairy cow in various management scenarios.

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12 SCIENTIFIC COMMUNICATIONS

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14 CURRICULUM VITAE

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15 APPENDIX

Appendix I	Schlamberger, G., S. Wiedemann, E. Viturro, H. H. D. Meyer, M. Kaske. 2010. Effects of different milking regimes on metabolism and productivity of dairy cows. Journal of Dairy Science 93:2471-85.
Appendix II	Schlamberger, G., S. Wiedemann, E. Viturro, H. H. D. Meyer, M. Kaske. 2010. Omission of dry period or milking once daily in early lactation affects metabolic status and is reflected by mRNA levels of enzymes in liver and skeletal muscle of dairy cows. Journal of Dairy Science (*in review)*.
Appendix III	Sigl, T., G. Schlamberger, H. Kienberger, S. Wiedemann, H. H. D. Meyer, M. Kaske. 2010. Conjugated linoleic acid supplementation of dairy cows in early lactation: effects on milk composition, milk yield, blood metabolites and gene expression in liver. Acta Veterinaria Scandinavica 52:16-24.
Appenidx IV	Klein, M. S., M. F. Almstetter, G. Schlamberger, N. Nürnberger, K. Dettmer, P. J. Oefner, H.H.D. Meyer, S. Wiedemann, W. Gronwald. 2009. NMR and mass spectrometry based milk metabolomics in dairy cows in early and late lactation. Journal of Dairy Science 93:1539-50.

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Effects of continuous milking during the dry period or once daily milking in the first 4 weeks of lactation on metabolism and productivity of dairy cows

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ABSTRACT

The objective was to compare the effects of 3 management systems in high-yielding dairy cows on metabolic profiles and milk production. Thirty-six multiparous Brown Swiss cows were randomly assigned to 1 of 3 treatment groups (n = 12 cows/group): the control (C) group, in which cows were dried off 56 d before calving and milked twice daily throughout next lactation (305 d); the once daily milking (ODM) group, in which cows were dried off 56 d before calving and milked once daily for the first 4 wk of lactation and twice daily for the remaining lactation; and the continuous milking (CM) group, in which cows were milked twice daily until calving and also during the subsequent lactation. Serum glucose concentrations decreased between wk 1 and 4 exclusively in C cows. Serum concentrations of NEFA and BHBA in the first 4 wk of lactation were highest in C cows compared with ODM and CM cows. Decreased backfat thickness during early lactation and reduction of body condition score were markedly more pronounced in C cows compared with ODM and CM cows. Mean lactational milk yield of C cows [11,310 ± 601 kg of energy-corrected milk (ECM)/305 d] was approximately 16% higher compared with ODM cows (9,531 ± 477 kg of ECM/305 d) and CM cows (9,447 ± 310 kg of ECM/305 d). The lactation curve of CM cows compared with C cows was characterized by a similar time of peak yield (wk 3), a reduced peak yield, and no obvious differences in persistency. Mean percentage of milk protein was significantly higher for CM cows (3.91%) compared with C cows (3.52%). In contrast, once daily milking was accompanied by a reduced and significantly delayed peak yield (wk 8) compared with the control treatment, whereas persistency was better and milk protein (3.79%) was higher in ODM cows than in C cows. In conclusion, continuous milking and once daily milking, targeting the interval before or after calving, respectively, substantially reduced the metabolic challenge of fresh cows and improved milk protein percentage. Continuous milking and once daily milking increased milk protein percentage markedly; furthermore, once daily milking during the first 4 wk of lactation improved the lactation curve.
Key words: continuous milking, once daily milking, metabolism, productivity

INTRODUCTION

The critical challenge in high-cost, high-return dairy production systems is attaining high lactational milk yield while simultaneously achieving sustainable production by maintaining an appropriate level of animal health and fertility. Producing milk with high protein percentage to meet market demands and addressing society's concerns regarding animal welfare are also priorities. Genetic selection for greater milk production is a risk factor for a variety of production diseases because increases in feed intake lag behind increases in milk production in early lactation (Ingvartsen and Andersen, 2000; Ingvartsen et al., 2003; Mulligan and Doherty, 2008). Therefore, efforts have been made to optimize housing, feeding, and management of high-yielding dairy cows (Distl, 1991; Drackley, 1999; Grummer et al., 2004).

A successful transition from late pregnancy to early lactation is essential for animal health and productivity (Grant and Albright, 1995; Drackley, 1999). One way to reduce the risk of metabolic imbalance during this is time is to omit the dry period (**DP**; Rémond and Bonnefoy, 1997; Andersen et al., 2005). In a study by Andersen et al. (2005), plasma concentrations of glucose and insulin were higher and plasma concentrations of NEFA and BHBA were lower in cows with an omitted DP than in control cows with a conventional DP during the first 5 wk of early lactation. However, in other studies, continuous milking compromised colostrum quality (Grummer and Rastani, 2004; Caja et al., 2006) and caused a 12 to 25% decrease in milk yield during the subsequent lactation (Swanson, 1965; Rémond et al., 1992; Rémond and Bonnefoy, 1997). Therefore, it was concluded that the DP is needed to enhance secretory

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function of the mammary gland (Rémond et al., 1997; Ingvartsen et al., 2003), enabling involution, proliferation, and redifferentiation of mammary tissue (Capuco et al., 1997; Wilde et al., 1997; Capuco et al., 2003). It was previously recommended that the DP last at least 6 to 8 wk because shorter intervals reduced milk yield in the following lactation (Schaeffer and Henderson, 1972; Coppock et al., 1974; O'Connor and Oltenacu, 1988). Conversely, it was recently established that mammary involution is completed within 25 d, suggesting that a 30-d DP was adequate to ensure high milk production (Capuco et al., 1997; Gulay et al., 2003). Moreover, cows assigned to a shortened DP (28 d) had an improved energy status for the first 10 wk of lactation compared with cows with a 56-d DP (Rastani et al., 2005). Primiparous cows assigned to a 35-d DP after the first lactation had lower peripheral NEFA concentrations immediately after parturition than those given a 56-d DP (Pezeshki et al., 2007). Multiparous cows with a 28-d DP had an improved energy status based on total milk production and BCS at 150 DIM (Pezeshki et al., 2008).

Another approach to reducing the metabolic challenge during early lactation is to alter milking frequency. The interval from calving to peak yield, its magnitude, and the rate of increase of milk yield per day during the first weeks of lactation are important risk factors for production diseases in early lactation (Ingvartsen and Andersen, 2000). However, the time that peak yield occurs can be influenced only slightly by the feeding regimen, but may depend more on milking frequency. In several studies, increased frequency enhanced milk yield (van der Iest and Hillerton, 1989; Rastani et al., 2007; Wall and McFadden, 2008). In contrast, in a study by Carruthers and Davis (1993), once daily milking for 2 wk in early lactation reduced milk yield by 16% compared with twice daily milking. Although milking thrice daily for the first 28 d of lactation increased milk yield by 19.6% compared with once daily milking, it was accompanied by more severe losses of body weight by d 60 of lactation (Davis et al., 1999; Patton et al., 2006). Energy status was improved in cows milked once daily during the first 3 wk of lactation (Patton et al., 2006), reducing the risk of metabolic disorders (Rauw et al., 1998).

To date, continuous milking has not been compared with once daily milking in early lactation as a way to easier cope with the metabolic challenge of high-yielding dairy cows. Thus, the objective of the present study was to compare the effects of continuous milking without DP and milking frequency during early lactation on the metabolic profile, fertility, and productivity of the subsequent lactation. The hypothesis to be tested was that metabolic profile and, as a consequence, parameters of productivity were different among cows that were milked either continuously or once daily for the first 4 wk of lactation compared with those managed with a traditional 56-d DP and twice daily milking after calving.

MATERIALS AND METHODS

Animals and Experimental Design

The study was conducted at the research farm Veitshof of the Technische Universitaet Muenchen (Freising, Germany) from December 2006 to December 2008 using 45 multiparous Brown Swiss dairy cows.

Cows were assigned at random to 1 of 3 treatment groups. In the control group (**C**; n = 16), cows were dried off 56 d before expected calving and milked twice daily throughout the entire lactation (305 d). Cows in the once daily milking group (**ODM**; n = 16) were dried off 56 d before expected calving and milked only once daily for the first 4 wk of lactation and twice daily for the remainder of lactation. Cows of the continuous milking group (**CM**; n = 13) were milked twice daily up to the day of calving and throughout the subsequent 305-d lactation (i.e., they were not dried off).

One cow from the CM group, 4 cows from the ODM group, and 4 cows from the C group did not finish the entire lactation; they were removed because of various health disorders. Ultimately, complete data sets (305-d lactation) for 12 cows in each treatment group were used for statistical analysis (Table 1).

Housing and Feeding

All cows were kept in a cubicle housing system fitted with rubber-coated slatted floors and bedded with wood shavings. Cows were milked in a 2 × 2 tandem milking parlor (GEA WestfaliaSurge GmbH, Boenen, Germany) either once (1545 h) or twice (0415 and 1545 h) daily. For drying-off (C and ODM cows only), milking was done once daily (1545 h) for 3 d and then an intramammary antibiotic treatment containing 1 g of cloxacillin (Orbenin, Pfizer, Berlin, Germany) was given 56 d before expected calving. Thereafter, C and ODM cows were transferred to a separate stable and fed the DP ration (Table 2). Approximately 1 wk before expected calving, all cows were moved to single calving pens bedded with barley straw. After calving, cows were moved to the lactating herd and fed the lactation diet. The CM cows were not separated from the herd during the last 2 mo of pregnancy because they were milked throughout the entire pregnancy–lactation cycle. All lactating cows were fed the partly mixed ration formulated to meet nutrient requirements, as

Table 1. Experimental design, animals excluded from the study and reasons for removal, and animals used for statistical analysis[1]

Item	C	ODM	CM
Cows assigned to study, n	16	16	13
Daily milking frequency[2]			
d −56 to 0	dry	dry	2
d 1 to 28	2	1	2
d 29 to 305	2	2	2
Cows removed from study, n	4	4	1
Downer cow syndrome	1 (4)[3]	1 (1)	
Severe injuries	1 (29)	1 (48)	
Lameness	1 (194)	1 (159)	1 (106)
Mastitis		1 (137)	
Abortion	1 (239)		
Cows with complete data sets, n	12	12	12
Previous energy-corrected 305-d milk yield,[4] kg	8,826 ± 377	8,725 ± 360	8,664 ± 336
Parity[4]	3.5 ± 1.6	3.8 ± 1.8	3.3 ± 2.1

[1]C = control group: cows were dried off 56 d before calving and milked twice daily throughout next lactation (305 d; n = 12); ODM = once daily milking group: cows were dried off 56 d before calving and milked once daily for the first 4 wk of lactation and twice daily for the remaining lactation (n = 12); CM = continuous milking group: cows were milked twice daily until calving and also during the subsequent lactation (n = 12).

[2]Numbers indicate days relative to calving.

[3]Values in parentheses represent days of lactation when cows were removed from the study.

[4]Mean ± SEM.

indicated by GfE (2007) (Table 2), and had ad libitum access to fresh water.

In addition, hay was fed immediately after milking (0600 h). The partly mixed ration, formulated on the basis of a milk yield of 22 kg/d, was delivered once daily (0700 h) and was intended to provide ad libitum intake (>5% residual feed). Cows producing more than 22 kg/d were fed additional concentrates (0.5 kg of concentrate/kg of milk, maximal 9 kg/d; Raiffeisen Kraftfutterwerke Süd, Würzburg, Germany) in feeding stations.

Health and Occurrence of Diseases

Health status was assessed daily. Retained fetal membranes, periparturient paresis, primary ketosis, mastitis, and lameness occurred during the study. Nine cows were removed from the study (Table 1) because they were severely affected with the following disorders: downer cow syndrome (n = 2; 1 C and 1 ODM cow), severe injuries (n = 2; 1 C and 1 ODM cow), lameness (n = 3; 1 C, 1 ODM, and 1 CM cow), mastitis (n = 1; 1 ODM cow), or abortion (n = 1; 1 C cow).

Records and Sampling

Milk yield was measured twice daily with electronic milk meters (Metatron P21, GEA WestfaliaSurge GmbH) and stored electronically (DairyPlan C21, GEA WestfaliaSurge GmbH). Daily milk yield was standardized to ECM (4.0% fat and 3.4% protein content) by ECM (kg/d) = [(0.38 × fat % + 0.21 × protein % + 1.05)/3.28] × milk yield (kg/d) (Fischer et al., 2002). Analysis of milk components (protein, fat, and SCC) was done on samples collected at the afternoon milking. Because milking intervals were similar between

Table 2. Ingredients and chemical composition of the partial mixed ration[1]

Item	LD	DPD
Ingredient, %		
Corn silage	60	43
Grass silage	23	25
Hay	4	32
Feed pellets[2]	12	—
Mineral mix[3]	1	—
Chemical composition, % unless noted		
DM	45.2	52.0
CP	12.2	12.9
Crude fiber	18.6	19.2
NFC	26.4	9.92
Ether extract	3.16	3.16
NE_L,[4] Mcal/kg	1.55	1.36

[1]Lactation diet (LD) was fed to all cows; dry period diet (DPD) was fed only to cows having a 56-d dry period (control and once daily milking cows, not continuous milking cows).

[2]Composition: corn gluten, 18.4%; turnips molasses chips, 13.8%; wheat, 10.0%; triticale, 10.0%; rape cake, 10.0%; maize, 8.8%; malt germ, 6.0%; grain distillation residual (ProtiGrain), 5%; rape extraction grist, 5%; rumen protected rape extraction grist, 5%; palm corn cake, 3.3%; soy extraction grist, 2.8%; sodium bicarbonate, 1.0%; calcium bicarbonate, 0.99%; plant oil (palm coconut), 0.40% (Raiffeisen Kraftfutterwerke Süd GmbH, Würzburg, Germany).

[3]Ingredients: Ca, 14%; Na, 10.0%; P, 5.0%; Mg, 5.0% (Josera, Kleinheubach, Germany).

[4]Estimates determined from tabulated values of feeds (GfE, 2007) and according to the following formula: NE_L (Mcal/kg) = 0.6 × [1 + 0.004 × (q − 57)] × ME(Mcal/kg), where q = ME/GE. Here, q = 88.9.

morning and afternoon milkings, measurements in afternoon samples were reliable estimations of the contents in daily milk in agreement with Lee and Wardrop (1984). Samples (~1 L) were collected during milking. The amount of milk separated into the sample pot was controlled by milk flow-rate and total amount of milk to get a proportional subsample (Metatron P21, GEA WestfaliaSurge GmbH). A 50-mL aliquot was taken and stored at 4°C for a maximum of 10 d with a preservative (acidiol) until analyses. Milk composition of cows in CM was analyzed twice weekly until calving. After parturition, milk composition of all cows was determined daily for the first week, twice weekly to 56 DIM, once weekly from 57 to 100 DIM, and then once every 2 wk to 305 DIM.

Blood samples from all cows were collected by jugular venipuncture at wk −4, −2, and −1 before expected calving, within 24 h postpartum (wk 0), and at wk 1, 2, 4, 6, 8, 12, 16, 24, 36, and 44. Samples were collected into 7-mL vacuum tubes (Vacuette, Greiner Bio-One, Kremsmünster, Austria) at 0700 (after milking); within 1 h, blood serum was separated by centrifugation (2,000 × g; 4°C; 15 min). Three aliquots (1.5 mL) of serum were stored at −20°C.

Backfat Thickness and BCS

Subcutaneous adipose tissue (backfat thickness) was assessed with ultrasonography (Sonovet 2000, Universal Ultrasound, Bedford Hills, NY) near the pelvic region (Schröder and Staufenbiel, 2006). The BCS was determined by the same person once every 2 wk from wk −8 to the end of the study (wk 44) using a scale from 1 to 5 (1 = emaciated, 5 = obese) in increments of 0.25 (Edmonson et al., 1989).

Analyses

Milk protein and fat were analyzed by infrared spectrophotometry (MilkoScan FT6000, Foss, Hillerød, Denmark), and SCC was determined with a fluorescence optical counting system (Fossomatic FC, Foss) in the laboratories of Milchprüfring Bayern e.V. (Wolnzach, Germany). Serum concentrations of glucose, NEFA, BHBA, and total bilirubin (**TB**) were determined with an automated clinical chemistry analyzer (ABX Pentra 400, Horiba, Montpellier, France); precision of 20 measurements of 1 sample was expressed as the respective relative coefficient of variation. Glucose concentrations were analyzed by using hexokinase method (Hoffmann La-Roche, Basel, Switzerland; CV = 2.3%), NEFA concentrations were measured using colorimetric enzymatic reactions (CV = 6.2%), BHBA was determined using a spectrophotometric enzymatic analysis (Sigma-Aldrich Diagnostics, Munich, Germany; CV = 7.1%), and TB was determined with the Jandrassik/Grof reaction (Jandrassik and Grof, 1938). Calibration and quality controls were done daily. The threshold values are based on Macrae et al. (2006).

Statistics

End points measured repeatedly (daily milk yield, milk composition) were pooled to weekly means before statistical analysis. For BCS and backfat, an average value for every 4 wk from wk −8 to wk 40 was used.

For any metabolic key parameters in blood, as for milk yield, percentage milk protein, milk fat content, and milk protein and fat yield, treatment effects, and differences among groups were determined using REML in the MIXED procedure in SAS (SAS Institute, 2002). The model contained fixed effects of treatment and week and random effects of cow within treatment. For the repeated measurements, the model also contained weeks relative to calving and the interaction between treatment and weeks relative to calving. The effects of time, group, and time × group interactions were tested.

For each variable analyzed, 3 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's Bayesian criterion closest to zero was used (Littell et al., 1998). Differences between treatments were determined using the PDIFF option.

Incidence of health disorders and the proportion of blood samples from wk 1 to 4 with low glucose, high NEFA, and high BHBA were tested for differences with PROC ANOVA using Dunnett's one-tailed t-test to locate differences. Results are reported as least squares means ± standard error of means. Means were considered to differ significantly in case of $P < 0.05$.

RESULTS

Milk Yield

Total lactational ECM yield (305 d) was highest for C cows (11,310 ± 601 kg of ECM). Compared with C cows, total ECM was 15.7 and 16.5% lower for ODM cows (9,531 ± 477 kg of ECM) and CM cows (9,447 ± 310 kg of ECM), respectively (Figure 1A; Table 3).

Total milk yield of CM cows from d −56 to parturition was 1,186 ± 82 kg of ECM. Daily ECM yield declined by 57.3% from wk −8 to wk −1 relative to calving. During the last week before calving, average daily milk yield of CM cows was 14.4 ± 2.1 kg of ECM.

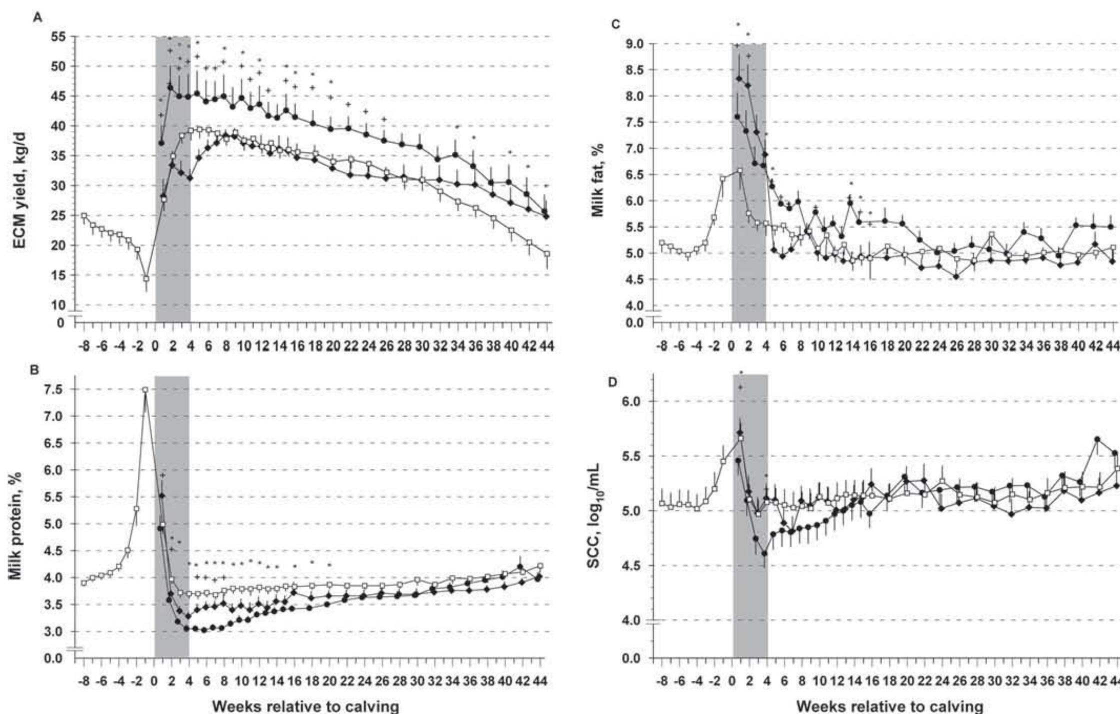

Figure 1. A) Energy-corrected daily milk yield (kg/d), B) milk protein concentration (%), C) milk fat concentration (%), and D) SCC pattern ($\log_{10}$/mL) from wk −8 before parturition up to wk 44 of lactation for Brown Swiss dairy cows assigned to 1 of 3 management strategies: 56-d dry period (DP) and twice daily milking after parturition (control, C; ●), 56-d DP and once daily milking (ODM) for the first 28 d of lactation (◆), and 0-d DP with continuous milking (CM) twice daily throughout lactation (□). Asterisks indicate differences between C and CM; plus signs indicate differences between C and ODM ($P < 0.05$); circles indicate differences between ODM and CM. Values are LSM ± SEM. The gray box indicates time of different milking regimens in early lactation from 1 to 28 DIM.

The average dry-off milk yield was 17.7 ± 2.0 kg/d for C cows and 17.4 ± 1.8 kg/d for ODM cows.

Within the first 28 DIM, average daily milk yield was highest for C cows (43.3 ± 1.8 kg of ECM/d), which was 38% more than for ODM cows (31.3 ± 1.1 kg of ECM/d; $P < 0.001$) and 24% more than for CM cows (35.0 ± 1.1 kg of ECM/d; $P = 0.005$). During this time, ECM yield did not differ between ODM and CM cows. Milk yield for ODM cows increased from the start of twice daily milking at wk 5 ($P < 0.001$), but remained approximately 8.1 kg/d of ECM (18%) lower than for C cows ($P = 0.01$) until wk 8. From wk 5 to wk 20, ODM cows produced about 7.3 kg/d (15.7%) and CM cows about 6.5 kg/d (13.7%) less ECM than C cows. Yield of ECM peaked at wk 2 for C cows (46.4 ± 3.6 kg of ECM/d), at wk 5 for CM cows (39.4 ± 1.5 kg of ECM/d), and at wk 8 for ODM cows (38.4 ± 1.2 kg of ECM/d).

Milk Composition

Milk protein and milk fat content increased for CM cows from wk −8 to wk −1 relative to calving (3.90 ± 0.06% to 7.49 ± 0.41%, $P < 0.001$, and 5.20 ± 0.18% to 6.42 ± 0.35%, $P = 0.001$, for milk protein and milk fat content, respectively; Figure 1B and C). From wk −8 to wk −1, daily fat yield decreased (1.02 ± 0.40 kg/d to 0.64 ± 0.31 kg/d; $P < 0.01$) in milk of CM cows, whereas daily protein yield remained constant (0.75 ± 0.26 kg/d to 0.71 ± 0.32 kg/d; $P = 0.19$).

Milk protein concentration decreased steeply for C cows from wk 1 (4.91 ± 0.8%) to wk 4 (3.05 ± 0.23%; $P < 0.001$). For the first 4 wk postpartum, milk protein concentration was higher in both ODM cows (3.97 ± 1.07%; $P = 0.041$) and CM cows (4.06 ± 0.63%; $P = 0.009$) compared with C cows (3.65 ± 0.12%). However, reduced milk volume for ODM cows resulted in lower

Table 3. Energy-corrected milk yield (LSM ± SEM) during the last 56 d before calving for CM cows and for the first, second, and third 100-d interval of a 305-d lactation period compared among experimental groups

Item	Treatment group[1] C	ODM	CM	P-value[2] C vs. ODM	C vs. CM	ODM vs. CM
56 to 0 DIM	—	—		—	—	—
Average kg/d			21.2 ± 1.5			
Total			1,186 ± 82			
1 to 100 DIM						
Average kg/d	44.9 ± 2.8	37.6 ± 1.2	37.8 ± 0.9	0.025	0.021	0.99
Total	4,349 ± 278	3,514 ± 90	3,706 ± 95	0.002	0.016	0.46
101 to 200 DIM						
Average kg/d	39.2 ± 1.9	32.5 ± 2.1	33.6 ± 1.3	0.017	0.005	0.6256
Total	3,987 ± 186	3,323 ± 212	3,434 ± 131	0.013	0.040	0.67
201 to 305 DIM						
Average kg/d	31.8 ± 2.3	28.5 ± 2.3	25.0 ± 1.6	0.043	0.003	0.31
Total	2,975 ± 230	2,694 ± 229	2,306 ± 154	0.29	0.012	0.14
305-d lactation yield						
Average kg/d	38.2 ± 0.56	32.4 ± 0.41	30.5 ± 0.45	0.04	0.005	0.12
Total	11,310 ± 601	9,531 ± 477	9,447 ± 310	0.013	0.009	0.90

[1]C = control group: cows were dried off 56 d before calving and milked twice daily throughout next lactation (305 d; n = 12); ODM = once daily milking group: cows were dried off 56 d before calving and milked once daily for the first 4 wk of lactation and twice daily for the remaining lactation (n = 12); CM = continuous milking group: cows were milked twice daily until calving and also during the subsequent lactation (n = 12).

[2]Means are different among groups ($P < 0.05$).

daily protein yield for these cows (0.83 ± 0.22 kg/d) compared with C cows (1.13 ± 0.3 kg/d; $P = 0.007$) and CM cows (1.15 ± 0.14 kg/d; $P = 0.007$). The CM cows had a higher weekly milk protein concentration for the first 20 wk of lactation compared with C cows (3.85 ± 0.07% vs. 3.34 ± 0.07%; $P = 0.005$).

During the first 4 wk of lactation, milk fat concentration was highest for ODM cows (7.67 ± 0.20%) compared with C cows (7.07 ± 0.20%; $P < 0.02$) and CM cows (5.84 ± 0.13%; $P < 0.001$; Figure 1C). Daily fat yield was 36% higher for C cows (2.23 ± 0.73 kg/d) compared with ODM cows (1.64 ± 0.47 kg/d; $P < 0.001$) and 33% higher compared with CM cows (1.68 ± 0.32 kg/d; $P < 0.001$; Table 4).

Somatic cell count differed only at wk 1 between cows in group C (5.34 ± 0.4 $\log_{10}$SCC) and group ODM (5.47 ± 0.4 $\log_{10}$SCC; $P = 0.042$). No differences were found for SCC among treatment groups at any other time points (Figure 1D).

Health and Occurrence of Diseases

Five C cows, 2 ODM cows, and 3 CM cows suffered from clinical mastitis; 2 of the latter had infections of the udder during the last week before calving. A retained placenta was observed in 2 C cows, 5 ODM cows, and 1 CM cow. One ODM cow suffered from clinical hypocalcemia, and primary ketosis was diagnosed in 3

Table 4. Milk protein yield, milk fat yield, milk protein concentration, and milk fat concentration (LSM ± SEM) compared among treatment groups for 305-d lactation[1]

Item	Treatment group[2] C	ODM	CM (1 to 305 DIM)	CM+ (−56 to 305 DIM)	P-value[3] ODM vs. CM	C vs. ODM	C vs. CM	C vs. CM+
Protein, %	3.52 ± 0.3 (100)	3.79 ± 0.41 (107)	3.91 ± 0.28 (110)	4.04 ± 0.27 (115)	0.07	0.06	<0.001	<0.001
Fat, %	5.50 ± 0.2 (100)	5.15 ± 0.1 (94)	4.70 ± 0.1 (85)	5.33 ± 0.30 (97)	0.03	0.05	<0.001	0.13
Protein yield, kg	342 ± 15 (100)	311 ± 16 (91)	325 ± 12 (95)	367 ± 14 (107)	0.52	0.14	0.39	0.26
Fat yield, kg	541 ± 37 (100)	437 ± 23 (81)	417 ± 16 (77)	484 ± 25 (89)	0.924	0.006	0.005	0.13
Daily fat yield, kg	1.87 ± 0.08	1.42 ± 0.08	1.38 ± 0.08	1.26 ± 0.08	0.78	0.002	0.004	<0.001
Daily protein yield, kg	1.08 ± 0.05	0.99 ± 0.05	1.10 ± 0.04	1.03 ± 0.05	0.09	0.81	0.15	0.07
SCC, $\log_{10}$ cells/mL	5.06 ± 0.08	5.10 ± 0.08	5.13 ± 0.08	5.15 ± 0.08	0.79	0.77	0.57	0.59

[1]Data in relation to control (set 100) are given in parentheses.

[2]C = control group: cows were dried off 56 d before calving and milked twice daily throughout next lactation (305 d; n = 12); ODM = once daily milking group: cows were dried off 56 d before calving and milked once daily for the first 4 wk of lactation and twice daily for the remaining lactation (n = 12); CM = continuous milking group: cows were milked twice daily until calving and also during the subsequent lactation (n = 12). CM+ = CM group, but for calculation of LSM of total lactation values, the extra days of milking (from wk −8 up to day of calving) were included. Values in parentheses represent the timeframe relative to calving from which data were obtained.

[3]Means are different among groups ($P < 0.05$).

Table 5. Occurrence of diseases (number of events) in the treatment groups during study[1]

Diagnosis	Treatment group[2] C	ODM	CM	P-value[3]
Mastitis	5 (3, 15, 109, 248, 263)	2 (61, 118)	3 (−7, −5, 60)	0.47
Retained placenta	2 (1)	5 (1)	1 (1)	0.61
Hypocalcemia	0	1 (1)	0	0.56
Ketosis	3 (13, 31, 32)	0	0	0.18
Lameness	2 (33, 53)	2 (86, 88)	0	0.21

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

[2]C = control group: cows were dried off 56 d before calving and milked twice daily throughout next lactation (305 d; n = 12); ODM = once daily milking group: cows were dried off 56 d before calving and milked once daily for the first 4 wk of lactation and twice daily for the remaining lactation (n = 12); CM = continuous milking group: cows were milked twice daily until calving and also during the subsequent lactation (n = 12).

[3]Means are different among groups ($P < 0.05$).

C cows. Two each of C and ODM cows became lame (Table 5).

Blood Analysis

Before Parturition. Before parturition there were no differences in serum concentration of glucose, NEFA, BHBA, and TB among treatment groups except during the last week before calving, when serum NEFA concentrations were higher for ODM cows compared with CM cows (467 ± 88 μmol/L vs. 216 ± 79 μmol/L; $P = 0.025$).

At Parturition. Serum glucose concentration did not differ between groups at parturition. The C cows and ODM cows had higher blood NEFA concentrations compared with CM cows. The C cows had similar blood BHBA concentrations compared with ODM cows but higher blood BHBA concentrations compared with CM cows. Total bilirubin was highest for ODM cows compared with C and CM cows (Figure 2A, B, C, and D; Table 6).

Wk 1 to 4 Postpartum. Blood glucose concentration was lowest for C cows for the first 2 wk of lactation (2.62 ± 0.82 mmol/L at wk 1 and 2.72 ± 0.81 mmol/L at wk 2) compared with CM cows (3.29 ± 0.27 mmol/L at wk 1, $P = 0.006$, and 3.40 ± 0.30 mmol/L at wk 2, $P < 0.001$) and ODM cows (3.16 ± 0.5 mmol/L at wk 1, $P = 0.032$, and 3.29 ± 0.32 mmol/L at wk 2, $P < 0.005$; Figure 2D; Table 6). On average during the first 4 wk of lactation, blood glucose concentration was lowest for C cows compared with ODM and CM cows. During the first 4 wk of lactation, blood serum NEFA concentrations were 44% lower for ODM cows and 66% lower for CM cows compared with C cows. Like NEFA, blood BHBA concentrations for C cows were approximately twice as high as for ODM cows and approximately thrice as high as for CM cows. Simultaneously, CM cows had about half the TB blood concentration of C cows. The latter was comparable to the TB concentration measured in blood serum of ODM cows (Figure 2A, B, and C; Table 6).

Furthermore, considering blood samples collected in wk 1, 2, and 4 of lactation, a proportion of samples was assessed in which established alarm levels of metabolic key parameters in herd medicine were exceeded. A higher percentage of C cows exhibited hypoglycemia compared with ODM and CM cows (47, 20, and 14%, respectively; $P < 0.05$). Moreover, the proportion of C cows with excessive lipomobilization and ketonemia was higher compared with those of the other treatment groups (Table 7).

From wk 5 to 8, glucose concentration did not differ between groups. Concentrations of NEFA and TB were higher for C cows than for ODM cows and CM cows. Blood serum BHBA concentrations were higher in C cows than in CM cows (Figure 2A, B, C, and D; Table 6). No differences between treatment groups were obvious for any of the analyzed blood parameters from wk 12 to 44.

Backfat Thickness and BCS

Backfat thickness was not different among treatments at any time point (Figure 3A). It declined from mo −2 before to mo 2 after calving by 24, 19, and 25% for C, ODM, and CM cows, respectively (i.e., a decrease of on average 5.8 ± 0.7 mm; $P < 0.001$). After calving, backfat thickness declined from mo 1 to 4 by approximately 40% for C cows (21.4 ± 2.3 to 13.1 ± 1.9 mm; $P < 0.001$) and by 17% for ODM cows (23.7 ± 3.3 to 19.6 ± 3.9 mm; $P = 0.06$). The C cows exhibited the most pronounced decrease between calving and mo 3 of lactation, followed by an increase of backfat thickness until mo 10 of lactation by 63% (15.6 ± 2.3 to 25.4 ± 3.3 mm; $P < 0.001$). Backfat thickness increased from mo 4 of lactation to mo 10 in ODM cows by approxi-

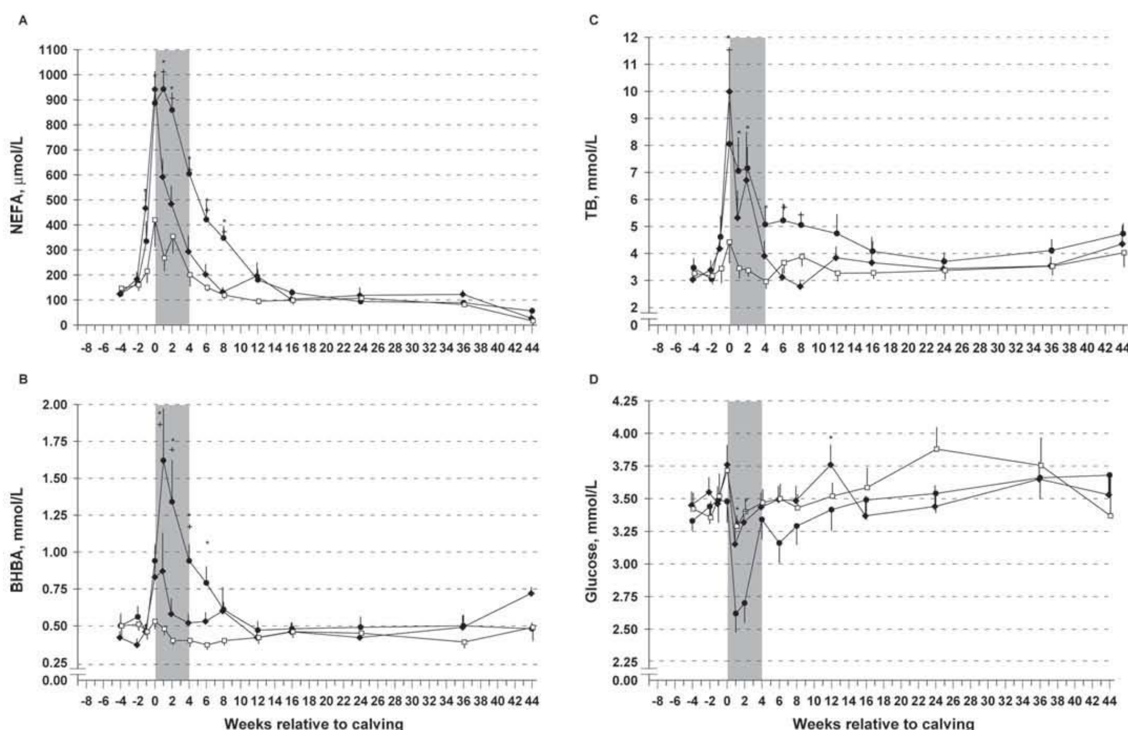

Figure 2. A) NEFA, B), BHBA, C) total bilirubin (TB), and D) glucose concentrations in blood of cows assigned to 1 of 3 different management strategies: 56-d dry period (DP) and twice daily milking after parturition (control, C; ●), 56-d DP and once daily milking (ODM) for the first 28 d of lactation (♦), and 0-d DP with continuous milking (CM) twice daily throughout lactation (□). Asterisks indicate that blood serum concentrations differed between C and CM; plus signs indicate that blood serum concentrations differed between C and ODM; circles indicate that blood serum concentrations differed between ODM and CM ($P < 0.05$). Values are LSM ± SEM. The gray box indicates time of different milking regimens.

mately 28% (22.0 ± 3.8 to 29.4 ± 3.2 mm; $P < 0.001$). For CM cows, increase of backfat thickness started 2 mo earlier compared with ODM cows already at wk 6; backfat thickness increased by 74% (15.7 ± 2.1 to 27.3 ± 2.7 mm; $P < 0.001$).

Before calving, BCS did not differ between C cows at mo −2 (3.7 ± 0.3) and mo −1 (4.0 ± 0.5) relative to calving compared with CM cows (3.5 ± 0.4, $P = 0.84$, and 3.6 ± 0.5, $P = 0.23$ for mo −2 and −1, respectively) and ODM cows (3.7 ± 0.51, $P = 0.51$, and 3.8 ± 0.4, $P = 0.11$ for mo −2 and −1, respectively; Figure 3B). The BCS was lower in the first month of lactation in C cows compared with ODM cows (2.9 ± 0.5 vs. 3.39 ± 0.5; $P = 0.022$) and CM cows (3.3 ± 0.5; $P = 0.03$). Decrease of BCS between the first and the third month of lactation was most pronounced in C cows (0.31 ± 0.09; $P < 0.001$). A smaller reduction in BCS was found for ODM cows (0.20 ± 0.09; $P = 0.92$) and CM cows (0.16 ± 0.09; $P = 0.08$). The BCS of CM cows steadily increased between the first (3.1 ± 0.2) and tenth (3.6 ± 0.1) month of lactation ($P = 0.004$). For C cows, BCS increased by 24% between the third (2.9 ± 0.1) and the tenth (3.6 ± 0.2) month of lactation ($P < 0.001$), whereas BCS for ODM cows increased in this timeframe by 15% (3.3 ± 0.5 to 3.8 ± 0.6; $P = 0.004$).

DISCUSSION

The objective was to characterize the effects of 2 different approaches adopted to reduce metabolic stress throughout the transition period in high-yielding dairy cows. Brown Swiss cows for the treatment groups were recruited from the Technische Universitaet Muenchen herd in which the breeding strategy for a decade was designed to maximize milk protein. Thereby, the protein percentage in the University herd's tank milk (mean during the last 15 yr: 3.69%) is somewhat higher than that of the German Holstein population

Table 6. Least squares means ± SEM of serum metabolites of dairy cows managed with different milking regimens during dry period and early lactation

Item	Treatment group[1] C	ODM	CM	P-value[2] C vs. ODM	C vs. CM	ODM vs. CM
Glucose, mmol/L						
Before parturition	3.50 ± 0.11	3.51 ± 0.12	3.44 ± 0.11	0.96	0.73	0.70
At parturition	3.56 ± 0.25	3.76 ± 0.15	3.74 ± 0.15	0.22	0.51	0.58
Wk 1 to 4	2.95 ± 0.1	3.29 ± 0.1	3.39 ± 0.05	0.04	0.01	0.63
Wk 5 to 8	3.23 ± 0.11	3.49 ± 0.07	3.46 ± 0.07	0.089	0.1	0.92
Wk 12 to 44	3.55 ± 0.08	3.56 ± 0.09	3.64 ± 0.08	0.89	0.39	0.50
NEFA, µmol/L						
Before parturition	177 ± 50	257 ± 51	170 ± 49	0.27	0.92	0.22
At parturition	831 ± 71	868 ± 71	414 ± 75	0.48	<0.001	<0.001
Wk 1 to 4	778 ± 45	435 ± 46	266 ± 45	<0.001	<0.001	0.008
Wk 5 to 8	389 ± 52	172 ± 51	137 ± 52	0.004	<0.001	0.62
Wk 12 to 44	110 ± 133	129 ± 38	91 ± 37	0.70	0.70	0.47
BHBA, mmol/L						
Before parturition	0.48 ± 0.07	0.40 ± 0.07	0.48 ± 0.07	0.80	0.52	0.86
At parturition	0.85 ± 0.09	0.72 ± 0.09	0.53 ± 0.09	0.06	<0.001	0.14
Wk 1 to 4	1.28 ± 0.06	0.65 ± 0.06	0.43 ± 0.06	<0.001	<0.001	0.02
Wk 5 to 8	0.72 ± 0.07	0.57 ± 0.07	0.39 ± 0.07	0.15	0.002	0.08
Wk 12 to 44	0.49 ± 0.04	0.47 ± 0.05	0.44 ± 0.05	0.77	0.49	0.71
TB,[3] mmol/L						
Before parturition	3.62 ± 0.46	3.56 ± 0.46	3.28 ± 0.45	0.92	0.59	0.67
At parturition	7.51 ± 0.58	9.59 ± 0.60	4.24 ± 0.60	0.026	<0.001	<0.001
Wk 1 to 4	6.38 ± 0.42	5.27 ± 0.42	3.23 ± 0.41	0.07	<0.001	0.007
Wk 5 to 8	5.23 ± 0.47	3.06 ± 0.47	3.78 ± 0.47	0.002	0.033	0.28
Wk 12 to 44	4.25 ± 0.29	3.68 ± 0.33	3.39 ± 0.34	0.2	0.06	0.54

[1]C = control group: cows were dried off 56 d before calving and milked twice daily throughout next lactation (305 d; n = 12); ODM = once daily milking group: cows were dried off 56 d before calving and milked once daily for the first 4 wk of lactation and twice daily for the remaining lactation (n = 12); CM = continuous milking group: cows were milked twice daily until calving and also during the subsequent lactation (n = 12).

[2]Means are different among groups ($P < 0.05$).

[3]TB = total bilirubin.

(roughly 3.38%). Furthermore, the implementation of a transponder-based concentrate feeding system and the availability of a superior maize silage induced a significant increase in milk yield during the experimental period compared with former years.

Nine out of 45 cows left the herd because of severe health problems during the study period; this culling rate (20%) was similar to that of previous investigations (Reist et al., 2003). Nevertheless, the remaining group size (n = 12 cows/group) allowed the investigation of treatment effects for blood serum parameters and parameters of milk yield and composition with a statistical power of 0.8 based on previously obtained standard deviations and generally accepted differences of means. However, the present experimental design did not provide sufficient statistical power to evaluate possible differences among groups for health disorders. Statistical power analysis revealed that at least 38 cows for each group were needed for unbiased statistical comparisons of health disorders.

Because of the limited group size, the lower incidence of diseased CM cows compared with C cows did not

Table 7. Percentage of blood samples (36 samples/group) collected in wk 1, 2, and 4 of lactation exceeding established alarm levels of metabolic key parameters in herd medicine of all treatment groups

Parameter	Threshold value[1]	Treatment group[2] C	ODM	CM	P-value[3] C vs. ODM	C vs. CM	CM vs. ODM
Glucose	≤3.0 mmol/L	47.1	20.0	13.9	<0.001	<0.001	0.81
BHBA	≥1.4 mmol/L	29.4	2.9	0	<0.001	<0.001	0.62
NEFA	≥700 µmol/L	41.2	17.1	8.3	0.012	<0.001	0.9

[1]Threshold values are based on our own experience and data of Macrae et al. (2006).

[2]C = control group: cows were dried off 56 d before calving and milked twice daily throughout next lactation (305 d; n = 12); ODM = once daily milking group: cows were dried off 56 d before calving and milked once daily for the first 4 wk of lactation and twice daily for the remaining lactation (n = 12); CM = continuous milking group: cows were milked twice daily until calving and also during the subsequent lactation (n = 12).

[3]Means are different among groups ($P < 0.05$).

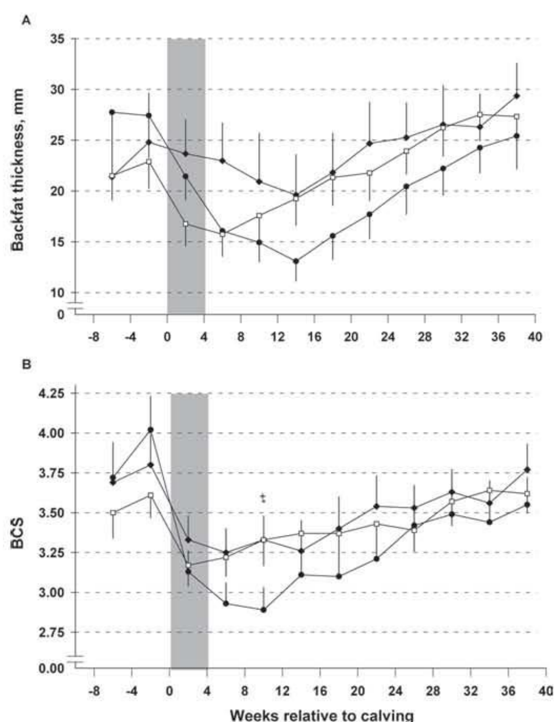

Figure 3. Changes of backfat thickness and BCS from month −2 before parturition to mo 10 of lactation for cows assigned to 1 of 3 different management strategies: 56-d dry period (DP) and twice daily milking after parturition (control, C; ●), 56-d DP and once daily milking (ODM) for the first 28 d of lactation (♦), and 0-d DP and continuous milking (CM) twice daily throughout lactation (□). Asterisks indicate differences between C and CM; plus signs indicate differences between C and ODM ($P < 0.05$).

allow the suggestion that this is a valid difference. Three C cows, but no CM cows, suffered from ketosis; however, with 12 animals used for each treatment, no differences could be confirmed at a significance level of 0.05.

Continuous Milking Compared with a 56-d DP and Twice Daily Milking During Lactation

Milk Yield. Yield of ECM from CM cows averaged 21.2 ± 1.5 kg/d during the last 8 wk before calving. For 1 cow, however, milk yield decreased to less than 3 kg/d in the last week before calving. Two cows developed clinical mastitis at d −7 and −5 and dried off independently at d −2 relative to calving. That contrasts with the observation that up to 50% of all cows assigned to CM dry off between 7 and 2 d prepartum (Andersen et al., 2005; Madsen et al., 2008).

In our study, the 305-d lactation ECM yield was 17% lower in CM cows compared with C cows. This result was comparable with the depression in milk yield systematically entailed by the omission of the DP, as described in the literature (Swanson, 1965; Farries and Hoheisel, 1989; Rémond et al., 1992). Rémond et al. (1997) assumed that high-producing cows are less sensitive to this management system compared with lower producing cows. Our results using cows of high genetic merit did not confirm this hypothesis. This result is in accordance with Andersen et al. (2005). In fact, involution, proliferation, and differentiation of mammary parenchyma during DP represent decisive preconditions for a high subsequent lactation yield (Capuco et al., 1997) independently of the genetic merit of the cow (Smith et al., 1966; Andersen et al., 2005). Accordingly, continuously milking over more than 2 successive lactations reveals more severe production losses (18–29%) in the subsequent lactation (Rémond et al., 1997). The depression of milk yield is caused primarily by an impaired functionality of mammary parenchyma (Annen et al., 2004).

Effects of Continuous Milking on Metabolic Profile. Glucose is a key molecule in ruminant metabolism (e.g., in lactogenesis; Guinard-Flament et al., 2006). Blood glucose concentration reflects the balance between glucose input and output. Hypoglycemia is prevented under physiologic conditions by the effects of different hormones including glucagon, catecholamines, glucocorticoids, and the GH-IGF-I system. In our study, 47% of serum samples collected between wk 1 and 4 from C cows revealed hypoglycemia (<3.0 mmol/L; Table 7). In contrast, only 14% of samples of CM cows collected from wk 1 to 4 revealed hypoglycemia (Table 7). The sustained physiological serum glucose levels in early lactation for CM cows indicate a more stable metabolic status and lower blood serum NEFA concentrations. Similar results were reported by Rastani et al. (2007) during the first week of lactation and by Andersen et al. (2005) and Madsen et al. (2008) during the first 5 wk of lactation for CM cows compared with C cows milked twice daily after a 56-d DP. Because higher NEFA serum concentrations of C cows reflect a higher rate of lipolysis in adipose tissue (Mashek and Grummer, 2003), continuous milking is accompanied by reduced lipomobilization in fresh parturient cows. Simultaneously, serum BHBA concentrations were higher in C cows compared with CM cows because, in case of large amounts of NEFA entering the hepatocytes, ketogenesis represents an alternative metabolic pathway in hepatic mitochondria (Heitmann and Fernandez, 1986). Differences in serum concentration of glucose and BHBA suggest an improved metabolic status for CM cows in early lactation.

The DMI of CM cows was found to be similar (Andersen et al., 2005) or even higher (Rastani et al., 2005) compared with 56-d dry cows. Usually, DP includes diet changes associated with facility and grouping changes. These management changes may contribute to a reduction of DMI. Feeding the same diet throughout the entire lactation–gestation cycle may reduce nutritional stress because of less frequent dietary changes at prepartum and may improve energy status in cows with short or no DP. Further on, lower postpartum milk yield of CM cows may contribute to reduced metabolic stress.

Effects of Continuous Milking on Milk Yield, Milk Composition, and Body Condition. The present study indicated an increased lactational milk protein concentration in CM cows (3.91 ± 0.28%) compared with C cows (3.52 ± 0.3%) in agreement with earlier results (Rémond et al., 2004). This difference was apparent throughout the entire lactation as indicated by an average milk protein content of more than 4.0% for the last 6 wk of lactation in CM cows. The increased milk protein content in early lactation indicates improved availability of energy attributable to a reduced milk yield (Rémond et al., 1997) because milk protein content is considered an indicator for energy supply in a herd monitoring system. It is indeed well established that improvement in the energy balance increases the protein content of milk. Regarding the total produced protein yield, CM cows produced about 25 kg more milk protein than C cows because of being milked in late pregnancy. During this time, while C and ODM cows were dried off, CM cows produced about 42 kg of milk protein. In addition to the high milk protein content throughout lactation, cows of this group produced the highest lactational milk protein yield from d 56 before parturition to the end of lactation.

However, the quality of the colostrum is poor in continuously milked goats (Caja et al., 2006) and cows (Annen et al., 2004) because an accumulation of immunoglobulin level requires prolonged secretion in the mammary gland before calving (Wheelock et al., 1965). Furthermore, there is concern about higher SCC levels in milk of CM cows because SCC was increased in late pregnancy for CM cows, particularly the last week before calving (Farries and Hoheisel, 1989; Rémond et al., 1992). In our study, SCC of CM cows did not differ from that of C cows in either early lactation or during the entire lactation. There were 2 cows with mastitis at d −7 and −1 relative to calving; this may have been caused by the very low milk yield for these cows (<2 kg/d) followed by a lack of intramammary pressure, longer stripping times, and, as a consequence, a higher risk for mastitis for these cows, which are known to be immunosuppressed before and after calving.

In late pregnancy, the milk was progressively enriched with fat and protein, as found by Wheelock et al. (1965) and Rémond and Bonnefoy (1997). Postpartum milk fat concentration was lower for CM cows compared with C cows for the first 5 wk of lactation. This is in contrast with Rastani et al. (2005), who observed that milk fat percentage increased from 3.86 to 4.08% when DP was reduced from 56 to 28 d. The higher milk fat content of C cows in our study may have been caused by a higher infiltration of long-chain fatty acids from blood into milk fat, as found for periparturient cows with a strong lipolysis (Bauman et al., 2006).

The moderate decrease of BCS and backfat in CM cows in early lactation may be the consequence of an improved metabolic status for these cows as indicated by higher glucose, lower BHBA and NEFA concentrations, and higher milk protein content. The C cows, on the other hand, started into early lactation with a BCS of more than 3.5. Thus, these cows seemed to have been overfed during the DP, which is a well-known explanation for postpartum depression of appetite causing stronger lipolysis and proteolysis. The resulting sharp decrease of backfat and BCS was clearly recognizable in C cows (Figure 3).

Once Daily Milking During the First 4 Wk of Lactation Compared with a 56-d DP and Twice Daily Milking During Lactation

Our approach to implementing once daily milking was to improve the metabolic situation of high-yielding dairy cows after calving in a high-cost, high-return production system. This is contrast to most other studies testing the effects of once daily milking that focus on the implementation of once daily milking to improve quality of life of the farmer or as a tool to increase labor productivity, for example, in low-cost, low-return dairy systems in New Zealand (Clark et al., 2006). Few studies exist in which once daily milking was implemented during early lactation (Carruthers and Davis, 1993; Stelwagen and Knight, 1997). Interestingly, high-producing cows do not necessarily exhibit greater reductions of milk yield because of once daily milking compared with substandard cows (Davis et al., 1999). During once daily milking compared with twice daily milking, a reduction in milk yield of 19% on average is well established (Davis et al., 1999).

Only 1 recent study compared the effects of once daily milking with thrice daily milking on the energy status of the dairy cow in early lactation (Patton et al., 2006). In that study, however, the production level was much lower compared with that of the high-yielding dairy cows used in our trial.

Effects of Once Daily Milking on Metabolism and Body Condition. Interestingly, in the study presented here, once daily milking had almost similar effects as continuous milking on metabolism and productivity in the subsequent lactation, but the effects were obviously attributable to different causes. The blood concentrations of metabolic key parameters did not differ during the first 4 wk of lactation from those of CM cows but were lower compared with C cows. In particular, the decrease of serum glucose concentration at wk 2 observed in C cows was not observed in ODM cows (Figure 2D). The higher blood serum glucose concentrations for ODM cows indicated less severe metabolic stress compared with those of C cows. The results are in agreement with previous studies that demonstrated that once daily milking for about 4 wk in early lactation results in reduced milk yield and less metabolic stress (Davis et al., 1999). At calving, ODM cows had blood NEFA concentrations more than twice as high as CM cows. At this time, ODM cows are in practically the same metabolic situation as C cows. However, just 1 wk after calving, ODM cows had higher blood glucose concentrations and lower NEFA and BHBA blood concentrations compared with C cows. The ODM cows lost less body reserves during early lactation than reported elsewhere (Rémond et al., 2002). Even after switching to twice daily milking starting at wk 5 of lactation, body weight loss in the subsequent months was less pronounced in ODM cows compared with C and CM cows. Rémond et al. (2004) found that BW and BCS of cows milked once daily were gradually improved compared with those of cows milked twice daily at wk 36 of lactation.

Effects of Once Daily Milking on Animal Welfare and Health. Animal well-being during once daily milking in fresh cows is an important issue. Generally, it is observed that high-yielding dairy cows wait near the milking parlor before milking time and seem to expect milking, probably because of high intramammary pressure. In our study, however, we did not observe in ODM cows any indicators revealing severe discomfort, a finding in agreement with other research (Davis et al., 1999; Rémond et al., 2004; Tucker et al., 2007). One of the 12 cows mooed and lowed during the first few days of once daily milking treatment before and during afternoon milking, but after 3 d the animal accepted once daily milking without problems. There were 2 leaking cows in this group; 1 leaked because of teat damage. A typical concern in respect to once daily milking is udder health because udder distension in cows milked once daily may lead to a higher probability of inflammatory responses. One study indicated a doubled SCC in comparison with twice daily milking when cows were milked once daily for a longer time (Lacy-Hulbert et al., 2005). However, no differences were obvious in the incidence of mastitis for ODM cows compared with C or CM cows in our study, which corresponded with others (Clark et al., 2006). Rémond et al. (2004) found no differences in mean SCC between once and twice daily milking despite a sharp increase noted for cows milked once daily during the last third of lactation. Loiselle et al. (2009) found differences neither for SCC nor for chemotaxis, phagocytosis, or oxidative burst activity during once daily milking in the first week postpartum compared with twice daily milking or thereafter up to wk 14 of lactation.

Despite the similar body condition of ODM and C cows at calving, ODM cows lost less body reserves at the onset of lactation (Figure 3B) than did C cows. The improved nutritional and metabolic condition status of ODM cows is also reflected by a lower frequency of hypoglycemia during the first 4 wk of lactation compared with C cows.

Effects of Once Daily Milking on Productivity. The obvious reduction of total lactation ECM yield by 16% for ODM cows compared with C cows was primarily attributable to the reduced yield during the first 4 wk of lactation, with a smaller carryover effect after resumption of twice daily milking during the following 4 wk. Losses reported in previous experiments ranged from 7 to 34% for once daily milking experiments, with an average yield loss of about 19% (Davis et al., 1999; Tucker et al., 2007). Rémond et al. (2004) found a reduction of total 305-d lactation milk yield of about 30% for cows milked once daily compared with those milked twice daily throughout a 305-d lactation. This reduction of milk yield may be caused by functional changes in the mammary gland: mammary cell number and metabolic activity, tight junction permeability, regulation of alveolar cell activity by feedback inhibitor of lactation, and apoptosis stimulating factors. Because these processes are essential to the proper functioning of the mammary gland, it has been suggested that milking frequency affects secretory alveolar cell activity (Vetharaniam et al., 2003) as epithelial cells begin to regress after a variable period of time and are more likely to senesce when longer intermilking intervals are imposed (Capuco et al., 2003). Changes in mammary cell activity are often measured as changes in the mRNA level and the activity of several key mammary enzymes. Furthermore, the reduced milk yield of ODM cows may be caused by a reduced alveolar capacity provoked by longer intermilking intervals (Davis et al., 1998). The effects of increasing and decreasing milking frequency on functionality of the mammary gland are excellently reviewed by Stelwagen (2001). Interestingly,

SCC was not affected by once daily milking compared with twice daily milking in C cows, in accordance with earlier reports (Rémond et al., 2004).

The lactation curve of ODM cows was remarkable compared with C cows in respect to a reduced peak yield, a 6-wk-delayed time of peak yield, and a better persistency throughout the remainder of lactation. This is exactly the lactation curve that is favorable for dairy farmers but difficult to achieve by breeding programs (Togashi and Lin, 2003; Togashi and Lin, 2004). Persistent cows are more desirable because they are more efficient in roughage usage and suffer less metabolic stress because of high peak yield, and thus are more resilient to production diseases (Jakobsen et al., 2002). This may be attributable to the fact that the risk for metabolic disturbances depends largely on the increase of milk yield during the first weeks of lactation (Drackley, 1999). Lower NEFA and BHBA concentrations combined with a later peak yield and a better persistency revoke the reduced milk production in early lactation.

Besides milk yield, milk protein content is a matter of growing economic interest to the dairy processing industry compared with all other milk components. It must be emphasized that continuous milking as well as once daily milking are useful tools for producing milk with higher milk protein content and a higher price compared with traditional management of dairy cows. Also, Patton et al. (2006) demonstrated a higher milk protein concentration (3.55%) and milk fat concentration (4.70%) during the first 28 d of lactation for cows milked once daily compared with cows milked thrice daily. In our study, once daily milking was associated with the highest fat concentration during the first 4 wk of lactation. Because of the lower milk yield, ODM cows produced only three-fourths of the daily fat yield produced by C cows. However, no changes in milk protein concentration were found in short-term studies (once daily milking for 1 wk; Guinard-Flament et al., 2007; Loiselle et al., 2009).

Comparisons Between Continuous Milking and Once Daily Milking. Both once daily milking and continuous milking exhibited profound effects on metabolism and productivity. For both treatments, an improved metabolic status in early lactation was obvious as indicated by a reduced incidence of hypoglycemia and lipomobilization. Extent of reduced lactational milk yield was comparable for both treatments.

Once daily milking is furthermore a management system often chosen in pasture-based, low-cost, low-return farming systems. There are many economical aspects and lifestyle choices involved in the choice of milking frequency because certain fixed costs are associated with milking, including consumables and power. Our results indicate that once daily milking is furthermore appropriate in high-yielding dairy cows. In contrast, the additional milk produced during the extra 56 d in the previous lactation for the CM cows (about 1,200 kg) and the higher milk protein yield compared with prepartum dried-off C cows added additional value to this management scheme, whereas problems related to the colostral supply of calves had a negative effect. In the present study, continuous milking was conducted during 1 gestation–lactation cycle. This approach is not practical for consecutive lactations because most cows dry off independently after more than 2 successive lactations without a DP (Rémond et al., 1997). The well-known challenges of maintaining health and well-being in high-yielding dairy cattle suggest that there may be management alternatives to a 56-d DP and twice daily milking.

CONCLUSIONS

The rapid increase of milk yield after calving represents an important risk factor for metabolic disturbances in conventionally reared, high-yielding dairy cows after a 56-d DP. Both continuous milking without DP and once daily milking during the first 4 wk of lactation represent management strategies to successfully reduce the metabolic stress in fresh cows, combined with an improved metabolic balance and increased milk protein content throughout the entire lactation. The lactational ECM yield (305 d) was decreased, however, in both the ODM and CM cows by roughly 20% compared with C cows. The proportions of hypoglycemic and ketotic cows were lower in the CM group than in the C and ODM groups. The lactation curve for ODM cows peaked later and proceeded flatter. The optimal duration of once daily milking in high-yielding dairy cows remains an issue for further investigation. Milk protein content was markedly increased in CM cows (+0.5%) and, to a lesser extent, in ODM cows (+0.3%). Thus, considering the actual pricing system for milk, continuous milking and once daily milking may be meaningful, economical alternatives for dairy farmers.

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1 **INTERPRETATIVE SUMMARY**

2 Omission of dry period or milking once daily affects metabolic status and is reflected by
3 mRNA levels of enzymes in liver and muscle of dairy cows. ***By Schlamberger et al***.
4 The study investigated the effect of poor or improved metabolic status of transition cows
5 triggered by different milking regimes in late gestation or early lactation on transcript
6 abundance of genes that are involved in central metabolic pathways such as glucose, lipid
7 and protein metabolism. Improved metabolic status was reflected by moderate changes of
8 gene expression in liver or muscle of dairy cows, cows with poor metabolic status showed
9 a stronger adaptation to lactation.
10

1 **Running title:**
2 EFFECTS OF MILKING REGIMES ON mRNA LEVELS IN LIVER AND MUSCLE OF
3 DAIRY COWS
4
5 **Omission of Dry Period or Milking Once Daily affects Metabolic Status and is**
6 **reflected by mRNA Levels of Enzymes in Liver and Muscle of Dairy Cows**
7
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1 **ABSTRACT**

2 The objective of this study was to profile mRNA expression of hepatic and muscular genes
3 of high yielding dairy cows with poor or improved metabolic status in early lactation
4 triggered by different milking regimes. C-cows (n=12) were dried off 56 days before
5 expected date of calving and were milked twice daily after calving, ODM-cows (n=12)
6 were dried off similar to C-cows, but these cows were milked only once daily during the
7 first four weeks of lactation. CM-cows (n=12) were milked twice daily throughout
8 gestation lactation cycle. Liver biopsy samples were obtained one week before expected
9 day of calving, within 24 hours after calving, at week 1, 2, 4, and 8. Muscle biopsy
10 samples were taken within 24 hours after calving, at week 6 and 16. Liver samples were
11 analyzed for mRNA abundance of genes related to carbohydrate metabolism
12 (phosphoenolpyruvate carboxykinase **PEPCK**, citrate synthase **CS**) and fatty acid
13 metabolism (carnitine palmitoyltransferase 1A **CPT 1A**; acylcoenzyme A dehydrogenase
14 very long chain **ACADvl,** Glycerol-3-phosphate acetyltransferase **GPAM**), protein
15 metabolism (tyrosin-amino-transferase **TAT**; cathepsin L β **CTSL β**), and glucose
16 transporter 2 (**Glut2**). Samples of muscle tissue were analyzed for mRNA abundance of
17 genes related to fatty acid oxidation (acylcoenzyme A dehydrogenase very long chain
18 **ACADvl**, EnoylCoA hydratase **EnoylCoA**), muscle function (lactate dehydrogenase
19 **LDH;** α actin **ACTA1**), glucose transporter 1 and 4 (**Glut1 and Glut4**), and the insulin
20 receptor β (**IRβ**). Metabolic status of cows based on blood glucose, NEFA and BHBA
21 concentrations was reflected by mRNA changes of ACADvl, CPT 1A, GPAM and PEPCK
22 in liver. In general, C-cows showed a stronger adaptation to lactation, whereas CM-cows
23 seemed easily to adapt to lactation, shown by moderate changes of transcript abundance of
24 hepatic genes. In skeletal muscle, mRNA levels of glucose transporters were not affected
25 by omission of dry period or reduced milking frequency in early lactation. Gene expression
26 related to fatty acid metabolism (ACADvl, EnoylCoA) showed no variation over time or

among groups, except that EnoylCoA was markedly upregulated in C-cows at wk 6 compared to ODM- and CM-cows and remained on a higher level at wk 16 for this group. In all cows mRNA levels of LDH were up-regulated at wk 16 compared to wk 6 and compared to calving. Significant negative correlations were found both for all cows, among NEFA blood concentration and mRNA level of LDH in muscle. Daily milk yield and mRNA level of LDH in skeletal muscle were positive correlated for all groups. Significant positive correlations were found among milk yield and hepatic PEPCK transcript abundance (r = 0.47) and among NEFA blood concentrations and CPT 1A and ACADvl transcript abundance (r = 0.49 and r = 0.43, respectively), but only for C-cows.

Our findings suggest that cows with a poor metabolic status experience more adaptative performance than cows without a dry period or milked only once daily 4 wks after calving. For these cows, especially CM-cows, metabolism seemed to be more adapted to lactation as indicated by moderate changes of transcript abundance of key enzymes related to glucose and lipid metabolism.

Key words: dairy cow, continuous milking, dry period, once daily milking, metabolism, gene expression.

1 **INTRODUCTION**

2 With the onset of lactation, an extensive array of physiological adaptations occur in a
3 coordinated manner to support the synthesis of large quantities of milk and preserve
4 metabolic homeostasis. The transition from pregnant and nonlactating to nonpregnant and
5 lactating can increase nutritional requirements of the high-producing dairy cow by 4-fold
6 (Drackley et al, 2001). However, these increased requirements are not accompanied by an
7 immediate or sufficient increase in feed intake because feed intake usually decreases at the
8 onset of parturition and the subsequent rate of increase is not as rapid as the increase in
9 milk yield (Bell and Bauman, 1997). Due to this, cows experience a period of significant
10 metabolic imbalance in early lactation (Bell and Bauman, 1997). Milk yield and
11 reproductive performance are compromised if the magnitude and duration of this period of
12 insufficient nutrient supply are prolonged. Health problems during the periparturient period
13 can easily erase the entire profit potential for an individual cow in that lactation (Drackley,
14 1999). So the importance of a successful transition from late pregnancy to early lactation is
15 unequivocal. There are two strategies to improve the metabolic status of the high yielding
16 dairy cow in early lactation besides enhancing energy density of diet and nutrient intake:
17 Omission of dry period (DP) or reducing milking frequency in early lactation. Omission of
18 DP resulted in improved metabolic imbalance in early lactation, elevated serum glucose
19 concentrations and reduced postpartum serum BHBA and NEFA concentrations (Andersen
20 et al., 2005; Schlamberger et al., 2010). However, 15 % loss of milk production in the
21 subsequent lactation has consistently been reported in cows that were continuously milked
22 compared to those having a 56-d DP (Schlamberger et al., 2010). Milking frequency is a
23 factor that affects both milk yield and metabolic disturbances around calving (Rémond and
24 Pomies, 2005). Milking high yielding dairy cows once daily during the first four weeks of
25 early lactation leads to lower rate of hypoglycemic blood serum concentrations compared
26 to those with a 56-d DP and milking twice daily postpartum (20 % vs. 47 %, respectively,

1 Schlamberger et al., 2010). The mentioned study showed that functional metabolic
2 parameters and productivity of dairy cows may be influenced by the pre- and postpartum
3 milking regimen. Continuously milked cows showed a lower ratio of hypoglycemic blood
4 serum samples from wk 1 to 4. It is not yet known, if this improved metabolic status after
5 calving is due to less dietary changes followed by moderate feed intake depression or only
6 is due to the lower nutrient output postpartum by lower milk yield compared to C-cows.
7 Metabolic status in early lactation was also improved by milking cows once daily for the
8 first four weeks of lactation.
9 During the transition period where large day-to-day changes in regulating factors are the
10 norm, we still have a limited body of data on these regulating processes that result in
11 improved or poor metabolic status. Therefore, the current paper is intended to clarify if
12 metabolic status in early lactation affected by different milking regimes is reflected by
13 mRNA levels of some key enzymes that are involved in central metabolic pathways in
14 liver or in muscle. Gluconeogenesis (PEPCK), β-oxidation (CPT 1A, ACADvl),
15 glycerolipid biosynthesis (GPAM) or protein metabolism (TAT, CTSLb) are central
16 pathways in liver tissue, accompanied by glucose release from hepatocytes into blood
17 (Glut2) and from blood into cells in peripheral tissue like skeletal muscle (Glut1 and
18 Glut4). Glut1 and Glut4 are well studied glucose transporters, as they act as shuttles to
19 move sugar across the cell surface. Glut4 which is thought to play a key role in glucose
20 homeostasis due to the regulation of its activity by insulin is an important rate limiting step
21 for glucose transport (Hocquette and Abe, 2000). Insulin independent glucose transporter 1
22 (Glut1) accounts for basal muscle glucose demands and is reported to be the predominant
23 facilitative glucose transporter in the mammary gland (Zhao et al., 1996). Other enzymes
24 like LDH (energy metabolism), ACTA 1 (muscle protein), and most important the insulin
25 receptor like IRβ are key factors of metabolic processes that are located in skeletal muscle,
26 but we still have a limited body of data for changes in expression of transcript abundance

of these enzymes during the transition period and as a subject to different milking regimes as described earlier (Schlamberger et al., 2010).

Our objective was to determine how management of DP and milking frequency in early lactation both to reduce metabolic stress in early lactation affect mRNA levels of hepatic genes, genes involved in β-oxidation and glucose transporters in liver and skeletal muscle. We hypothesized that changes in hepatic gene expression associated with the homeorhetic coordination of metabolic adaptation to the onset of lactation would be altered by improved metabolic status triggered by milking during late gestation or milking once daily for the first four weeks of lactation. We focused on genes associated with the lipid, protein and glucose metabolism.

1 **MATERIAL AND METHODS**

2 Experiments were performed on 45 multiparous Brown Swiss Dairy Cows at the Research
3 farm Veitshof of the Technische Universitaet Muenchen, Freising, Germany.
4
5 ***Animals and Feeding***
6 The experiment from which samples were obtained has been reported previously
7 (Schlamberger et al., 2010). In short, exclusively multiparous cows were assigned at
8 random to one of three treatment groups: cows of the control group (control, **C;** N = 12)
9 were dried off 56 d before expected calving and milked twice daily throughout the whole
10 lactation (305 days). Cows of the second group (once daily milking; **ODM,** N = 12) were
11 dried off 56 d before expected calving, milked only once a day for the first 4 wks of
12 lactation and thereafter twice daily for the rest of lactation. Cows of the third group
13 (continuously milking, **CM;** N = 12) were not dried off, i.e. these cows were milked twice
14 daily up to the day of calving and thereafter throughout the subsequent 305-d lactation.
15
16 All cows were kept in a cubicle housing system fitted with rubber coated slatted floors and
17 bedded with wood shavings. Cows were milked in a 2 x 2 tandem milking parlour (GEA
18 WestfaliaSurge GmbH, Boenen, Germany), either once (1545 h) or twice daily (0415 and
19 1545 h). For drying off (C-cows and ODM-cows only), milking was done once daily (1545
20 h) for 3 d, and then an intramammary antibiotic treatment containing 1 g cloxacillin
21 (Orbenin®, Pfizer, Berlin, Germany) was given 56 d before expected calving. Thereafter,
22 C-cows and ODM-cows were transferred to a separate stable and fed the DP ration
23 (**Table 1**). Approximately 1 wk before expected calving, all cows were moved to a single
24 calving pen bedded with barley straw. After calving, cows were moved to the lactating
25 herd and fed the lactation diet (**Table 1**). The CM-cows were not separated from the herd
26 during the last 2 mo of pregnancy, as they were milked throughout the entire pregnancy-

1 lactation cycle. All lactating cows were fed the partly mixed ration (**PMR**) formulated to
2 meet nutrient requirements, as indicated by GfE (2007), and had *ad libitum* access to fresh
3 water. In addition, hay was fed immediately after milking (0600 h). The PMR, formulated
4 on the basis of a milk yield of 22 kg/d, was delivered once daily (0700 h) and intended to
5 provide *ad libitum* intake (> 5% residual feed). Cows producing more than 22 kg/d were
6 fed additional concentrates (0.5 kg concentrate per kg milk; maximal 9 kg/d, Raiffeisen
7 Kraftfutterwerke Süd, Würzburg, Germany) in feeding stations.
8
9 ***Sampling and Analyzing***
10 Muscle tissue samples of musculus semitendinosus (approximately 600 mg) were removed
11 from the animals by an open muscle biopsy procedure at three times of lactation: within 24
12 hours after parturition, at week 6 and 16 of lactation. Cows received a local subcutaneous
13 anesthesia (5 ml, Procasel®, Selectavet, Weyarn, Germany) and caudal epidural anesthesia
14 (7 ml Procainhydrochlorid (2 %), Procasel®, Selectavet, Weyarn, Germany). Samples
15 were cut free of visible connecting tissue and divided into two aliquots, frozen in liquid
16 nitrogen, and stored at -80° until analysis of mRNA levels.
17
18 Liver biopsies were obtained from all cows at wk -1 relative to calving, within 24 hours
19 after calving and at wk 2, 4, and 8 of lactation by blind percutaneous needle biopsy
20 (Bard®Magnum™, Covington, USA). A field of 15 x 15 cm² was shaved, washed and
21 degreased with 70 % ethanol and disinfected with iodine solution. Local anesthetic (5 ml
22 Procasel ®, Selectavet, Weyarn, Germany) was used to desensitize skin and underlying
23 body wall and muscle. A small incision was made through the skin at the intersection of a
24 line running from the tuber coxae to the shoulder joint with the 11th and 12th intercostal
25 space (Buckley et al., 1986) and was just large enough to admit the trocar. Liver tissue

1 (nearly 100 mg) was directly given into cryo-cups, frozen in liquid nitrogen and stored at -
2 80°C until RNA extraction.
3
4 Blood samples from all cows were collected by jugular venipuncture at wk -4, -2, and -1
5 before expected calving, within 24 h postpartum (wk 0), and at wk 1, 2, 4, 6, 8, 12, 16, 24,
6 36, and 44. Samples were collected into evacuated tubes (Vacuette®, Greiner Bio-One,
7 Kremsmünster, Austria) at 0700 (after milking) and within 1 h blood serum was separated
8 by centrifugation (2,000 x *g*, 4 °C, 15 min). Three aliquots (1.5 mL) of serum were stored
9 at -20 °C.
10 Total ribonucleic acid (RNA) was isolated from 50 mg tissue samples according to the
11 manufacturer's instructions of peqGOLD TriFast^{TM} (peqlab Biotechnologie GmbH,
12 Erlangen, Germany). RNA was quantified by spectrophotometry (Nanodrop, Eppendorf,
13 Hamburg) obtaining an OD 260/280 ratio of 1.7 to 2.0 for all samples and diluted in 50 µl
14 RNase-free water. Degradation of the RNA was measured with the Agilent 2100
15 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) in connection with the RNA
16 6000 Nano Assay.
17
18 Constant amounts of 1 µg RNA were reverse transcribed respectively to cDNA using the
19 following RT master mix: 12 µL 5×Buffer (Promega, Mannheim, Germany), 3 µL Random
20 Hexamer Primers (50 mM; Invitrogen, Carlsbad, USA), 3 µL dNTP Mix (10 mM;
21 Fermentas, St Leon-Rot, Germany) and 200 U of MMLV H- Reverse Transcriptase
22 (Promega, Mannheim, Germany).
23 The reverse transcription reaction was carried out according to the manufacturer in a 60-µL
24 reaction volume in a PCR thermocycler (Biometra, Goettingen, Germany) and was
25 achieved by successive incubations at 21 °C for 10 min and 48 °C for 50 min, finishing
26 with enzyme inactivation at 90 °C for 2 min.

Target genes muscle

The enzymes selected and measured involved in lipid metabolism of muscle were acyl-coenzyme A dehydrogenase very long chain (**ACADvl**), EnoylCoA hydratase (**EnoylCoA**), both involved in β-oxidation of fatty acids. Lactate dehydrogenase (**LDH**) is part of energy metabolism. It converts pyruvate, the final product of glycolysis to lactate when oxygen is absent or in short supply, and it performs the reverse reaction during the cori cycle in the liver. Glucose transporters **GLUT1** and **GLUT4** are involved in glucose transport: The insulin independent glucose transporter **GLUT1** is predominantly located in the muscle cell plasma membrane, and accounts for the basal glucose supply of the myocyte. The insulin-regulated glucose transporter **GLUT4** recycles between the muscle cell plasma membrane and an intracellular tubulovesicular pool, where it is associated with cytoplasmic vesicles. Actin alpha 1 (**ACTA1**) is a protein that is involved in cell motility, structure and integrity. It is a major constituent of the contractile apparatus. The insulin receptor β (**IRβ**) is a transmembrane receptor that is activated by insulin. Insulin receptor β substrates binding and phosphorylation eventually leads to an increase in the high affinity glucose transporter (**GLUT4**) molecules on the outer membrane of insulin-responsive tissues, including muscle cells and adipose tissues, and therefore to an increase in the uptake of glucose from blood into these tissues.

Target genes liver

Most important for hepatic adaptation is phosphoenolpyruvate caboxykinase (**PEPCK**), which is described as ratelimiting enzyme for hepatic gluconeogenesis (Greenfield et al., 2000). Hepatic oxidation of long chain-fatty acids occurs in mitochondria and peroxisomes (Drackley et al., 2001). Mitochondrial fatty acid oxidation involves 4 key steps: 1) uptake and activation of fatty acids to fatty acylCoA, 2) translocation of fatty acyl-CoA into the

mitochondria, 3) β-oxidation of fatty acyl-CoA, and 4) ketogenesis. Carnitine palmitoyltransferase 1A (**CPT 1A**), an integral protein located on the outer mitochondrial membrane, catalyzes the formation of fatty acyl-carnitine from fatty acyl-CoA and carnitine and is believed to be a key regulatory step in metabolism of long-chain fatty acids (McGarry and Brown, 1997). Glycerol-3-phosphate acetyltransferase (**GPAM**) catalyzes the initial and committing step in glycerolipid biosynthesis and is predicted to play a pivotal role in the regulation of cellular triacylglycerol and phospholipid levels. Before glycerol can enter the pathway of glycolysis or gluconeogenesis (depending on physiological conditions), it must be converted to the intermediate glyceraldehyde 3-phosphate, which is mediated by GPAM. Acyl-coenzyme A dehydrogenase, very long chain (**ACADvl**) catalyzes the initial step of each cycle of fatty acid β-oxidation. The protein encoded by Cathepsin Lb (**CTSLb**) is lysosomal cysteine proteinase that plays a major role in intracellular protein catabolism. Tyrosin-amino-transferase (**TAT**) is involved in the frist step of AA degradation. Glucose transporter 2 (**GLUT2**) is a transmembrane carrier protein that enables passive glucose movement across cell membranes. It is the principal transporter for transfer of glucose between liver and blood. The enzyme citrate synthase (**CS**) stands as a pace-making enzyme in the first step of the citric acid cycle. Oxalacetate will be regenerated after the completion of one round of the citric acid cycle.

Intron-spanning primers were designed (**Table 2**) using Primer3 online-software and synthesized by Metabion International AG (Martinsried, Germany) to amplify cDNA from the above mentioned genes except for Glut1 and Glut4, which were according to Komatsu et al. (2005). For the LightCycler reactions a master mix of the following reaction components was prepared to the indicated end concentration: 6.4 µl water, 1.2 µl $MgCl_2$ (4 µM), 0.2 µl Forward Primer (0.4 µM), 0.2 µl Reverse Primer (0.4 µM) and 1.0 µl LightCycler DNA Master SYBR Green I (1À). 9 ml of LightCycler master mix was filled

1 in the LightCycler glass capillaries and 25 ng reverse transcribed total RNA in 1 µl was
2 added as PCR template. The capillaries were closed, centrifuged in a microcentrifuge, and
3 placed in the LightCycler rotor (Roche). A conventional LightCycler amplification cycle
4 contains three segments: In the 1st segment DNA is denaturized at 95 °C. In the 2nd
5 annealing segment the primer annealing takes place and the chosen temperature should be
6 as high as possible to improve specificity. Within the following 3rd elongation segment at
7 72 °C, the elongation time should be adapted to the length of the desired product, which is
8 limited by Taq Polymerase processing rate (1000 bp/min elongation time). The following
9 LightCycler protocol was used for real-time PCR: *denaturation program* [95 °C for 30 s],
10 a 3 segment *amplification and quantification program* repeated 40 times [95 °C for 3 s;
11 60 °C for 10 s; 72 °C for 10 s]; *melting curve program* [60 °C to 95 °C with a heating rate
12 of 0.1 °C/s] and a final *cooling program* down to 40 °C. The display mode and the
13 fluorimeter gains of channel 1 were set to 5. Amplified products underwent melting curve
14 analysis after the last cycle to ensure integrity of amplification. Interassay and intraassay
15 CV for qRT-PCR of target and reference genes were lower than 1 %.
16
17 ***Statistical Analysis***
18 For valid comparisons between functional parameters of metabolism and productivity on
19 the on side and mRNA levels of genes encoding for specific metabolic pathways on the
20 other side, data for milk yield and fat yield presented here originate from day of sampling
21 and are not pooled weekly means. So, corresponding daily milk yield and daily fat yield as
22 well as NEFA, BHBA and glucose concentrations were available at each biopsy time
23 point. Blood serum data were presented for time points with a corresponding sample of
24 biopsy.
25 Data of mRNA were analyzed using the second derivate maximum method described in
26 the LightCycler Relative Quantification Software, obtaining a crossing-point (Cq) value

for each gene on each animal at each time point. These values were translated to normalized expression quantities (ΔCq) using two reference genes for each tissue in a form of normalization index (glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and Histone in liver tissue samples, and Histone and Ubiquitine in muscle tissue samples (Table 2 for primer sequences). Δ quantitative Cycle (Cq)-values were calculated as:

$$\Delta Cq = Cq_{\text{target gene}} - \text{mean}Cq_{\text{reference genes}}$$

For **liver tissue**, a relative expression index ($\Delta\Delta$Cq-values) was calculated according to:

$\Delta\Delta Cq = \Delta Cq_{\text{timepoint (calving to wk 8)}} - \text{mean}\Delta Cq_{\text{(week -1)}}$ to detect regulations within groups between times (normalized to precalving) or

$\Delta\Delta Cq = \Delta Cq_{\text{treatment group}} - \text{mean}\Delta Cq_{\text{control group}}$ to detect regulations within times between groups.

For **muscle tissue**, a relative expression index ($\Delta\Delta$Cq-values) was calculated according to:

$\Delta\Delta Cq = \Delta Cq_{\text{time point (calving or wk 6)}} - \text{mean}\Delta Cq_{\text{week 16}}$ to detect regulations within groups between times.

In order to avoid negative numbers in the table that shows mRNA abundance while allowing an estimation of a relative comparison between two genes, data are presented as means ± SEM subtracted from the arbitrary value 5 for liver samples and 10 for muscle samples (ΔCq). Thus, a high ΔCq resembles high transcript abundance (Livak and

1 Schmittgen, 2001). An increase of one ΔCq represents a two-fold increase of mRNA
2 transcripts.
3 Data of mRNA abundance (log_2) of hepatic and muscular genes were analyzed with a
4 MIXED procedure of SAS (SAS Institute, 2002) as described earlier (Schlamberger et al.,
5 2010), but instead of including week, biopsy time point was included in the same model.
6 The effects of time, group, and time x group interactions were tested.
7 Correlation and linear regression coefficients used to describe relationships between gene
8 expression and plasma metabolites and parameters of productivity were estimated using
9 the CORR procedure. If data were normal distributed Pearson correlation coefficients are
10 shown, if data were not normal distributed spearman rank-order correlation coefficients are
11 shown in the tables. Graphs were plotted, if correlation coefficients and coefficients of
12 determination were > 0.40.
13

1 **RESULTS AND DISCUSSION**

2 ***Reference genes used in liver or muscle samples***

3 The two genes tested to be used as a reference in the relative expression calculations were
4 GAPDH and Histone for liver tissue samples, and Histone and Ubiquitin in muscle tissue
5 samples. In each tissue, both had consistent level of expression across all time points;
6 therefore these genes were used as reference for calculating relative expression. Other
7 studies demonstrated that expression of traditionally used housekeeping genes from bovine
8 liver during late pregnancy and lactation was affected by physiological state, feed intake,
9 and dietary treatment (Janovick-Guretzky et al., 2007). In this study of Janovick-Guretzky
10 et al (2007) β-actin, GAPDH, β-glucuronidase, peptidyl-prolyl isomerase A, polyubiquitin,
11 ribosomal protein S9, ribosomal protein L32, and 18S ribosomal RNA were examined and
12 ribosomal S9 protein was found to be the most stably expressed gene across varying
13 physiological conditions. However, these physiological changes were due to dietary
14 supplement, varying amounts of energy of diet prepartum or suffering from ketosis. In our
15 study, for the above mentioned genes we observed constant transcript abundance for
16 biological samples of all cows despite of differences in metabolic status among groups.
17

18 ***Expression of GLUT4 in musculus semitendinosus:***

19 Under hyperglycemic conditions, insulin is secreted from the pancreas and increases the
20 translocation of cytoplasmatic Glut4 vesicles towards and into the plasma membrane, thus
21 stimulating the transport of glucose into the cells (Kahn, 1996). Glut4 was identified as the
22 main glucose transporter in fat and muscle cells (James et al., 1989). Insulin triggers the
23 movement of the sugar transporter that is found in these cells from an intracellular store to
24 the plasma membrane (Bryant et al., 2002).

25 In our study, Glut4 mRNA levels in skeletal muscle did not differ among groups, but
26 changed over time in all groups. Transcript abundance was higher at wk 6 compared to

1 calving (1.69 fold) and similar to calving at wk 16 (**Table 3**). This is in contrast to findings
2 of Komatsu et al. (2005), who did not detect changes of mRNA level in muscle tissue
3 among the various stages of lactation, e.g. peak, late and non lactating state. One reason for
4 this discrepancy may be the fact that in the study of Komatsu et al. (2005) the whole
5 pregnancy lactation cycle was included, while in our study the first 16 weeks of lactation
6 were in focus. The most metabolic adaptation to lactation occurs within the first third of
7 lactation. While Komatsu et al. (2005) compared peak- and late lactating cows, samples in
8 our study were obtained at time points accompanied by daily milk yield more than 33 kg
9 ECM/d. Peak lactating cows of Komatsu et al. (2005) had a daily milk yield of 26.6 kg/d.
10 It is considered that insulin resistance increases during lactation (Rose et al., 1997) and an
11 insulin deficiency with regard to glucose utilization has been reported in lactating goats
12 (Debras et al., 1989). Insulin-resistance in peripheral tissue may be caused by inhibition of
13 Glut4 translocation to the cytomembrane, not by inhibition of Glut4 expression or Glut4
14 content in general (Komatsu et al., 2005). In humans suffering from insulin-independent
15 diabetes mellitus, muscle cell Glut4 contents are like those of healthy persons, but the
16 ability of insulin to provoke Glut4 translocation into the myocyte plasma membrane is
17 interrupted (Zierath et al., 1998). In our study, mRNA levels of Glut4 were increased at wk
18 16 compared to calving, for ODM-and C-cows, but not for CM-cows. A possible
19 explanation for these findings is that the activity of insulin did not increase the Glut4
20 translocation in myocytes in CM-cows, but in C- and ODM-cows. For this reason, the
21 bovine insulin-independent glucose transport via Glut1 may compensate for the smaller
22 insulin-induced Glut4 translocation.
23 Furthermore, in our study muscle tissue samples were obtained from musculus
24 semitendinosus which is metabolically characterized to be a glycolytic muscle by
25 Duehlmeier et al. (2005). Glut4 contents were 3.0 times lower in musculus semitendinosus
26 (glycolytic) than in musculus masseter and diaphragm (oxidative) of bovine skeletal

1 muscle. They found higher Glut1 contents and lower Glut4 contents in glycolytic muscles.
2 However, we detected higher mRNA levels of Glut4 than of Glut1 in (bovine glycolytic)
3 skeletal muscle, but these findings were exclusively based on transcript abundance of these
4 genes related to glucose transport quantified by qRT-PCR. Although physiological
5 mechanisms cannot be completely explained through RNA abundance, protein expression
6 data have been extensively demonstrated to be largely concordant with gene transcription
7 profiling (Mootha et al., 2003). Higher Glut1 and a lower Glut4 level in glycolytic muscles
8 of lactating cows could be an effective mechanism for maintaining a basic glucose supply,
9 which prevents insulin-sensitive excessive glucose transport into these muscle cells, thus
10 facilitating a preferential insulin-independent glucose supply for the mammary gland.
11 Insulin-dependent glucose transport is mediated through changes in compartmentalization
12 of Glut4 within cells and Glut4 gene expression. The fact that Glut4 is not expressed in
13 bovine mammary epithelial cells supports a lack of insulin-responsive glucose uptake by
14 mammary tissue (Zhao and Keating, 2007). Duehlmeier et al. (2007) found that Glut1 may
15 be of greater importance in the whole body glucose utilization in herbivores with
16 forestomachs than in monogastric omnivores. It would be worthwhile to test the hypothesis
17 of such an interaction between lactation and glycolytic muscle in further experiments on
18 cows.
19
20 ***Expression of Glut1 in musculus semitendinosus:***
21 Insulin independent glucose transporter Glut1 is expressed in many tissues, including
22 brain, kidney, and mammary gland (Burant et al., 1991). It is generally assumed that Glut1
23 accounts for basal muscle glucose demands (Duehlmeier et al., 2005), as it is
24 predominantly located in the muscle cell plasma membrane. Komatsu et al. (2005) could
25 not detect Glut1 protein or mRNA in muscle of lactating or non-lactating cows, but in the

1 mammary gland. There they could detect no difference between Glut1 protein and mRNA
2 abundance at peak and late lactation. In dry cows, Glut1 protein and mRNA were barely
3 detectable in the mammary gland (Komatsu et al., 2005). However, qRT-PCR analysis
4 showed that mRNA levels of bovine Glut1 did not change from calving up to wk 16 of
5 lactation in skeletal muscle in our own study (**Table 3**). It is well known, that Glut1 mRNA
6 in mammary gland increases from at least 5-fold to several hundred-fold from late
7 pregnancy to early lactation and peaks during lactation (Zhao et al., 2007). However, Glut1
8 mRNA levels in skeletal muscle tissue remained unchanged at wk 6 and 16 of lactation,
9 and was unchanged by DP management or milking once daily in early lactation. In our
10 study, transcript abundance of Glut1 seemed to be lower than of Glut4. That´s in contrast
11 to findings of Duehlmeier et al. (2005) who found higher levels of Glut1 content than of
12 Glut4 content in bovine glycolytic muscles. Data from the study of Duehlmeier et al.
13 (2005) strongly indicate that Glut1 is the predominant glucose transporter of bovine
14 glycolytic skeletal muscle. However, Duehlmeier et al. (2005) indicated that fluctuations of
15 plasma insulin and glucose levels seem to require a glucose transport pathway partly
16 independent from an insulin-stimulated uptake to maintain glucose levels in large
17 locomotor muscles of dairy cows. In ruminants, the insulin-independent glucose utilization
18 is of greater importance than in monogastric omnivores, and it may compensate at least in
19 part of the impaired in vivo insulin sensitivity in adult ruminants (Debras et al., 1989).
20 Most important of our findings is that transcript abundance of Glut1 was unchanged by
21 improved metabolic status triggered by milking regimes in late gestation and early
22 lactation.
23
24 ***Expression of LDH in musculus semitendinosus:***:
25 Pyruvate is a central intermediate for energy metabolism, and partitioning of pyruvate
26 metabolism can greatly affect energy supply to the cell. Instead of accumulating inside the

1 muscle cells, lactate produced by anaerobic fermentation is taken up by the liver. If muscle
2 activity has stopped, the glucose is used to replenish the supplies of glycogen through
3 glycogenesis. So LDH is part of the energy metabolism of cells.
4 No previous studies known by the authors have investigated the expression of LDH related
5 to pyruvate metabolism in bovine skeletal muscle. In our study, transcript abundance of
6 the gene encoding LDH was quantified. At the onset of lactation, mRNA levels of LDH in
7 muscle were down-regulated compared with later times of lactation (wk 16), especially for
8 C- and ODM-cows. But we could not detect differences for transcript abundance among
9 treatments (**Table 3**). So, transcripts of LDH constantly rose from calving up to week 16 in
10 control and treatment groups (3.6 fold for control animals and 2.7 fold for ODM and 2.6
11 fold for CM-cows). Higher transcript abundance at later times of lactation (wk 16)
12 compared to earlier times (wk 6) or compared to calving may suggest, that after the first
13 100 days of lactation, the metabolic state of peripheral tissue like skeletal muscles is more
14 stable than at earlier times.
15
16 ***Expression of ACADvl and EnoylCoA in musculus semitendinosus***:
17 ACADvl and EnoylCoA are two enzymes involved in catalyzing the first step of the β-
18 oxidation of long chain fatty acids. We found effects of group on EnyolCoA mRNA levels
19 and effects of time on mRNA levels of ACADvl, but fatty acid metabolism in muscle was
20 not markedly influenced by treatments, as shown by similar and unchanged mRNA levels
21 of these two enzymes (**Table 3**). However, C- and ODM-cows exhibited an increased
22 transcript abundance of EnoylCoA compared to CM-cows at time of calving (1.8 and 1.6
23 fold for C and ODM-cows, respectively). At wk 6, expression of EnoylCoA was highest
24 for C-cows compared to ODM- and CM-cows suggesting that these cows appropriately
25 adjust their metabolism to support increased nutrient requirements of early lactation. At wk
26 16, transcript abundance in muscle of C-cows was higher compared to CM-cows, but not

1 to ODM-cows. These differences may be caused by the improved metabolic status and the
2 lower milk yield of these cows, which did not need the utilization of body fat as energy
3 fuel in the range as C-cows did.
4
5 ***Expression of IRβ in musculus semitendinosus***
6 The insulin receptor (**IRβ**) is a transmembrane receptor that is activated by insulin.
7 Transcript abundance of Insulin receptor beta showed marked variation among groups and
8 across time (**Table 3**). Around calving, mRNA levels were higher in ODM- and CM-cows
9 compared to C-cows. Surprisingly the higher levels of mRNA encoding for the IRβ in
10 ODM-cows at calving were not expected whereas for CM-cows higher levels of IRß might
11 have caused an improved metabolically situation as determined by a higher blood glucose
12 concentration. These cows easily adapt to lactation perhaps by an efficient translocation of
13 glucose into cells by the insulin dependent glucose transporter Glut4. For all groups,
14 transcript abundance of IRβ decreased from calving to wk 6 (1.8 fold) and 16 (2.6 fold).
15 However, mRNA levels of Glut4 in muscle were down-regulated at wk 6 and up-regulated
16 at wk 16 of lactation. That result emphasizes the widely accepted doctrine that in
17 ruminants early lactation is characterized by distinctive metabolic changes including the
18 development of insulin resistance in skeletal muscles and adipose tissue (Bell and Bauman,
19 1997) to maintain mammary gland lactose production. In contrast, Duehlmeier et al. (2007)
20 suggested that this insulin resistance is rather due to a decreased Glut4 expression than a
21 down-regulation of insulin receptors as suggested in our study. Sano et al. (1991) found
22 that during late gestation and early lactation, lowered responsiveness and sensitivity of
23 extra hepatic tissues to insulin facilitate partitioning of nutrients for the rapidly growing
24 fetus and the mammary gland. Significant changes of mRNA levels of IRβ suggest that
25 ODM and CM may improve the basal glucose supply in musculus semitendinosus.
26 However, the question is, if the major site of differences concerning the insulin sensitivity

1 occurs with the insulin receptor or with GLUT4 that recycles between membrane structures
2 and an intracellular tubulovesicular pool.
3
4 ***Expression of ACTA1 in musculus semitendinosus***
5 Actins are highly conserved proteins that are involved in various types of cell motility and
6 are ubiquitously expressed in all eukaryotic cells. Transcript abundance of Acta1 did not
7 differ among groups but was increased at wk 16 compared to day of calving for all groups
8 (**Table 3**). The increase of mRNA levels of muscle protein expression was not unexpected
9 despite of the observed mobilization of body reserves during the first three months of
10 lactation. At wk 16, cows of all groups replenish body reserves as time of peak of milk
11 yield lags behind. The higher transcript abundance at wk 16 compared to day of calving
12 was significant for all groups (3.8 fold for C-cows, 2.5 fold for ODM- and 3.3 fold for
13 CM-cows) and showed that catabol status was altered to anabol status. However, these
14 results might only be valid for the musculus semitendinosus as muscle types respond
15 differently to metabolic status, as has been shown earlier by biochemical approaches
16 (Hoquette and Abe, 2000).
17
18 ***Expression of CPT 1A in liver***:
19 Uptake of NEFA into hepatic mitochondria where oxidation occurs is regulated by CPT
20 1A. Activity of CPT 1A and sensitivity of CPT 1A to malonyl-CoA and methylmaloyl-
21 CoA inhibition during different physiological and pathological states have been
22 investigated by Dann and Drackley (2006). Aiello et al. (1984) showed that CPT 1A
23 activity in dairy cows was greater at d 30 than at d 60, 90, or 180 of lactation. The higher
24 activity of CPT 1A in early lactation was associated with higher rates of gluconeogenesis
25 and ketogenesis, possibly due to a greater negative energy balance in early lactation.
26 Similar to Aiello et al. (1984), Dann and Drackley (2005) showed that CPT 1A activity

1 peaked at 1 DIM and decreased at 21 and 65 DIM. Mizutani et al. (1999) compared CPT
2 1A activity of cows in early (0 to 110 DIM), mid (111 to 220 DIM), and late (220 DIM)
3 lactation and found no difference among stages of lactation. Energy status of the cows at
4 the various stages was not reported by Mizutani et al. (1999). Energy balance among
5 groups may have been similar and therefore no difference in CPT 1A activity would be
6 expected. That's in contrast to our findings where mRNA levels of CPT 1A were higher
7 for CM-cows than for C- and ODM-cows especially before calving (**Table 4**). That's may
8 be the result of an improved metabolic status in early lactation for these cows. We found
9 that transcript abundance was down-regulated for CM-, but not for C- and ODM-cows
10 relative to one week before calving. Oxidation of NEFA and other substrates provides ATP
11 needed for gluconeogenesis. For CM-cows, lower serum NEFA levels combined with
12 reduced mRNA levels of CPT 1A relative to calving compared with C-cows and ODM-
13 cows suggest that there was no need to use body fat as energy fuel in CM-cows. The influx
14 of NEFA from the adipose tissue mobilization 1d after parturition was not in a range that
15 up-regulates the carnitine palmitoyltransferase system, likely be due to the absence of
16 elevated NEFA concentration around calving.
17 Loor et al. (2005) observed increased mRNA expression of CPT 1A 1d after parturition
18 compared to prepartum. That's in accordance to results we found for C-cows for the first 8
19 weeks of lactation and for ODM-cows for the first 4 weeks of lactation. Surprisingly, these
20 cows had the lowest energy corrected milk yield during this time compared with the other
21 groups due to once daily milking. CPT 1A mRNA levels increased for ODM-cows in this
22 group 2 weeks after calving.
23
24 ***Expression of PEPCK in liver***:
25 It is the major criterion of glucose metabolism in ruminants that the majority of dietary
26 carbohydrate is fermented to volatile fatty acids the rumen. Thus, gluconeogenesis

1 represents a major metabolic activity required to maintain glucose homeostasis and is the
2 primary source of glucose for maintenance and productive processes. In our study, levels
3 of PEPCK mRNA were elevated after calving compared to precalving more pronounced in
4 CM-cows. For these cows, we quantified markedly higher levels one week before calving
5 and at wk 2 after calving compared to C- and ODM-cows. At other time points, there were
6 no significant differences with respect to milking regimen (**Table 4**). Similar findings in
7 the literature suggest differences in gluconeogenic enzyme regulation of PC and PEPCK in
8 the transition cow (Greenfield et al., 2000). We found the strongest and rapidly increase of
9 PEPCK transcript abundance for CM-cows around calving, which may suggest that these
10 cows were able to cover demands for gluconeogenesis. We found constantly rising mRNA
11 levels of PEPCK up to wk 8 for C- and ODM-cows, whereas for CM-cows already at wk 4
12 mRNA levels of PEPCK turned to levels lower than one week precalving. Greenfield et al.
13 (2000) observed an increased abundance of PEPCK mRNA following the transition period,
14 but it increased only slowly and only by about 50 % in samples at day 28 postpartum
15 compared with values compared to day -28 prepartum. In our study, mRNA expression
16 was highest for CM-cows before calving and at wk 2 postpartum compared to C- and
17 ODM-cows. Especially C- and ODM-cows showed lower transcript abundance of PEPCK
18 after calving with significant lower levels at wk 2. This time was associated with the
19 highest daily milk yield for C-cows (Figure 1A). This observation could imply an
20 inhibition of PEPCK mRNA expression in hypoglycemic and ketotic cows, possibly by
21 insulin (Hanson and Reshef, 1997). The increasing PEPCK mRNA level with time after
22 calving may represent the incremental glucose demand for milk production (Greenfield et
23 al., 2000). For C-cows, transcript abundance rosed 1.65 fold, but 2.0 fold for ODM and
24 2.94 fold for CM-cows at wk 2 compared to time of calving. For these latest cows, the
25 most obvious up-regulation of gluconeogenesis regulating enzyme occurred and may be
26 followed by an enhanced gluconeogenesis and therefore an improved metabolic status.

1 Gene expression of PEPCK may be affected by propionate as well as lactate and amino
2 acid supply and was therefore not different at all times among groups, but changed
3 significantly over time.
4
5 ***Expression of GPAM in liver***:
6 In our study, GPAM mRNA levels were down-regulated with the onset of lactation in all
7 groups, but up-regulated earlier during succeeding lactation in CM-cows than in C-cows.
8 This is in accordance to Loor et al. (2005), who detected differential expression of
9 glycerol-3-phosphate acyltransferase at specific times during the DP and through peak
10 lactation. Concerted down-regulation of GPAM around parturition channels NEFA toward
11 fatty acid oxidation. Differences among groups are obvious at wk 8 of lactation, when CM-
12 and ODM cows have already higher mRNA levels compared to precalving, although
13 mRNA levels of GPAM were still reduced compared to precalving (wk -1). Glycerol
14 released from adipose tissue during lipid mobilization also represents recycling of glucose,
15 but this recycling occurs over the course of a lactation cycle, not on minute-to-minute basis
16 as for lactate (Drackley et al., 2001). For that reason we found significant changes over
17 time for GPAM mRNA levels in hepatic tissue, especially the higher mRNA levels of this
18 enzyme at wk 8 compared to day of calving showed that glycerol may be an important
19 gluconeogenic precursor as the cow adapts to lactation. During extensive mobilization of
20 adipose triglyceride of approximately 3.2 kg/d, glycerol may provide maximally 15 to
21 20 % of the glucose demand at 4 d postpartum (Bell, 1995). Accordingly dietary
22 circumstances and particularly the metabolic status in early lactation that decrease or
23 increase the amount of adipose tissue mobilized will increase or decrease the contribution
24 of glycerol to gluconeogenesis during early lactation. Furthermore up-regulation of GPAM
25 is a necessary response in mice to accommodate the greater influx of NEFA into liver, i.e.
26 there is an imbalance between fatty acid oxidation and lipid synthesis. The improved

1 metabolic status in CM-cows is reflected by higher mRNA levels of these enzymes in early
2 lactation compared to C- and ODM-cows (**Table 4**).
3
4 ***Expression of ACADvl in liver***:
5 Transcript abundance of ACADvl rose from one week precalving to time of calving 2.04
6 fold and from one week precalving to wk 2 of lactation 0.78 fold for C-cows and 2.56 fold
7 and 0.75 fold, respectively, for ODM-cows ($P < 0.01$). For CM-cows transcript abundance
8 returned to levels similar to precalving at wk 2. Higher mRNA levels of GPAM channels
9 NEFA toward fatty acid oxidation as described above. This was accompanied by increased
10 mRNA abundance of ACADvl in hepatocytes for cows of all groups with the onset of
11 lactation. Milking cows continuously did not really change transcript abundance of this
12 enzyme of fatty acid oxidation in liver. Blood serum NEFA concentration differed at wk 2
13 and 4 between groups after calving and was in a critical range only at calving for C-cows
14 and ODM-cows and at wk 2 only for C-cows. Serum NEFA concentration is followed by
15 an increased β-oxidation which was reflected by expression of genes encoding for this
16 metabolic pathway.
17
18 ***Expression of CS in liver***:
19 The CS catalyzes the condensation of acetyl-CoA and oxalacetate to form citrate, which
20 represents the first step in the citric acid cycle (Voet and Voet, 2004). Transcript
21 abundance revealed no variation for CS subjected either to time during lactation or to
22 group. Surprisingly neither C-cows neither ODM- or CM-cows did show any changes in
23 mRNA encoding for CS (**Table 4**), suggesting that an increase in the activity of the citric
24 acid cycle in response to an increase in acetyl-CoA did not occur as shown by higher levels
25 of BHBA for C-cows at wk 2 and 4 after calving compared to ODM- and CM-cows. We
26 suggest that the lack of difference of mRNA expression of CS among groups and over time

1 in our study showed that for this enzyme no functional regulation was necessary to adapt to
2 metabolic stress. Perhaps other mechanisms work to adapt to requirements of lactation.
3
4 ***Expression of TAT in liver***:
5 The gene tyrosin-amino-transferase catalyzes the decomposition of L-tyrosine. At the end
6 of this reaction where two deoxygenases are necessary, fumarate and acetoacetate were
7 liberated, which can enter into the citric acid cycle or fatty acid synthesis. Despite the
8 observed adaptations to lactation for genes of gluconeogenesis or β-oxidation we did not
9 find marked changes in transcript abundance of TAT in liver for all groups (**Table 4**). Most
10 interestingly is the elevated transcript abundance of TAT for ODM- compared C-cows
11 (2.23 fold) at wk 4. Perhaps effects of once daily milking were stronger after these four
12 weeks of lactation resulting in the lowest daily milk yield compared to C- and CM-cows.
13 In general, breakdown of protein did not occur even in C-cows where daily milk yield
14 exceeded the nutrient input.
15 In the present study we measured the abundance of mRNA sequences that encode for
16 major proteolytic systems. In lactating sows, a greater mRNA abundance for key
17 components of the ubiquitin-mediated, ATP-dependent pathway was associated with
18 increased muscle protein catabolism (Clowes et al., 2005).
19
20 ***Expression of CTSLβ in liver***:
21 The protein encoded by Cathepsin Lb (**CTSLb**) is lysosomal cysteine proteinase that plays
22 a major role in intracellular protein catabolism. Transcript abundance of CTSLb did not
23 change over time, but mRNA levels were different among groups, showing higher levels
24 for ODM-cows compared to C- and CM-cows before calving and at wk 2 and 4 after
25 calving (**Table 4**). The elevated transcript abundance precalving for ODM-cows is
26 doubtful as both ODM- and C-cows were dried off in late gestation, so there is no reason

1 available why this gene encoding intracellular protein metabolism was more intense
2 prepartum in ODM-cows compared to C- and CM-cows. Most obvious are higher mRNA
3 levels of CTSLb at wk 2 (1.7 fold for ODM- and 2 fold for CM-cows) and wk 4 after
4 calving (1.8 fold for ODM- and 1.6 for CM-cows, respectively) compared to C-cows.
5
6 ***Expression of GLUT2 in liver***:
7 Glut2 is one of the facilitative glucose transporters. It mediates the uptake and release of
8 glucose by hepatocytes based on a high-capacity low affinity transport and release of
9 absorbed glucose across the basolateral surface of epithelial cells of the kidney and small
10 intestine and it is thought to be involved in the regulation of insulin secretion from β-cells.
11 In liver samples of the study presented here we could not detect any changes of Glut2
12 expression among groups and across time (**Table 4**). During the first 8 weeks of lactation
13 we found a tendency for a lower transcript abundance compared to precalving. Glucose
14 supply to hepatocytes is an important condition for functionality of these cells especially
15 gluconeogenesis. More important is the release of this hepatic glucose into the
16 bloodstream. Lower serum glucose concentrations of C-cows especially at wk 2 of
17 lactation seem to be more the result of a dramatic supply of glucose for lactose synthesis in
18 the mammary gland than an impaired availability of glucose perhaps due to restricted
19 hepatic glucose release induced by downregulation of GLUT2. The unchanged mRNA
20 levels of GLUT2 provide sufficient glucose supply to hepatocytes even in early lactation.
21

1 ***Metabolic status of cows: functional parameters and mRNA levels of key enzymes:***

2 Metabolic regulation in complex organisms relies partly on transcriptional control as a
3 long-term mechanism affecting the level of expression of several key enzymes (Desvergne
4 et al., 2006). Furthermore, a cellular gene network can be defined as a collection of DNA
5 segments that interact with a regulator such as a transcription factor or nuclear receptor, but
6 also with each other through their RNA and protein products and with other molecules in
7 the cell (Wittkopp, 2007).

8 It must be acknowledged that changes in gene expression do not necessarily indicate
9 differences in protein abundance or activity caused by post-translational regulation. Gene
10 expression data provide only a snapshot of information regarding the quantity of a given
11 transcript in a cell, but its assessment strengthens the functional parameters measured in
12 this study. However, there is high correlation between mRNA expression of target genes
13 and recruitment of lipogenic transcription factors or nuclear receptors and their co-
14 regulatory proteins to promoter regions in rodents, suggesting that gene expression analysis
15 is useful for inferring transcriptional activity (Bennett et al., 2008).

16 In the following chapter, possible relationships between functional parameters of blood
17 serum and transcript abundance of genes coding for regulating enzymes in liver and
18 muscle should be estimated. In general, it should be clarified if adaptations to lactation
19 were reflected by changes in mRNA abundance of genes related to central metabolic
20 pathways. So we associated mRNA levels of hepatic genes and genes of muscle with
21 functional parameters like BHBA- and NEFA concentration in blood serum.

22 Detailed information about improved metabolic situation postpartum triggered by different
23 milking regimes was described earlier (Schlamberger et al., 2010). In short, in C-cows
24 having a 56-d DP and two times daily milking after calving showed the highest percentage
25 of hypoglycemic blood glucose levels (47 %) compared to ODM-cows (20 %) and CM-
26 cows (14 %, $P < 0.001$) from wk 1 to wk 4. C-cows peaked at much higher level (43.7 ±

2.6 kg ECM/d) at wk 4, CM-cows at wk 4 (38.2 ± 2.5 kg ECM/d), and ODM-cows at wk 8 (37.3 ± 2.2 kg ECM/d) (**Figure 1 A**). As a consequence, C-cows experienced marked stress with the onset of lactation accompanied by enhanced lipolysis of adipose tissue and ketogenesis as proven by higher levels of BHBA and NEFA in blood serum compared to ODM- and CM-cows (**Figure 2 B, C**).

The enhanced lipolysis was also reflected by the highest daily milk fat yield for these cows (2.24 ± 0.14 kg/d) at wk 4, compared to ODM-cows (1.64 ± 0.14 kg/d) and CM-cows (1.81 ± 0.15 kg/d) at wk 4 and 2, respectively (**Figure 1 B**). The higher milk fat yield of C-cows might have been caused by a higher infiltration of long chain fatty acids stored in the body from blood into milk fat (Bauman et al., 2006) and also Pullen et al. (1989) found a positive correlation between milk fat content and plasma NEFA. C-cows experienced the largest drop in serum glucose at wk 2 and 4 (**Figure 2 A**) which might have been a consequence of a high mammary uptake of glucose for lactose synthesis, followed by an increased availability of fat used as energy fuel. The decreased glucose concentrations postpartum are probably related to low DMI, and the concomitant reduction in propionate absorption. This reduced blood serum glucose concentration was followed by higher NEFA and BHBA concentrations in blood serum of these cows (**Figure 2 B, C**). Serum concentrations of BHBA were correlated with mRNA abundance of LDH in muscle. These correlations were negative for all groups (**Table 6**), but most obvious for C-cows, with a significant correlation (r = -0.49, $P < 0.01$**, Figure 3 A**). LDH transcript abundance was found to be positive correlated with daily milk yield for all groups (r = 0.45, **Table 6, Figure 4**), and was negative correlated with blood serum NEFA concentrations (r = -0.41, **Table 6, Figure 5**). It was suggested that energy metabolism in muscle was impaired with increasing BHBA and NEFA concentrations in blood serum. In addition with increasing BHBA and NEFA, mRNA levels of ACTA1 decreased with the plainest correlation for C-cows (r = -0.42 for NEFA) and ODM-cows (r = -0.42 for BHBA, $P < 0.01$, **Figure 6**).

1 The high mammary uptake of glucose soon after parturition especially for C-cows with
2 peak of daily milk yield at wk 4 (**Figure 1 A**) of lactation resulted in low serum
3 concentrations of glucose (**Figure 2 A**). As we have not measured endogenous glucose
4 production in this study, we are not able to conclude whether a greater gene expression of
5 gluconeogenic enzymes (like PEPCK) implied increased hepatic glucose production. But
6 higher blood serum concentrations postpartum in CM- cows compared to C-cows were
7 rather the result of an enhanced gluconeogenesis immediately postpartum than of a reduced
8 demand of glucose for lactose synthesis in the mammary gland. CM-cows had a daily milk
9 yield similar to C-cows at wk 2 but with blood serum BHBA and glucose concentrations
10 out of critical range. Most interestingly gluconeogenesis indicated by transcript abundance
11 of PEPCK seemed to start earlier in CM- than in C-cows. Metabolism of CM-cows seemed
12 to be ready for the onset of lactation as shown by higher transcript abundance of PEPCK
13 precalving, at calving and thereafter compared to C-cows (**Table 4**). Also mRNA levels of
14 CPT1A antepartum were higher for CM- cows compared with those of ODM- and C-cows.
15 We suggest, that twice daily milking in late gestation up to day of calving may influence
16 well ahead of parturition hepatic adaptation to the onset of parturition. Perhaps other
17 factors besides the hormonal environment characteristic of the periparturient period
18 regulate hepatic adaptations to lactation. Most important is that hepatic gene expression
19 changes are more pronounced in C- and ODM-cows than in CM-cows. That is also
20 reflected by downstream activation of genes with key functions in fatty acid oxidation
21 (CPT1A, ACADvl, EnoylCoA) that were also influenced by the milking regimen.
22 Transcript abundance of ACADvl in hepatocytes showed a marked increase at time of
23 calving and wk 2 of lactation followed by a marked decrease up to wk 8 of lactation for C-
24 and ODM-, but not for CM-cows. Upregulation of ACADvl with calving was more
25 pronounced in C- and ODM- than in CM-cows, suggesting that regulation of metabolic
26 adaptation was moderate in CM-cows compared with C-cows. Upregulation of hepatic

1 ACADvl transcript abundance around calving compared to precalving was also found by
2 Loor et al (2005). In our study, serum concentrations of BHBA and NEFA were correlated
3 positive with transcript abundance of ACADvl in liver (**Table 5**), but this correlation was
4 really marked for NEFA blood serum concentration and mRNA levels of ACADvl for C-
5 cows (r = 0.43, P = 0.007, **Figure 7 B**).
6 We could not detect any changes of transcript abundance of the GLUT 2 transporter in
7 liver tissue, suggesting that hepatic glucose release remained unaffected by treatments and
8 might not, therefore, be regulated by metabolic status of cows (Drackley, 2001). For
9 enzymes of protein catabolism we could not detect any differences among groups
10 prepartum in liver. The onset of lactation is associated with increased AA uptake by the
11 mammary gland and liver (Verbeke et al., 1972). The increased protein supply either
12 originate through increases in DMI and muscle protein degradation, as muscle stores large
13 amounts of AA and release them for gluconeogenesis and milk protein synthesis. Despite
14 of this we could not measure any changes in protein catabolism in liver. Most important is
15 that all the adaptations to lactation seem to occur in CM-cows before calving perhaps over
16 a long timeframe, while adaptions especially for C-cows seem to occur within a short
17 timeframe, at worst within two weeks, as milk yield peaked at this early time point of
18 lactation.
19 One week before calving, levels of blood serum NEFA and BHBA concentrations did not
20 differ among groups, but transcript abundance of CPT1A, GPAM, PEPCK in liver differed
21 among groups. Higher levels of mRNA abundance for CM-cows compared to C and
22 ODM-cows were found in enzymes of liver (CPT1A, PEPCK, GPAM). Metabolism of
23 these cows seemed to be more adapted to lactation compared to C-cows and ODM-cows.
24 Serum concentrations of NEFA correlated positive with mRNA levels of hepatic CPT1A
25 (**Table 5,**) but correlation was significant only for C-cows, suggesting that these cows
26 showed a greater adaptive performance than others (r = 0.49, **Figure 7 A**). The correlation

1 between NEFA and CPT1A mRNA level was also observed by (Loor et al., 2005). For C-
2 cows we observed a positive correlation among daily milk yield and transcript abundance
3 of hepatic PEPCK (**Table 5**), showing an increased glucose demand with increasing milk
4 yield which directly affects this gluconeogensis regulating enzyme ($r = 0.49$,
5 **Figure 7 C**).The metabolic imbalance of these cows compared to ODM- and CM-cows
6 was reflected by changes in relative mRNA expression of specific genes in liver and
7 muscle, measured at different time points.
8 In skeletal muscle, EnoylCoA transcript abundance was significantly lower in CM-cows
9 than in C-cows at time of calving and wk 16. At wk 6 transcript abundance of EnoylCoA
10 were lower for both, CM- and ODM-cows compared to C-cows. These C-cows showed a
11 stronger lipolysis marked by higher mRNA levels of this regulatory step of the β-
12 oxidation. Levels of ACTA1 were upregulated at wk 6 and 16 of lactation for cows of all
13 groups, suggesting that metabolism returns more and more from catabol to anabol status.
14 Blood serum concentrations of BHBA were negative correlated with ACTA1 transcript
15 abundance for ODM-cows ($r = -0.42$, $P < 0.001$, **Figure 6 B**) and NEFA blood serum
16 concentrations were negative correlated with ACTA1 mRNA levels in C-cows ($r = -0.42$,
17 $P < 0.01$, **Figure 6 B**). We could not detect any relationships between transcript abundance
18 of insulin receptor β or glucose transporter Glut4 with any of functional parameters in
19 blood serum. However Glut1 mRNA abundance was positive correlated with BHBA blood
20 serum concentrations (**Table 6**). But this correlation was more obvious in C-cows ($r =$
21 0.45, **Figure 3 B**). Perhaps changes in transcript abundance of several key enzymes are
22 more pronounced in liver than in skeletal muscle of dairy cows. Despite of this we found
23 changes in the expression of IRβ in skeletal muscle showing decreasing levels from
24 calving to wk 16 for all groups, interestingly more pronounced for ODM- and CM-cows.
25 Therefore, interactions between hepatic glucose and lipid metabolism and productivity of
26 dairy cows especially in the transition period need further investigations. In conclusion,

milking in late pregnancy or once daily in early lactation affects metabolic status of cows. This was shown by a lower rate of hypoglycemic and ketotic blood serum samples during the first four weeks of lactation. Improved metabolic status was reflected by moderate changes of transcript abundance of key enzymes of glucose and lipid metabolism, while C-cows showed a stronger adaptation to lactation.

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Table 1. chemical composition and ingredients of diet

	LD[1]	DPD[1]
Ingredient, %		
Corn silage	60	43
Grass silage	23	25
Hay	4	32
Feed pellets[2]	12	-
Mineral mix[3]	1	-
Chemical composition, %		
DM	45.2	52.0
CP	12.2	12.9
CF	18.6	19.2
NFC	26.4	9.92
Ether extract	3.16	3.16
NE_L (Mcal/kg)[4]	1.55	1.36

[1] Lactation Diet (LD) was fed to all cows, dry period diet (DPD) was fed only to cows having a 56-d dry period (control and once daily milking cows) but not to continuously milking cows.

[2] Composition: corn gluten 18.4 %, turnips molasses chips 13.8 %, wheat 10.0 %, triticale 10.0 %, rape cake 10.0 %, maize 8.8 %, malt germ 6.0 %, grain distillation residual (ProtiGrain) 5 %, rape extraction grist 5 %, rumen protected rape extraction grist 5 %, palm corn cake 3.3 %, soy extraction grist 2.8 %, sodium bicarbonate 1.0 %, calcium bicarbonate 0.99 %, plant oil (palm coconut) 0.40 % (Raiffeisen Kraftfutterwerke Süd GmbH, Würzburg Germany)

[3] Ingredients: calcium 14 %, sodium 10.0 %, phosphorous 5.0 %, magnesium 5.0 % (Josera, Kleinheubach, Germany).

[4] Estimates determined from tabulated values of feeds (GfE, 2007) and according to the following formula: NEL (Mcal/kg) = 0.6*[1+0.004*(q-57)]*ME (Mcal/kg), with q = ME/GE, here q = 88.9

1 **Table 2.** Primer information, annealing temperature, and reference:

Primer[1]	Primer sequence 5´-3´	tissue	function	Annealing temperature	Accesion no
PEPCK, for PEPCK, rev	TACGAGGCCTTCAACTGGCGT AGATCCAAGGCGCCTTCCTTA	liver	gluconeogenesis	60°	XM_583200
CPT 1A for CPT 1A rev	CCATACTCACATAATTGGTAGCC GCAACTAGTGAAGCCTCTTATGAA	liver	β-oxidation	54°	BF_039285
GPAM for GPAM rev	TCTGACTGAAGATGGGGATG ATGGGGAATTTGCCGCTTAT	liver	glycerolipid-biosynthesis	54°	AF_469047
GLUT1 for GLUT1 rev	GTGCTCCTGGTTCTGTTCTTCA GCCAGAAGCAATCTCATCGAA	muscle	glucose transport	61°	NM_174602
GLUT4 for GLUT4 rev	GGACCGCGAATAGAAGAAAGA CAACTTCATCATCGGCATGG	muscle	glucose transport	61°	NM_174604
ACADvl for ACADvl rev	CGTACATGGTGAGTGCCAAC GTCATTTGTCCCCTCGAAGA	muscle/ liver	β-oxidation	60°	NM_174494
EnoylCoA for EnoylCoA rev	GCTGCTGTCAATGGCTATGC ACCAGTGAGGACCATCTCCA	muscle	β-oxidation	60°	NM_001025206
GAPDH for GADPH rev	GTCTTCACTACCATGGAGAAGG TCATGGATGACCTTGGCCAG	liver	glycolysis, reference gene	60°	NM_001034034
Histone H3 for Histone H3 rev	ACTTGCCTCCTGCAAAGCAC ACTTGCTACAAAAGCCGCTC	muscle/ liver	ordering DNA, reference gene	54°	NM_001014389
Ubiquitin for Ubiquitin rev	AGATCCAGGATAAGGAAGGCAT GCTCCACCTCCAGGGTGAT	muscle	post-translational modification, reference gene	60°	Z18245
LDH for LDH rev	GTGGCTTGGAAGATAAGTGG ACTAGAGTCACCATGCTCC	muscle	pyruvate metabolism	62°	NM_005566
ACTA1 for ACTA1 rev	TATTGTGCTCGACTCCGGCGA GTCACGAAGGAGTAGCCACG	muscle	muscle protein cell motility	60°	NM_174225
IRß for IRß rev	TCCTCAAGGAGCTGGAGGAGT TAGCGTCCTCGGCAACAGG	muscle	insulin receptor β	60°	M_37211
Glut2 for Glut2 rev	GGACCTTGGTTTTGGCTGTC CACAGACAGGGACCAGAACA	liver	glucose transport	60°	XM_614140
CS for CS rev	TGGACATGATGTATGGTGG AGCCAAGATACCTGTTCCTC	liver	citric acid cycle	60°	BC_114138
CTSLb for CTSLb rev	CAC TGG TGC TCT TGA AGG ACA TAA GAT TCC TCT GAG TCC AGG C	liver	protein metabolism (degradation)	60°	NM_174032
TAT for TAT rev	ACC CTT GTG GGT CAG TGT TC ACA GGA TGG GGA CTT TGC TG	liver	AA metabolism (degradation)	60°	NM_001034590

2

3 [1] PEPCK = Phosphoenolpyruvate carboxykinase; CPT 1A = Carnitine palmitoyltransferase 1A; GPAM =
4 Glycerol-3-phosphate acetyltransferase; Glut1 = Glucose transporter 1; Glut4 = Glucose transporter 4;
5 ACADvl = Acyl-coenzyme A dehydrogenase very long chain; EnoylCoA = EnoylCoA hydratase; GAPDH =
6 Glyceraldehyde 3-phosphate dehydrogenase; LDH = Lactatdehydrogenase; ACTA1 = Actin, alpha 1; IRβ =
7 Insulin receptor β; Glut2 = Glucose transporter 2; CS = Citratsynthase; CTSLb = Cathepsin Lb; TAT =
8 Tyrosin-amino-transferase;

Table 3. mRNA abundance[1] (log_2) of Glut1, Glut4, LDH, ACADvl, EnoylCoA, IRβ and ACTA1 in muscle of dairy cows managed with different milking regimen in early lactation or milked continuously.

		Group[2]				ANOVA		
Item	Week[3]	C	ODM	CM	SEM	Milking regimen	Time	Milking regimen x time
Glut1	0	2.39	2.59	2.77	0.19	0.1078	0.06	0.1355
	6	2.15	1.71	2.94	0.18			
	16	1.70	2.19	2.12	0.19			
Glut4	0	6.70	6.44	6.21	0.17	0.0742	0.0103	0.8556
	6	7.23	7.44	6.94	0.16			
	16	7.04	6.82	6.22	0.18			
LDH	0	9.08*	9.22*	8.99*	0.16	0.70	<0.01	0.6933
	6	10.41	10.30	10.39	0.15			
	16	10.92	10.67	10.35	0.16			
ACADvl	0	5.72	5.99	5.60	0.14	0.38	0.05	0.70
	6	6.04	5.76	5.81	0.14			
	16	5.68	5.41	5.16	0.14			
EnoylCoA	0	3.81[a]	3.66[a]	2.96[b]	0.14	<0.01	0.84	0.58
	6	4.12[a]	3.34[b]	3.10[b]	0.14			
	16	3.99[a]	3.47[ab]	2.81[b]	0.15			
IRβ	0	3.78*[a]	4.99*[b]	4.53*[ab]	0.23	0.01	<0.01	0.96
	6	3.02	4.02	3.65	0.23			
	16	2.39[a]	3.81[b]	2.99[ab]	0.24			
ACTA1	0	11.56*	11.05*	11.37*	0.27	0.14	<0.01	0.92
	6	12.67	11.66	12.62	0.26			
	16	13.49	12.36	13.08	0.27			

[1] Data are presented as means ± SEM subtracted from the arbitrary value 10 (10 - ΔCq). Thus, a high ΔCq resembles high transcript abundance (Livak and Schmittgen, 2001). An increase of one ΔCq represents a two-fold increase of mRNA transcripts.

[2] Groups are C = 56-d dry period and twice daily milking after parturition (N = 12), ODM = 56-d dry period and once daily milking for the first 28 d of lactation (N = 12), CM = 0-d dry period and continuously milking twice a day throughout lactation (N = 12).

[3] weeks are relative to calving.

[a, b] Means within a row with different superscripts differ ($P < 0.05$). Means with * indicate a difference within one group compared to wk 16 ($P < 0.05$).

1 **Table 4**. mRNA abundance[1] (log $_2$) of CPT 1A, GPAM, PEPCK, TAT, Glut2, CS, CTSLβ
2 and ACADvl in liver of dairy cows managed with different milking regimen in early
3 lactation or milked continuously.

		Group[2]				ANOVA		
Item	Week[3]	C	ODM	CM	SE	Milking regimen	Time	Milking regimen x time
Liver								
CPT 1A	-1	4.34[a]	4.44[a]	5.36[b]	0.09	0.002	<0.001	0.0147
	0	4.56	4.35	4.49*	0.09			
	2	4.66	4.81	5.00	0.09			
	4	4.56[ab]	4.38[b]	4.86[a]*	0.10			
	8	4.37[a]	4.20[a]	4.89[b]*	0.10			
GPAM	-1	5.59[a]	5.18[b]	5.75[a]	0.08	<0.001	<0.001	0.54
	0	4.98[a]*	4.52[b]*	5.19[a]*	0.08			
	2	5.46[ab]	5.14[a]	5.53[b]	0.08			
	4	5.21[a]*	5.29[ab]	5.65[b]	0.07			
	8	5.33[ab]	5.17[a]	5.64[b]	0.08			
PEPCK	-1	5.78[a]	5.71[a]	7.79[b]	0.47	0.092	0.001	0.57
	0	5.06	4.39	6.48	0.45			
	2	5.79[a]	5.39[a]	8.04[b]	0.45			
	4	5.95	5.72	7.73	0.45			
	8	6.01	5.90	6.17	0.46			
TAT	-1	7.48	7.33	6.93	0.22	0.67	0.85	0.08
	0	7.44	7.82	7.08	0.21			
	2	7.82	7.26	7.19	0.20			
	4	6.88[a]	8.04[b]	7.29[ab]	0.21			
	8	7.41	7.32	8.08*	0.21			
Glut2	-1	5.34	5.89	5.70	0.22	0.12	0.05	0.74
	0	5.41	5.30	5.41	0.21			
	2	5.18	5.61	6.14	0.21			
	4	5.22	5.86	5.95	0.20			
	8	4.53	4.77*	5.50	0.21			
CS	-1	3.11	2.64	2.88	0.17	0.52	0.64	0.31

	0	2.94^{ab}	2.52^{a}	3.40^{b}	0.16			
	2	2.79	3.14	2.89	0.16			
	4	2.68	2.55	3.11	0.16			
	8	2.53	2.80	2.53	0.17			
CTSLβ	-1	6.04^{a}	7.07^{b}	6.93^{b}	0.17	<0.001	0.26	0.89
	0	6.57	7.09	6.74	0.16			
	2	6.56^{a}	7.32^{b}	7.56^{b}	0.16			
	4	6.46^{a}	7.32^{b}	7.19^{ab}	0.16			
	8	6.59	7.17	7.27	0.16			
ACADvl	-1	5.66	5.40	6.09	0.15	0.97	<0.001	0.26
	0	6.69*	6.76*	6.32	0.15			
	2	6.33*	6.34*	6.08	0.14			
	4	6.09	5.78	6.05	0.14			
	8	5.84	6.05	5.89	0.15			

[1] Data are presented as means ± SEM subtracted from the arbitrary value 5 (5 - ΔCq). Thus, a high ΔCq resembles high transcript abundance (Livak and Schmittgen, 2001). An increase of one ΔCq represents a two-fold increase of mRNA transcripts.

[2] Groups are C = 56-d dry period and twice daily milking after parturition (N = 12), ODM = 56-d dry period and once daily milking for the first 28 d of lactation (N = 12), CM = 0-d dry period and continuously milking twice a day throughout lactation (N = 12).

[3] weeks are relative to calving.

[a, b] Means within a row with different superscripts differ ($P < 0.05$). Means with * indicate a difference within one group compared to wk -1 precalving ($P < 0.05$).

1 **Table 5**. Correlations[1] (Pearson and Spearman rank-order correlation coefficients) among
2 mRNA abundance of target genes in liver and blood serum concentrations of metabolic
3 parameters and parameters of productivity of dairy cows managed with different milking
4 regimen in early lactation or milked continuously. Correlations were based on data at time
5 of calving, wk 2, wk 4, and wk 8.

	TAT[2]	**Glut2**	**CTSLb**	**CS**	**ACADvl**	**PEPCK**	**CPT 1A**	**GPAM**
Milk kg/d								
All cows	0.05	-0.049	0.013	-0.14	-0.22	0.12	-0.051	0.21
C[3]	-0.127	-0.273	-0.050	-0.22	-0.31	0.47***	-0.027	0.35
ODM	-0.04	0.01	0.13	-0.001	-0.32	0.13	-0.85	-0.001
CM	0.21	0.17	0.14	-0.15	-0.16	0.09	-0.004	0.10
Fat %								
All cows	0.09	-0.06	0.06	0.08	0.16	-0.15	-0.01	-0.11
C	0.25	-0.07	0.04	0.07	0.13	-0.27	0.21	-0.10
ODM	0.17	0.11	0.15	0.28	0.26	-0.07	0.13	0.10
CM	-0.07	-0.24	0.04	-0.08	0.08	-0.13	-0.16	-0.19
Protein %								
All cows	-0.05	0.005	0.04	0.01	0.11	-0.06	-0.03	-0.30
C	-0.06	0.01	0.12	0.03	0.36	-0.35	0.09	-0.29
ODM	0.01	0.01	-0.06	-0.06	0.08	-0.01	-0.09	-0.25
CM	-0.14	-0.11	0.01	0.05	0.03	-0.11	-0.14	-0.22
Glucose mmol/L								
All cows	-0.04	-0.01	-0.06	-0.05	-0.12	-0.09	-0.22*	-0.10
C	-0.07	0.14	-0.02	0.01	-0.15	0.01	-0.23	-0.02
ODM	0.14	-0.06	-0.34	-0.14	-0.03	-0.05	-0.36*	-0.24
CM	-0.21	-0.18	-0.05	-0.06	-0.17	-0.21	-0.22	-0.09
NEFA µmol/L								
All cows	0.04	-0.04	-0.01	-0.03	0.26*	-0.16	0.11	-0.26*
C	0.14	-0.04	0.08	-0.19	0.43***	-0.09	0.49***	-0.24
ODM	-0.04	-0.004	-0.07	0.14	0.22	-0.14	0.08	-0.33*
CM	-0.03	0.09	0.21	0.05	0.22	-0.14	0.03	-0.07
BHBA mmol/L								
All cows	0.03	-0.12	-0.06	0.06	0.28*	-0.15	0.13	-0.13
C	0.21	-0.03	0.05	-0.00	0.34*	-0.25	0.31	-0.14
ODM	-0.14	-0.13	0.10	0.07	0.11	-0.04	0.22	-0.18
CM	-0.11	-0.08	-0.10	0.31*	0.37*	-0.12	0.10	0.07

6 [1]* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

1 [2] Target genes in liver are: TAT = Tyrosin aminotransferase; Glut2 = Glucose transporter 2;CTSLb =
2 Cathepsin Lb; CS = Citratsynthase; ACADvl = Acyl-coenzyme A dehydrogenase very long chain; PEPCK =
3 Phosphoenolpyruvate carboxykinase; CPT 1A= Carnitine palmitoyltransferase 1A; GPAM = Glycerol-3-
4 phosphate acetyltransferase;
5 [3]Groups are C = 56-d dry period and twice daily milking after parturition (N = 12), ODM = 56-d dry period
6 and once daily milking for the first 28 d of lactation (N = 12), CM = 0-d dry period and continuously milking
7 twice a day throughout lactation (N = 12).
8

1 **Table 6**. Correlations[1] (Pearson and Spearman rank-order correlation coefficients) among
2 mRNA abundance of target genes in muscle and blood serum concentrations of metabolic
3 parameters and parameters of productivity of dairy cows managed with different milking
4 regimen in early lactation or milked continuously. Correlations were based on data at time
5 of calving, wk 6, and wk 16.

	IRβ[2]	ACTA1	Glut1	Glut4	LDH	ACADvl	EnoylCoA
Milk kg/d							
All cows	-0.26	0.32	-0.18	0.25	0.45**	0.04	0.02
C[3]	-0.21	0.22	-0.21	0.20	0.48*	-0.33	-0.04
ODM	-0.24	0.20	-0.22	0.27	0.44**	-0.19	-0.11
CM	-0.22	0.32	-0.09	0.27	0.42***	0.12	0.05
Fat %							
All cows	0.09	-0.26	0.14	0.13	-0.25*	0.08	0.06
C	0.18	-0.28	0.35*	-0.09	-0.23	0.22	0.04
ODM	0.17	-0.29	-0.11	-0.41*	-0.39*	0.16	-0.01
CM	0.03	-0.15	0.19	-0.09	-0.13	-0.21	-0.03
Protein %							
All cows	0.20*	-0.21	0.11	-0.15	-0.38*	-0.03	-0.04
C	0.09	-0.11	0.01	-0.13	-0.18	-0.18	-0.01
ODM	0.21	-0.12	0.14	-0.25	-0.41*	0.08	-0.015
CM	0.25	-0.29	0.29	-0.07	-0.38*	-0.11	-0.18
Glucose mmol/L							
All cows	-0.08	-0.07	-0.08	-0.02	-0.07	-0.24	-0.16
C	-0.10	0.01	-0.32	-0.00	0.09	-0.13	-0.13
ODM	-0.37*	-0.02	0.11	-0.18	-0.01	-0.13	-0.06
CM	-0.07	-0.14	0.03	0.09	-0.19	-0.35*	-0.14
NEFA µmol/L							
All cows	0.27*	-0.37*	0.09	-0.05	-0.41**	0.15	0.10
C	0.33*	-0.43***	0.22	-0.14	-0.58***	0.15	-0.05
ODM	0.38*	-0.39*	0.17	-0.21	-0.41**	0.34	0.08
CM	0.21*	-0.39*	0.10	0.09	-0.36***	-0.07	0.08
BHBA mmol/L							
All cows	0.19	-0.26*	0.20	0.03	-0.34*	0.09	0.15
C	0.35	-0.30	0.45*	-0.14	-0.49**	0.06	-0.00
ODM	0.32	-0.42***	0.25	-0.06	-0.28	0.12	0.15
CM	-0.13	-0.21	0.17	-0.08	-0.37*	-0.23	0.02

6 [1]* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

7 [2]Target genes in muscle are: IRβ = Insulin receptor β; ACTA1 = Actin, alpha 1; Glut1 = Glucose transporter
8 1; Glut4 = Glucose transporter 4; LDH = Lactatde hydrogenase; ACADvl = Acyl-coenzyme A
9 dehydrogenase very long chain; EnoylCoA = EnoylCoA hydratase.

10 [3]Groups are C = 56-d dry period and twice daily milking after parturition (N = 12), ODM = 56-d dry period
11 and once daily milking for the first 28 d of lactation (N = 12), CM = 0-d dry period and continuously milking
12 twice a day throughout lactation (N = 12).

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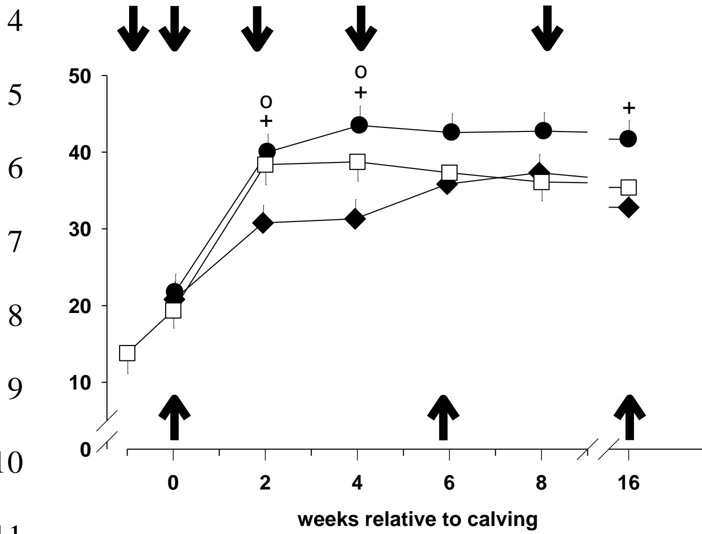
1 Figure 1

2 A B



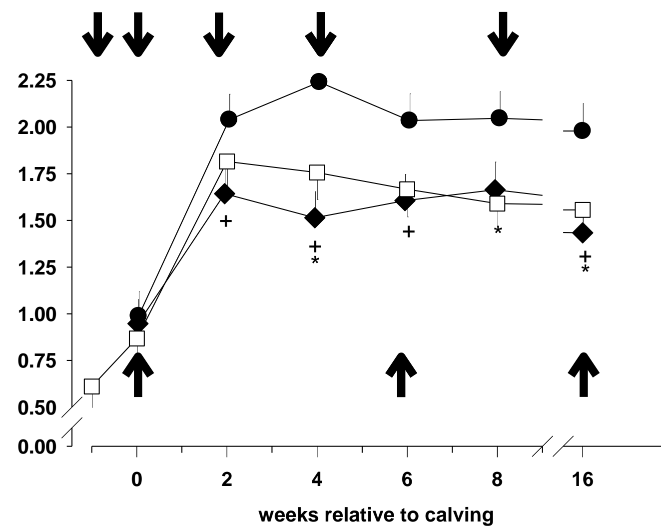


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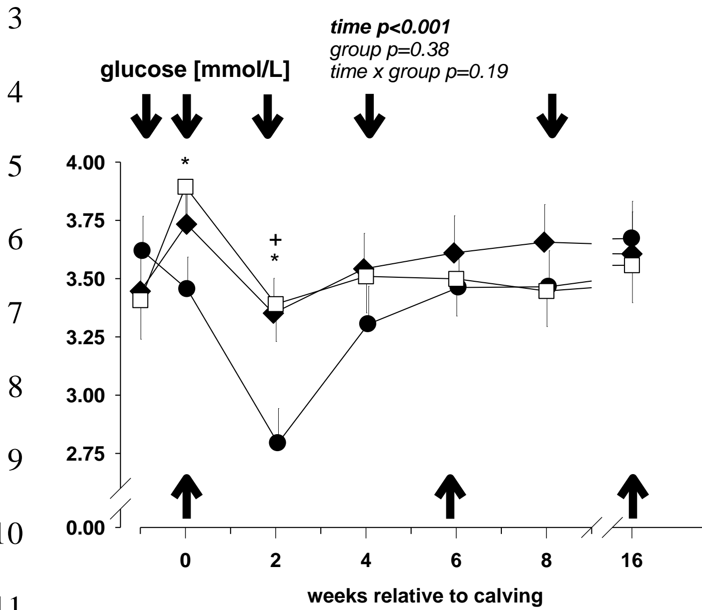
Figure 2

A



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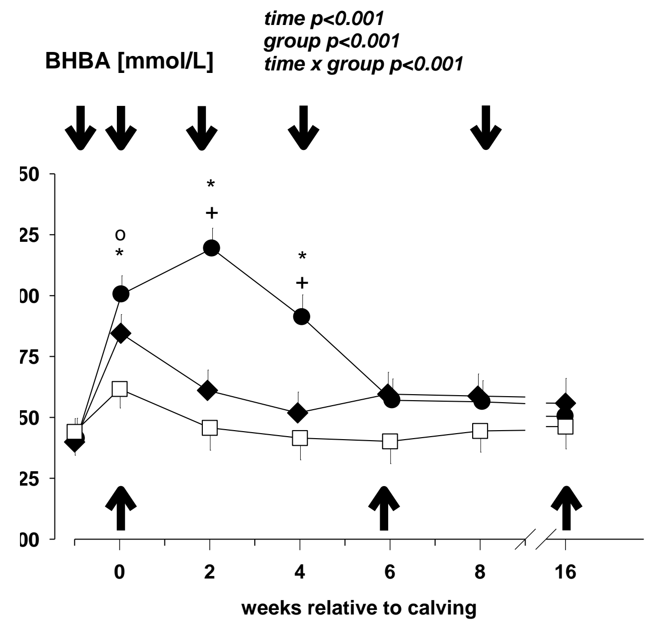

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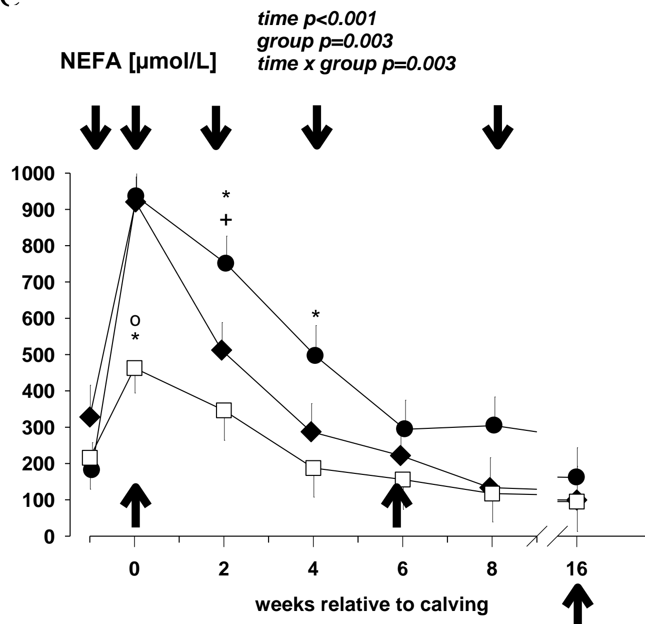
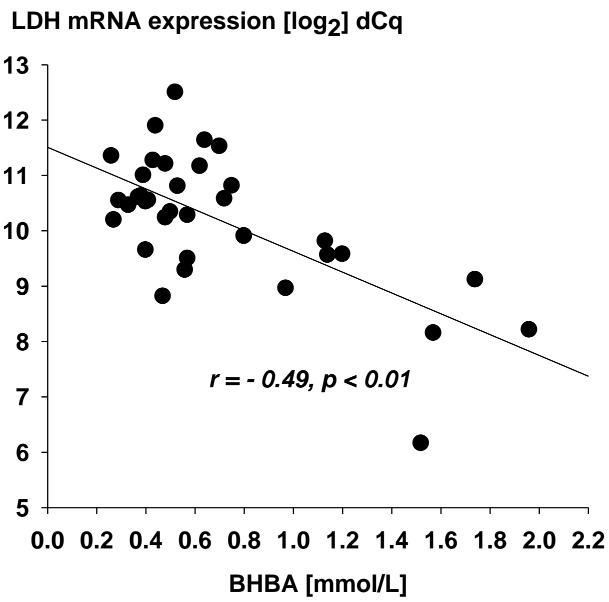
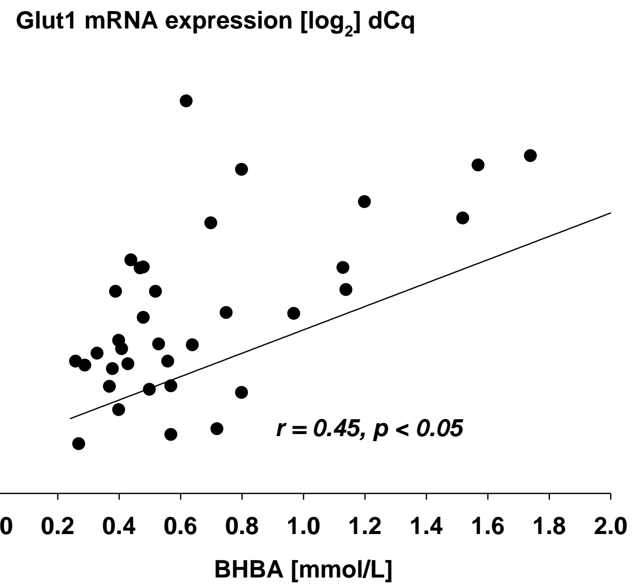


1 Figure 3

2 A B





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1 Figure 4

2 A B

LDH mRNA expression [log_2] dCq

r = 0.48, p < 0.05

LDH mRNA expression [log_2] dCq

r = 0.44, p < 0.01

milk [kg/d]

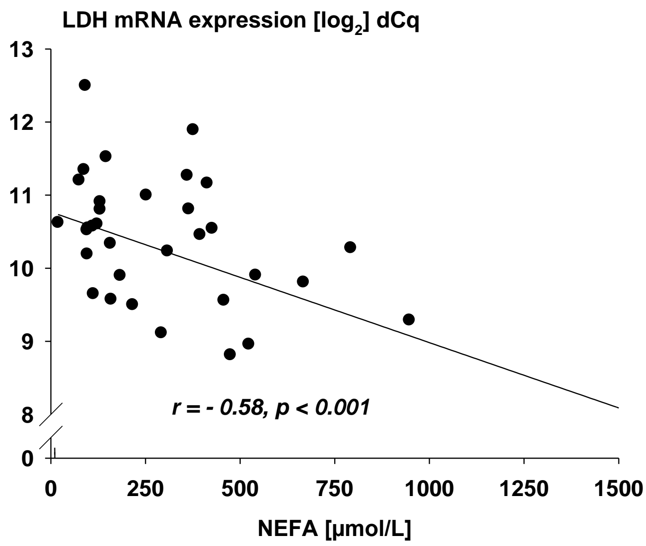
LDH mRNA expression [log_2] dCq

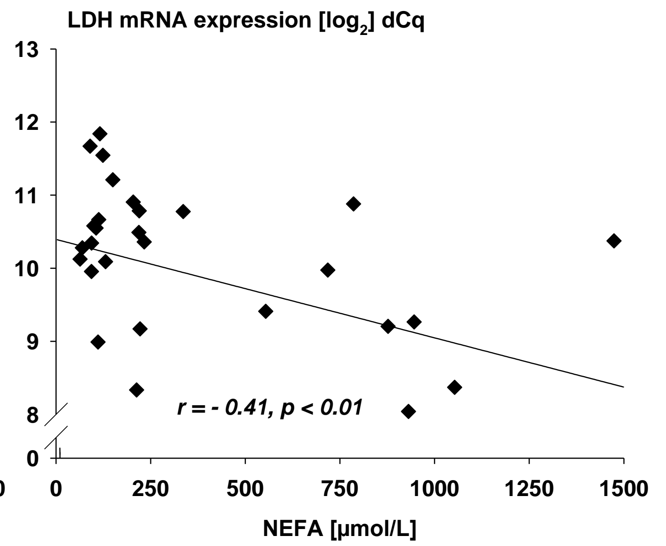
r= 0.42, p < 0.001

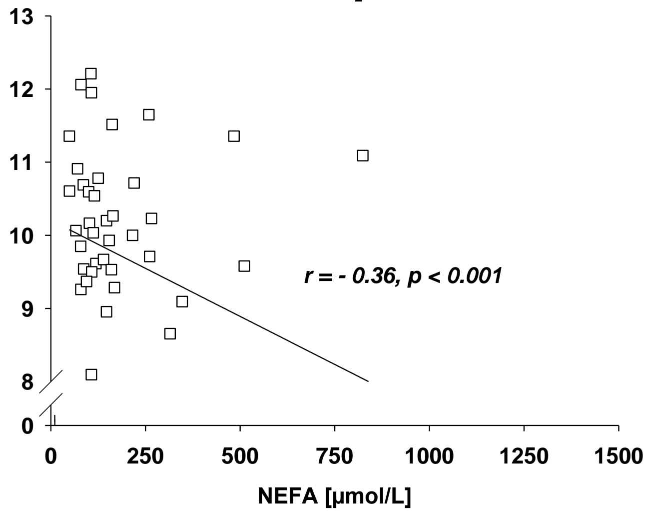
milk [kg/d]

1 Figure 5

2 **A** **B**







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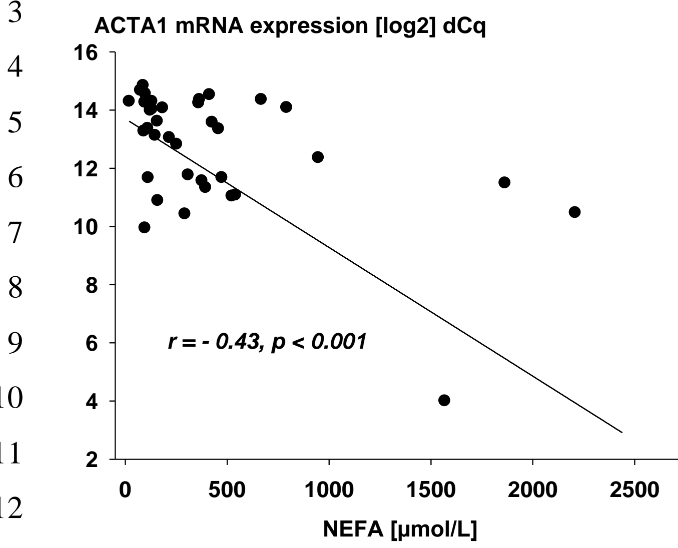
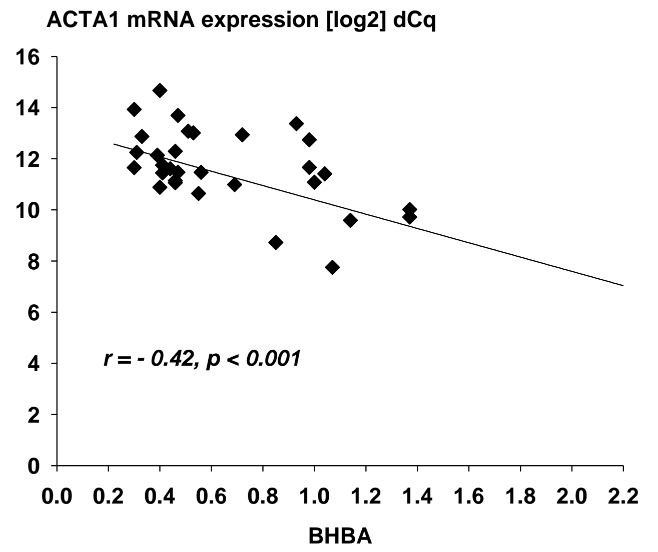
1 Figure 6

2 A B





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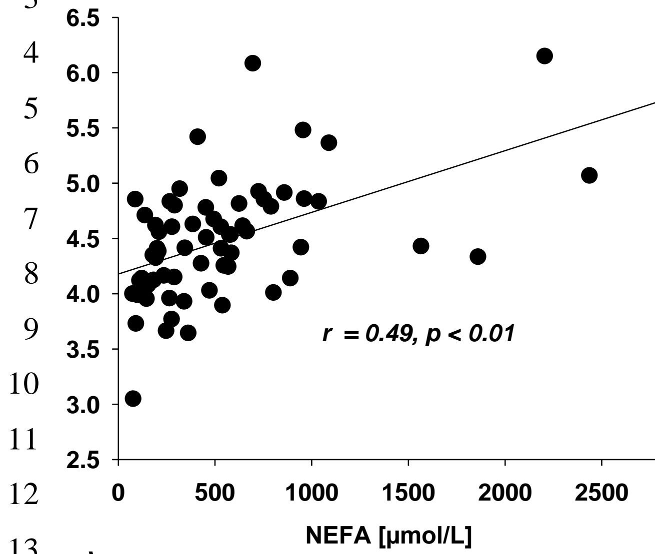
1 Figure 7

2 A



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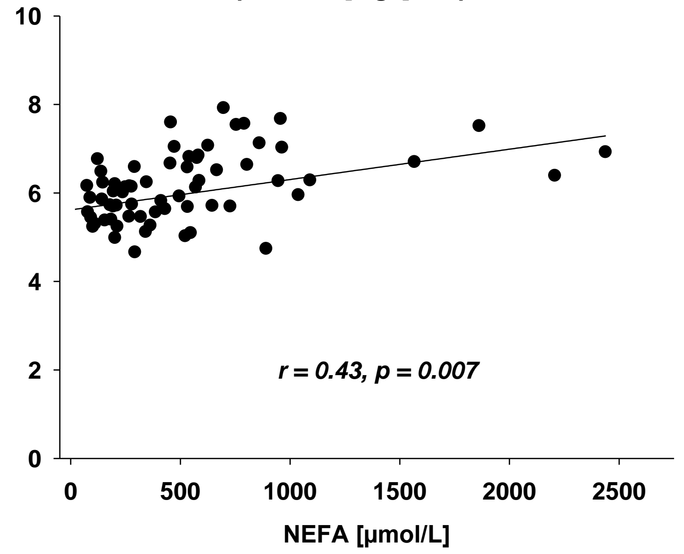

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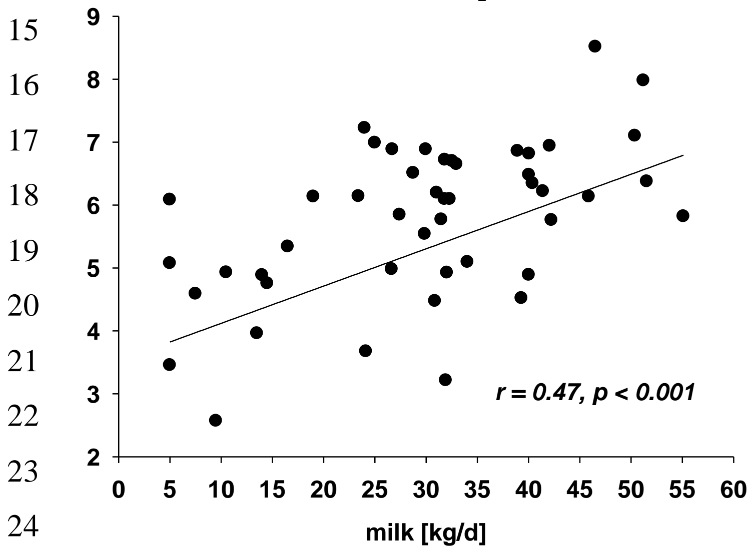

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Figure 1. Energy corrected daily milk yield (ECM)(**A**) and daily fat yield (kg/d)(**B**) from calving up to wk 16 of lactation for Brown Swiss dairy cows assigned to one of three management strategies: 56 d dry period (DP) and twice daily milking after parturition (controls, C, ●), 56 d DP and once daily milking (ODM) for the first 28 days of lactation (♦), and zero days dry with continuously milking (CM) (□) twice a day throughout of lactation. Data are presented for each group at given day of biopsy time point and are not pooled weekly means. So for each time point of biopsy the corresponding daily milk yield or daily fat yield was available. Arrows on the top shows biopsy time points of liver biospy, arrows on the bottom show time points of muscle biopsies. Asterisks (*) indicate differences between C and CM; plus signs (+) indicate differences between C and ODM ($P < 0.05$); circle signs (°) indicate differences between ODM and CM. Values are LSMeans ± SEM.

Figure 2. Blood serum metabolites glucose (A), BHBA (B), and NEFA (C) patterns from calving up to wk 16 of lactation for Brown Swiss dairy cows assigned to one of three management strategies: 56 d dry period (DP) and twice daily milking after parturition (controls, C, ●), 56 d DP and once daily milking (ODM) for the first 28 days of lactation (♦), and zero days dry with continuously milking (CM) twice a day throughout of lactation. Arrows on the top shows biopsy time points of liver biospy, arrows on the bottom show time points of muscle biopsy. Asterisks (*) indicate differences between C and CM; plus signs (+) indicate differences between C and ODM ($P < 0.05$); circle signs (°) indicate differences between ODM and CM. Values are LSMeans ± SEM. (□)

Figure 3. Correlations (Spearman order-rank correlation coefficients) among BHBA blood serum concentration and mRNA abundance of LDH (A) and Glut1 (B) in skeletal muscle for C-cows (● , 56 d dry period and twice daily milking after parturition).

1 **Figure 4.** Correlations (Pearson correlation coefficients) among daily milk yield and
2 mRNA abundance of LDH in muscle for C-cows (A, symbol ●, 56 d dry period and twice
3 daily milking after parturition), ODM-cows (B, symbol ◆, 56 d DP and once daily milking
4 for the first 28 days of lactation) and CM-cows (C, symbol □, zero days dry with
5 continuously milking twice a day throughout of lactation).
6 **Figure 5.** Correlations (Spearman rank-order correlation coefficients) among NEFA blood
7 serum concentration and mRNA abundance of LDH in muscle C-cows (A, symbol ●, 56 d
8 dry period and twice daily milking after parturition), ODM-cows (B, symbol ◆, 56 d DP
9 and once daily milking for the first 28 days of lactation) and CM-cows (C, symbol □, zero
10 days dry with continuously milking twice a day throughout of lactation).
11 **Figure 6.** Correlations (Spearman rank-order correlation coefficients) among mRNA
12 abundance of ACTA1 in muscle and blood serum concentration of NEFA for C-cows (A,
13 symbol ●**,** 56 d dry period and twice daily milking after parturition), and BHBA for
14 ODM-cows (B, symbol ◆, 56 d DP and once daily milking for the first 28 days of
15 lactation).
16 **Figure 7**. Correlations (Pearson correlation coefficients) among NEFA blood serum
17 concentration and mRNA abundance of CPT 1A (A) and ACADvl (B) and daily milk yield
18 and mRNA abundance of PEPCK in liver (C) for C-cows (symbol ●, 56 d dry period and
19 twice daily milking after parturition).
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RESEARCH Open Access

Rumen-protected conjugated linoleic acid supplementation to dairy cows in late pregnancy and early lactation: effects on milk composition, milk yield, blood metabolites and gene expression in liver

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Abstract

Background: Conjugated linoleic acid (CLA) is a collective term for isomers of octadecadienoic acid with conjugated double-bond system. Thus, it was the objective to investigate whether milk composition and metabolic key parameters are affected by adding CLA to the diet of dairy cows in the first four weeks of lactation.

Methods: A study was carried out with five primiparous cows fed a CLA supplemented diet compared to five primiparous cows without CLA supplementation. CLA supplemented cows received 7.5 g CLA/day (i.e. 50% *cis*(c)9, *trans*(t)11- and 50% *t*10,*c*12-CLA) starting two weeks before expected calving and 20 g CLA/day (i.e. 50% *c*9,*t*11- and 50% *t*10,*c*12-CLA) throughout day 1 to 28 of lactation.

Results: The CLA supplement was insufficiently accepted by the animals: only 61.5% of the intended amount was ingested. Fed CLA were detectable in milk fat, whereas contents of *c*9,*t*11-CLA and *t*10,*c*12-CLA in milk fat were higher for CLA supplemented cows compared to the control group. On average over the entire treatment period, there was a decrease of saturated fatty acids (FA) in milk fat of CLA supplemented cows, combined with a higher content of monounsaturated and *trans* FA.
Our study revealed no significant effects of *c*9,*t*11- and *t*10,*c*12-CLA supplementation either on milk yield and composition or on metabolic key parameters in blood. Furthermore the experiment did not indicate significant effects of *c*9,*t*11- and *t*10,*c*12-CLA-supplementation on gene expression of peroxisome proliferator-activated receptor-alpha (PPARα), PPARγ, sterol regulatory element-binding protein-1 and tumor necrosis factor-alpha in liver tissue.

Conclusions: Feeding *c*9,*t*11- and *t*10,*c*12-CLA during the first weeks after calving did not affect metabolic key parameters of blood serum or milk composition of fresh cows. Milk fatty acid composition was changed by feeding *c*9,*t*11- and *t*10,*c*12-CLA resulting in higher contents of these isomers in milk fat. High contents of long chain FA in milk fat indicate that CLA supplementation during the first four weeks of lactation did not affect massive peripheral lipomobilization.

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Background

Within the European Union, a system with fixed milk quotas per farm is applied, aiming to control total milk production and avoid surplus of milk. The quota is expressed as kg milk with a defined milk fat content, which allows the farmer to market a higher milk volume if milk fat content is low. Therefore, the possibilities to decrease milk fat content have gained interest from farmers. Furthermore, the dairy industry tries to enhance consumers trust in dairy fat by increasing the conjugated linoleic acid (CLA) content. Finally, the interest in CLA in research today is to a large extent driven by a general interest in the mechanisms of milk fat synthesis.

Extensive basic research, established predominantly by the group of Bauman and colleagues in Cornell, has shown that *trans*(t)10,*cis*(c)12-CLA reduces milk fat synthesis in the alveolar epithelial cells of the mammary gland [1] and, consequently, milk fat percentage.

Moreover, a reduction of milk fat production was considered as an option to reduce the metabolic imbalance of transition cows. The transition period, i.e. 21 days before calving until day 21 after calving [2], is a critical time frame in which the animals have to cope an enormous metabolic challenge due to the rapid onset of high milk production and a reduced dry matter intake. Managing and feeding the transition cow affects health and productivity during lactation and is the basis for economical and sustainable milk production. To meet energy requirements at the beginning of lactation, an increase of energy density of the diet by adding rumen-protected fat may be assumed. Furthermore, the energy deficit also could be reduced if the fat content of milk is lowered by a feed supplement.

It is hypothesized that a reduction of milk fat percentage may improve the metabolic resilience of high yielding dairy cows in early lactation. Thus, it was the objective to investigate whether milk composition and metabolic key parameters are affected by adding rumen-protected CLA to the diet of dairy cows in the first four weeks of lactation.

Materials and methods

Animals, treatments, and sampling

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 Bavarian State animal welfare committee. Ten primiparous Brown Swiss cows were allocated to one of two groups before calving. Cows of control group (n = 5) were fed a lactational diet with concentrates according to milk yield at the onset of lactation, cows of treatment group (n = 5) were fed the same lactational diet and concentrates. However additional CLA supplement was fed during the first four weeks of lactation to cows of the CLA-group. Group arrangement was based on how well cows accepted the fat supplement. Cows that consumed the fat concentrate during five successive test days before milk stasis (56 days before expected calving date) immediately after submission were arranged to CLA-treatment group. Due to the well known feed intake depression at the beginning of lactation, especially related to the mealy and powdery supplement, a good acceptance of the supplement by each cow of treatment group was required. The study was performed with primiparous cows exclusively to avoid lactation number as a confounding factor and due to the fact that the overall effects of CLA on milk synthesis do not depend on the lactation number [3]. All cows were housed in free stall barns fitted with rubber mats and fed the same basal diet (corn silage 43%, grass silage 25%, hay 32%; NE_L 1.36 Mcal/kg, Table 1) during the prepartum period, and then switched to a lactational diet (corn silage 60%, grass silage 23%, concentrates 12%, hay 4%, and minerals 1% of wet weight; NE_L 1.55 Mcal/kg, Table 1) immediately after parturition. The basal diet, formulated on the basis of a milk yield of 22 kg/day, was delivered once daily (0700 h) and intended to provide *ad libitum* intake (> 5% residual feed). If daily milk yield exceeded 22 kg additional concentrates were fed (0.5 kg concentrates per kg milk). Dry matter intake could not be assessed. Water was available at all

Table 1 Ingredients and chemical composition of lactational and basal diet[1].

Variable	Lactational diet	Basal diet
Ingredient, %		
Corn silage	60	43
Grass silage	23	25
Hay	4	32
Concentrates[2]	12	-
Mineral mix[3]	1	-
Chemical analysis, %		
DM	45.2	52.0
CP	12.2	12.9
CF	18.6	19.2
NFC	26.4	9.92
Ether extract	3.16	3.16
NE_L, Mcal/kg	1.55	1.36

[1]Lactational diet was fed to all cows postpartum and basal diet was fed to all cows prepartum.
[2] Composition: corn gluten 18.4%, turnips molasses chips 13.8%, wheat 10.0%, triticale 10.0%, rape cake 10.0%, maize 8.8%, malt germ 6.0%, grain distillation residual (ProtiGrain) 5%, rape extraction grist 5%, rumen protected rape extraction grist 5%, palm corn cake 3.3%, soy extraction grist 2.8%, sodium bicarbonate 1.0%, calcium bicarbonate 0.99%, plant oil (palm coconut) 0.40%.
[3] The mix contained calcium 14%, sodium 10%, phosphorous 5%, magnesium 5%.

Table 2 Ingredients of CLA supplement[1].

Variable	
Ingredient, %	
Soybean	52
Glucose	10
CLA	15
There of c9, t11-CLA	7.5
t10, c12-CLA	7.5
Biscuit flour	4
Wheat bran	4
Cornflakes	3.5
Magnesium phosphate	3.5
Malt sprouts	2.5
Brewer's yeast	1.5
lactalbumin powder	0.8
Soybean oil	0.2
NE_L, Mcal/kg	3.35

[1] Cows received 7.5 g CLA/day (50% *c*9,*t*11- and 50% *t*10,*c*12-CLA) starting two weeks before expected calving and followed by 20 g CLA/day (50% *c*9, *t*11- and 50% *t*10,*c*12-CLA) during the first 28 days of lactation

times. Five cows were additionally fed a special supplement including rumen-protected CLA (encapsulation technology: spray freezed; BEWITAL GmbH & Co. KG, Südlohn-Oeding, Germany) (Table 2). They received 7.5 g CLA/day (50% *c*9,*t*11- and 50% *t*10,*c*12-CLA) starting two weeks before expected calving and followed by 20 g CLA/d (50% *c*9,*t*11- and 50% *t*10,*c*12-CLA) during the first 28 days of lactation. CLA supplement was offered at the bunk once daily immediately after milking (0600 h) while cows were fixed in the feed fence. Refusals of CLA were weighed and recorded.

After parturition, cows were milked twice daily (0415 and 1545 h) and milk yields were recorded at each milking until day 56. Milk samples were taken at the evening milking. Samples were separated during milking into sample vessels (capacity about 1 liter) controlled by milk flow rate and total amount of milk (Metatron P21, GEA WestfaliaSurge, Boenen, Germany). One aliquot was stored at 4°C for a maximum of 10 days with a preservative (acidiol) until analyses of milk composition. A second aliquot was stored at -20°C until analyzed for fatty acid composition. Milk composition was analyzed daily during the first week of lactation and thereafter twice a week for 7 weeks (from week 2 to week 8 postpartum). Fatty acid (FA) composition was examined twice a week during the first 4 weeks postpartum and weekly during the following 4 weeks (from week 5 to week 8 postpartum).

Jugular blood samples were collected in the morning (0700 h) at calving and at weeks 1, 2, 4, 6, and 8 of lactation. Blood serum was harvested following centrifugation (2000 × *g*, 15 min at 4°C) and stored in three aliquots at -20°C until analyzed for total bilirubin (TB), glucose, non-esterified fatty acids (NEFA) and betahydroxybutyrate (BHB). Liver biopsies were obtained (Bard® MAGNUM™, Covington, USA) in the morning (0800 h) at weeks 1, 2, 4 and 8 of lactation. Liver tissue (approx. 100 mg) was frozen immediately in liquid nitrogen, and stored at -80°C until analyzing for gene expression.

Milk composition analysis

Milk protein, fat, lactose, urea and pH were analyzed by infrared-spectrophotometric technique (MilkoScan™ FT6000) and somatic cell count was determined by fluorescence-optical counting system (Fossomatic™ FC) in the laboratories of Milchprüfring Bayern e.V., Germany.

Milk fatty acid analysis

The FA composition of milk samples was determined using FA methyl esters (FAME) prepared by transesterification with TSMH (trimethylsulfonium hydroxide) at room temperature. FAMEs were analyzed using gas chromatography (GC 6890, AgilentTechnologies, Waldbronn, Germany) to determine isomer distribution patterns. FA were quantified by use of Chromeleon® 6.8 Chromatography Software (Dionex, USA).

Blood serum analysis

Glucose, NEFA, BHB, and TB were analyzed with an automated clinical chemistry analyzer (ABX Pentra 400, Horiba, Montpellier, France). The hexokinase method was applied for glucose analysis and NEFA concentrations were determined with the enzymatic reactions (both Hoffmann La-Roche, Basel, Switzerland). BHB measurement was performed by using an enzymatic analysis (Sigma-Aldrich Diagnostics, Munich, Germany). TB was analyzed via Jendrassik/Grof reaction [4]. The clinical chemistry analyzer was calibrated and controls assayed daily according to the manufacturer's instructions to ensure acceptable assay performance.

Gene expression

Total RNA was isolated from liver tissue samples according to the manufacturer's instructions of peqGOLD TriFast™. RNA was quantified by spectrophotometry (BioPhotometer, Eppendorf, Hamburg) and diluted in RNase-free water. Degradation of the RNA was measured with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) in connection with the RNA 6000 Nano Assay. Gene expression was measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR) (SuperScript™ III Platinum® SYBR® Green One PCR Kit, Invitrogen, Karlsruhe, Germany) using the RotorGene3000 (Corbett Research,

Table 3 Sequences of PCR primers[1].

Primer		Sequence (5' → 3')
Histone	forward	ACT TGC TAC AAA AGC CGC TC
	reverse	ACT TGC CTC CTG CAA AGC AC
Ubiquitin	forward	AGA TCC AGG ATA AGG AAG GCA T
	reverse	GCT CCA CCT CCA GGG TGA T
PPARα	forward	GGA TGT CCC ATA ACG CGA TTC G
	reverse	TCG TGG ATG ACG AAA GGC GG
PPARγ	forward	CTC CAA GAG TAC CAA AGT GCA ATC
	reverse	CCG GAA GAA ACC CTT GCA TC
SREBP1	forward	CCA GCT GAC AGC TCC ATT GA
	reverse	TGC GCG CCA CAA GGA
TNFα	forward	CCA CGT TGT AGC CGA CAT C
	reverse	CCC TGA AGA GGA CCT GTG AG

[1] Primers were designed using Primer3 online software and synthesized by metabion international AG (Martinsried, Germany).

Cambridge, United Kingdom). Primer for proliferator-activated receptor-alpha (PPARα), PPARγ, sterol regulatory element-binding protein-1 (SREBP1) and tumor necrosis factor-alpha (TNFα) were designed using Primer3 online-software and synthesized by Metabion International AG (Martinsried, Germany, Table 3). The mean of the two housekeeping genes, histone and ubiquitin, was calculated for the reference index and used for normalization. Δ quantitative Cycle (Cq)-values were calculated as $\Delta Cq = Cq_{target\ gene}$ - $meanCq_{refence\ genes}$ and ΔΔCq-values were calculated according to $\Delta\Delta Cq = \Delta Cq_{target\ gene}$ - $mean\Delta Cq_{refence\ genes}$.

Statistical analysis

Endpoints measured repeatedly (milk yield, milk composition and milk FA profile) were reduced to weekly means before statistical analysis. Differences among treatments (group) and comparisons between times (week) were analyzed by repeated measures ANOVA using Bonferroni's t-test (Sigma-Stat v.3.00 and the PASW Statistics 17, both SPSS Inc., Chicago, USA).

The effects of group and week were considered as fixed effects with week of experiment as a repeated measurement and with cow within dietary treatment (group) as the subject. Orthogonal polynomial contrast was used to describe linear, quadratic or cubic trends over time (week by group interaction) and group effects. All data are presented as mean ± standard deviation (SD). Means were considered to differ significantly in case $P < 0.05$.

Results

The CLA supplement was insufficiently accepted by the animals after parturition: on average, only 61.5% of the intended amount of 20 g CLA per day (10 g *c*9,*t*11- and 10 g *t*10,*c*12-CLA) was ingested. In the first week postpartum cows ingested 10.1 ± 7.8 g CLA per day, 11.5 ± 7.7 g CLA at week 2 postpartum, 15.2 ± 6.4 CLA at week 3 postpartum and 12.4 ± 6.5 g CLA at week 4 of lactation (50% *c*9,*t*11 and 50% *t*10,*c*12-CLA). On average, cows ingested 12.3 ± 4.7 g CLA/day during the treatment period. Results were calculated for two timeframes: from day 1 postpartum until day 28 postpartum (CLA supplemented period) and from day 29 postpartum until day 56 postpartum. Milk yield, milk protein, milk fat, and urea content did not differ between the two groups (Table 4).

Metabolic key parameters did not differ between groups during treatment and from week 5 to week 8 (Table 5).

Absorbed CLA was detectable in milk fat during the supplementation timeframe, resulting in a shift in the

Table 4 Means ± SD for milk yield and milk composition during and after the treatment period[1].

	Control (n = 5)				CLA (n = 5)			
	1 - 28 DIM		29 - 56 DIM		1 - 28 DIM		29 - 56 DIM	
Variable	mean[5]	SD	mean	SD	mean	SD	mean	SD
milk yield[2], kg/day	24.5	2.8	28.8	2.3	24.5	3.3	28.9	2.1
3.5% FCM[3], kg/day	30.9	5.4	34.7	5.7	30.4	2.6	33.0	3.5
milk fat[4], %	6.10	0.99	5.27	0.78	5.77	0.14	4.97	0.30
milk fat[4], kg/day	1.49	0.15	1.52	0.12	1.41	0.16	1.44	0.12
milk protein[4], %	3.81	0.12	3.31	0.13	3.82	0.15	3.42	0.13
milk protein[4], kg/day	0.93	0.09	0.95	0.07	0.94	0.09	0.99	0.08
milk lactose[4], %	4.75	0.28	5.02	0.03	4.72	0.25	4.94	0.02
milk lactose[4], kg/day	1.17	0.25	1.43	0.03	1.14	0.28	1.42	0.02
urea[4], mmol/L	6.25	1.14	5.28	1.27	5.63	1.26	5.38	0.96

[1]CLA supplemented timeframe: d 1 till d 28 of lactation; five primiparous cows received a special supplement including rumen-protected CLA (10 g *c*9,*t*11-CLA/day and *t*10,*c*12-CLA/day).
[2]Milk yield was recorded at each milking.
[3]FCM was calculated like following: (fat [%] × 0.15 + 0.4) × milk yield [kg/day]
[4]Milk composition was analyzed once daily in the first week after parturition and twice a week from week 2 until week 8 postpartum.
[5]Milk yield and milk composition values were reduced to weekly means, means from wk 1 till 4 are pooled to timeframe 1 - 28 DIM, means from week 5 till 8 are pooled to timeframe 29 - 56 DIM

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Table 5 Means ± SD for blood serum metabolites[1] during and after the treatment period[2].

	Control (n = 5)				CLA (n = 5)			
	1 - 28 DIM		29 - 56 DIM		1 - 28 DIM		29 - 56 DIM	
Variable	mean	SD	mean	SD	mean	SD	mean	SD
TB[3], μmol/L	7.01	4.52	3.58	0.74	4.51	0.73	3.10	0.70
Glucose, mmol/L	3.40	0.11	3.53	0.06	3.47	0.28	3.53	0.19
NEFA[4], μmol/L	483	174	165	105	378	119	152	61.1
BHB[5], mmol/L	0.54	0.14	0.22	0.08	0.49	0.28	0.39	0.04

[1]Jugular blood samples were collected at week 0, 1, 2, 4, 6, 8. Values from week 0, 1, 2, 4 were calculated for 1 - 28 DIM, values from week 6 and 8 were calculated for 29 - 56 DIM.
[2]CLA supplemented timeframe: d 1 until d 28 of lactation; five primiparous cows received a special supplement including rumen-protected CLA (10 g *c*9,*t*11-CLA/day and *t*10,*c*12-CLA/day).
[3]total bilirubin.
[4]non-esterified fatty acids.
[5]betahydroxybutyrate.

FA composition of milk fat (Table 6). On average over the entire treatment period, there was a reduction in the yield of saturated fatty acids in the milk fat of cows receiving CLA, together with a higher content of monounsaturated and *trans* FA. Contents of *c*9,*t*11-CLA were higher in milk fat of CLA supplemented cows compared to the control group (0.73 ± 0.04 g/100 g fat *vs.* 0.64 ± 0.01 g/100 g fat). In addition, contents of *t*10,*c*12-CLA in milk fat of CLA supplemented cows were significantly higher compared to the control group (0.02 ± 0.01 g/100 g fat *vs.* 0.00 ± 0.00 g/100 g fat, $P = 0.002$).

Total RNA quantity and RNA integrity number (RIN) values were similar for cows in the control group (RNA concentrations: 1276 ± 1016 ng/μl; RIN values: 6.5 ± 2.3) and for cows in CLA supplemented group (RNA concentrations = 1109 ± 986 ng/μl; RIN values: 6.6 ± 1.9). mRNA levels of histone and ubiquitin were tested for normal distribution. Constant mRNA levels of histone and ubiquitin was manifested by analysis of variance. ΔCq-values and ΔΔCq-values of the genes PPARα, PPARγ, SREBP1 and TNFα did not differ between the two groups and over the weeks (Table 7).

Discussion

The transition period between late pregnancy and early lactation is characterized by a shift in nutrient partitioning that requires extensive coordination of metabolism to ensure an adequate supply of nutrients to support milk synthesis [5]. Due to this metabolic adaptation, the CLA supplementation in this project was designed to start before parturition and to take place along the whole transition period.

In the present study, CLA supplementation did not affect milk fat content. This differs from other studies conducted with cows during established lactation, in which feeding rumen-protected CLA [6-9] or abomasal infusion of CLA [10-13] or intravenous infusions of CLA [14] resulted in a reduction of milk fat content. An explanation for the lack of a CLA response in milk fat during the first few weeks postpartum is unknown [8]. Our analysis indicates that *c*9,*t*11- and *t*10,*c*12-CLA were consistently transferred to milk fat throughout the treatment period. Contents of *c*9,*t*11- and *t*10,*c*12-CLA in milk of CLA supplemented cows were significantly higher compared to the *c*9,*t*11- and *t*10,*c*12-CLA content in milk of cows of the control group.

Peterson *et al.* [3] found that *t*10,*c*12-CLA was also transferred to milk fat and that the milk fat content of *t*10,*c*12-CLA are curvilinearly related to reduced milk fat yield, according to de Veth *et al.* [15]. However in our study we could not demonstrate such a decrease of milk fat yield. It can be speculated that at the onset of lactation the essential cellular signaling systems are attenuated such that *t*10,*c*12-CLA is unable to elicit the coordinated reduction in the expression of genes for key lipogenic enzymes.

Furthermore in milk of CLA supplemented cows, contents of short chain (< C 10) and middle chain FA (C 10 - C 16) were reduced and the amount of long chain FA (> C 16) was increased. These results go in line with previous results from studies conducted during early and mid lactation [8,9,16,17].

Results from the present study with transition cows are partially similar to those observed after administration of CLA to cows in established lactation. In established lactation milk yield and milk protein content were relatively unaffected by abomasal administration of CLA or CLA feeding [3,11,13]. In our study CLA supplementation did not affect milk yield and milk protein content.

Bernal-Santos *et al.* [8] presented the first study in which rumen-protected CLA was fed during the prepartum period. Supplementation had no effect on measured performance variables and plasma metabolites. In our study, CLA supplementation had no effects on concentrations of TB, glucose, NEFA and BHB in blood serum.

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Table 6 Means ± SD for fatty acid composition of milk fat from cows received *c*9,*t*11- and *t*10,*c*12-CLA and for the control group, during treatment period[1] and post-treatment.

	Control (N = 5)				CLA (N = 5)				*P*-value Control	*P*-value CLA	*P*-value Control vs. CLA	*P*-value Control vs. CLA
	1 - 28 DIM		29 - 56 DIM		1 - 28 DIM		29 - 56 DIM					
	mean	SD	mean	SD	mean	SD	mean	SD	1-28 vs. 29-56	1-28 vs. 29-56	1-28	29-56
Fatty acid, g/100 g fat												
4:0	1.40	0.10	1.20	0.12	1.38	0.11	1.24	0.04				
6:0	1.14	0.06	1.18	0.06	1.13	0.13	1.23	0.02				
8:0	0.84	0.03	0.94	0.02	0.86	0.10	1.01	0.04	0.003			0.038
10:0	1.91	0.12	2.39	0.20	1.95	0.20	2.55	0.13	0.011	0.012		
10:1	0.15	0.03	0.25	0.02	0.15	0.02	0.28	0.03	0.09	0.003		
11:0	0.05	0.02	0.08	0.08	0.04	0.01	0.07	0.01		0.013		
12:0	2.90	0.18	3.79	0.44	2.87	0.41	4.04	0.30	0.013	0.016		
12:1 *cis*-9	0.04	0.01	0.07	0.01	0.03	0.01	0.08	0.01	0.007	0.002		
iso-13:0	0.04	0.01	0.08	0.01	0.04	0.01	0.09	0.02	0.008	0.03		
13:0	0.09	0.02	0.14	0.09	0.07	0.01	0.12	0.02		0.013		
iso-14:0	0.09	0.03	0.11	0.04	0.08	0.00	0.10	0.02				
14:0	9.14	0.42	11.35	0.45	8.84	0.39	11.27	0.37	0.001	0.001		
14:1 *cis*-9	0.57	0.08	0.86	0.13	0.57	0.05	0.88	0.13	0.013	0.0021		
iso-15:0	0.10	0.00	0.11	0.00	0.09	0.01	0.10	0.01	0.012			
anteiso-15:0	0.27	0.04	0.36	0.04	0.26	0.03	0.36	0.04	0.034	0.023		
15:0	0.85	0.10	1.15	0.48	0.70	0.09	1.01	0.07		0.01		
iso-16:0	0.23	0.03	0.23	0.07	0.21	0.02	0.22	0.01				
16:0	26.17	0.82	27.90	0.95	25.74	1.57	26.62	0.95	0.05			
16:1 *trans*-9	0.17	0.00	0.17	0.01	0.17	0.01	0.16	0.01			0.04	
16:1 *cis*-9	1.48	0.04	1.40	0.06	1.65	0.12	1.38	0.13				
iso-17:0	0.36	0.02	0.42	0.04	0.36	0.04	0.43	0.03				
anteiso-17:0	0.43	0.04	0.45	0.04	0.42	0.05	0.44	0.04				
17:0	0.71	0.05	0.63	0.11	0.64	0.06	0.58	0.01				
17:1 *cis*-9	0.32	0.02	0.27	0.02	0.34	0.04	0.24	0.02	0.014	0.016		
iso-18:0	0.09	0.01	0.07	0.01	0.09	0.01	0.06	0.00		0.011		
18:0	11.84	0.44	9.52	0.46	11.59	0.54	10.13	0.29	0.001	0.014		
18:1 *trans*-9	0.59	0.12	0.61	0.03	0.63	0.03	0.66	0.05				
18:1 *trans*-10	0.30	0.02	0.36	0.05	0.31	0.05	0.39	0.05				
18:1 *trans*-11	1.48	0.43	1.29	0.13	1.49	0.11	1.29	0.08				
18:1 *cis*-9	25.78	1.26	22.19	0.94	26.45	1.48	22.44	0.17	0.009	0.01		
18:1 *cis*-11	1.09	0.06	1.01	0.17	1.10	0.11	1.00	0.04				
18:1 *cis*-12	0.24	0.04	0.27	0.02	0.27	0.05	0.28	0.02				
18:1 *cis*-13	0.11	0.02	0.07	0.01	0.11	0.05	0.08	0.03	0.03			
18:2 *trans*-9, *trans*-12	1.83	0.08	1.82	0.10	2.11	0.20	1.97	0.13			0.048	
18:2 *cis*-9, *trans*-11	0.64	0.10	0.69	0.05	0.73	0.04	0.68	0.07				
18:2 *trans*-10, *cis*-12	0.00	0.00	0.00	0.00	0.02	0.01	0.00	0.00		0.008	0.002	
18:2 *trans*-9, *trans*-11	0.02	0.01	0.01	0.00	0.03	0.00	0.01	0.00		0.002		
18:3 *cis*-9, *cis*-12, *cis*-15	0.43	0.03	0.40	0.06	0.43	0.01	0.36	0.01		0.003		
19:0	0.06	0.00	0.05	0.00	0.04	0.01	0.05	0.01			0.015	
20:0	0.14	0.01	0.13	0.00	0.13	0.00	0.14	0.00		0.004	0.029	0.027
20:1 *cis*-11	0.11	0.00	0.11	0.02	0.10	0.01	0.10	0.02				
20:2 *cis*-11, *cis*-14	0.03	0.01	0.03	0.01	0.05	0.01	0.05	0.03				
21:0	0.02	0.00	0.02	0.00	0.01	0.00	0.02	0.00		0.026	0.004	
20:3 *cis*-8, *cis*-11, *cis*-14	0.08	0.01	0.11	0.01	0.10	0.01	0.13	0.00	0.014	0.008		
20:4 *cis*-5, *cis*-8, *cis*-11, *cis*-14	0.13	0.03	0.12	0.01	0.11	0.01	0.10	0.02				
22:0	0.03	0.01	0.03	0.00	0.02	0.00	0.03	0.00		0.01		

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Table 6: Means ± SD for fatty acid composition of milk fat from cows received *c*9,*t*11- and *t*10,*c*12-CLA and for the control group, during treatment period[1] and post-treatment. *(Continued)*

20:5 *cis*-5. *cis*-8, *cis*-11, *cis*-14, *cis*-17	0.03	0.01	0.02	0.00	0.02	0.00	0.02	0.00				0.036
24:0	0.03	0.01	0.02	0.00	0.01	0.00	0.02	0.01			0.005	
22:5 *cis*-7, *cis*-10, *cis*-13, *cis*-16, *cis*-19	0.12	0.02	0.10	0.01	0.09	0.01	0.08	0.01				
Other	5.36		5.43		5.35		5.44					
Summation, g/100 g fat												
SFA[2]	58.9	0.87	62.3	1.01	57.5	1.95	61.9	0.07	0.005	0.018		
MUFA[3]	29.9	1.23	26.5	0.87	30.8	1.67	26.7	0.27	0.01	0.014		
PUFA[4]	0.39	0.06	0.38	0.03	0.39	0.01	0.38	0.03				
*t*FA[5]	4.37	0.58	4.25	0.17	4.71	0.34	4.46	0.21				
CLA[6]	1.12	0.07	1.14	0.10	1.20	0.05	1.08	0.06		0.046		

[1]CLA supplemented timeframe: d 1 till d 28 of lactation; five primiparous cows received a special supplement including rumen-protected CLA (10 g *c*9,*t*11-CLA/day and *t*10,*c*12-CLA/day). Post-treatment timeframe: 29 - 56 DIM.
[2]saturated fatty acids
[3]monounsaturated fatty acids
[4]polyunsaturated fatty acids
[5]*trans* fatty acids
[6]conjugated linoleic acids

Table 7 ΔCq[1]-values (mean ± SD) of hepatic mRNA expression of the genes PPARα[2], PPARγ[3], SREBP1[4], TNFα[5]in CLA supplemented cows vs. control group before (week -1), during (week 2 and 4) and after (week 8) treatment[6].

	Control (n = 5)								CLA (n = 5)							
	week -1		week 2		week 4		week 8		week -1		week 2		week 4		week 8	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
ΔPPARα	1.5	0.3	1.4	0.2	1.9	0.3	1.7	0.5	2.0	0.1	1.4	0.3	1.9	0.7	2.0	0.5
ΔPPARγ	7.8	0.8	9.6	2.3	9.8	2.4	9.4	0.8	9.1	0.3	8.6	0.2	9.2	0.7	9.2	0.2
ΔSREBP1	2.3	1.6	4.0	2.9	4.0	1.8	4.2	1.6	3.0	0.2	3.7	0.7	4.1	1.2	3.1	0.5
ΔTNFα	9.3	0.6	8.9	0.4	9.4	0.7	9.4	1.2	8.5	0.4	8.0	1.1	8.5	1.5	8.4	0.4

[1] ΔCq-values were calculated as $\Delta Cq = Cq_{target\ gene} - meanCq_{refence\ genes}$
[2] peroxisome proliferator-activated receptor-alpha.
[3]peroxisome proliferator-activated receptor-gamma.
[4]sterol regulatory element-binding protein-1.
[5]tumor necrosis factor-alpha.
[6]CLA supplemented timeframe: day 1 till day 28 of lactation; five primiparous cows received a special supplement including rumen-protected CLA (10 g *c*9,*t*11-CLA/day and *t*10,*c*12-CLA/day).

Because of sustained lipomobilization the metabolic situation could not be improved by feeding CLA. Comparable results were obtained by Perfield *et al.* [7]. In addition, CLA supplementation had no effects on hepatic mRNA levels of PPARα, PPARγ, SREBP1 and TNFα. Comparable studies, in which effects of CLA supplementation on hepatic gene expression in dairy cows were measured, do not exist to our knowledge. Previous studies in rodents revealed effects of *c*9,*t*11- and *t*10,*c*12-CLA on gene expression of fatty acid synthesis, fatty acid oxidation and drug detoxification-associated enzymes in liver tissue [18-20].

Our results go in line with previous results stating that *t*10,*c*12-CLA affects primarily the *de novo* synthesis of FA in the alveolar epithelial cells of the mammary gland but does not inhibit peripheral lipomobilization. The transition period is associated with an increased mobilization of body fat reserves, which results in an increased mammary uptake of circulating NEFA and their use to synthesize milk fat triglycerides [13]. This is one explanation of the considerably higher fat percentage of bovine milk in the first weeks of lactation when precursors for milk synthesis are not completely available from feed [1].

Obviously, during the first weeks of lactation, the milk fat depressing effects of *t*10,*c*12-CLA intake are less pronounced compared to mid or late lactation. This may be explained by a low contribution of long-chain FAs originating from lipomobilization to milk fat in mid lactation while the proportion of *t*10,*c*12-CLA-dependend *de novo* synthesized FAs is high compared to the first weeks of lactation. The study did not provide evidence that CLA

affected substrate partitioning in the body of the cows which may be explained by the comparatively small amounts fed compared to rodent studies.

The present study is the first describing supplemention of primiparous cows with CLA during the first four weeks of lactation. However, due to the limited number of animals per treatment group, the small amount of supplemented CLA and the restricted supplementation period during early lactation, it is difficult to make any definitive conclusions about the metabolic benefits of CLA supplementation.

Conclusions

Supplementation of *c*9,*t*11- and *t*10,*c*12-CLA during the first four weeks of lactation resulted in an increase of these specific CLA isomers provided during treatment. Therefore all the CLA isomers were taken up by the mammary gland and incorporated into milk fat. During the first four weeks of lactation, however, CLA supplementation did not affect milk yield, milk composition, blood serum metabolites and gene expression in liver of primiparous cows.

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Authors' contributions

TS was responsible for the CLA feeding as well as for all sample obtention, fatty acid composition analysis, mRNA extraction from liver tissue, RT-qPCR performance, and statistical analysis of the results. GS assisted in blood sampling. HK briefed TS in working with the HPLC. MK created the experimental design and supervised the study. SW performed liver biopsies. HHDM was the project leader and supervised the study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Nuclear magnetic resonance and mass spectrometry-based milk metabolomics in dairy cows during early and late lactation

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ABSTRACT

Milk production in dairy cows has dramatically increased over the past few decades. The selection for higher milk yield affects the partitioning of available nutrients, with more energy being allocated to milk synthesis and less to physiological processes essential to fertility and fitness. In this study, the abundance of numerous milk metabolites in early and late lactation was systematically investigated, with an emphasis on metabolites related to energy metabolism. The aim of the study was the identification and correlation of milk constituents to the metabolic status of the cows. To investigate the influence of lactation stage on physiological and metabolic variables, 2 breeds of different productivity were selected for investigation by high-resolution nuclear magnetic resonance spectroscopy and gas chromatography-mass spectrometry. We could reliably quantify 44 different milk metabolites. The results show that biomarkers such as acetone and β-hydroxybutyrate are clearly correlated to the metabolic status of the individual cows during early lactation. Based on these data, the selection of cows that cope well with the metabolic stress of early lactation should become an option.
Key words: milk metabolomics, nuclear magnetic resonance, mass spectrometry

INTRODUCTION

Measurement of selected milk constituents to monitor the udder health or the metabolic status of cows has attracted much attention in dairy research and is widely used on dairy farms. Milk SCC and *N*-acetyl-β-D-glucosaminidase activity are well-known indicators of intramammary infections (Pyorala, 2003). Furthermore, the biochemical milk profile has been related to the health status of the cow and, in particular, to imbalances during early lactation (Duffield et al., 2009). Ketone bodies such as acetone, acetoacetate, and BHBA in milk are biomarkers for subclinical ketotic conditions (Geishauser et al., 2000; Enjalbert et al., 2001). For example, BHBA concentrations ≥0.10 mmol/L indicate subclinical ketosis yielding a sensitivity, specificity, and a positive and negative predictive value of 69, 95, 75, and 93%, respectively. Enjalbert et al. (2001), on the other hand, proposed thresholds for milk acetone and BHBA of 0.16 and 0.07 mmol/L, respectively. On the basis of the close interaction between blood circulation and milk secretion, changes in additional milk metabolites that have yet to be defined can be assumed to reflect the metabolic and health conditions of the cow and the mammary gland.

High-resolution nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for the analysis of physiological fluids that contain complex mixtures of organic molecules (Lindon et al., 2006). Requiring little sample preparation, NMR allows the direct and reliable determination of analytes in biological specimens (Wishart, 2008). The one drawback of NMR is its limited detection sensitivity compared with mass spectrometry (MS). The latter also offers the advantage of excellent selectivity when operated in selected ion monitoring mode. Coupled to gas chromatography (GC), a high-resolution separation technique, hundreds of metabolites can be identified and quantified in a single run (Almstetter et al., 2009).

Previously, both NMR (Belloque and Ramos, 2002; Hu et al., 2007) and GC-MS (Toso et al., 2002; Boudonck et al., 2009) have been applied successfully to the analysis of compounds in commercially available milk samples. Here, NMR and GC-MS were applied to the determination of a total of 44 milk compounds in milk specimens obtained from individual dairy cows of 2 different breeds during early and late lactation, with an emphasis on metabolites related to energy metabolism. The ultimate aim of the study was to identify the compounds that are highly correlated to the metabolic status of the indivdual animals.

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Table 1. Ingredients and chemical composition of the partial mixed rations[1]

Item	Farm 1	Farm 2
Ingredient (%)		
Corn silage	60	59
Grass silage	24	27
Hay	4	3
Concentrate	11[2]	11[3]
Mineral mix	1[4]	—
Chemical composition		
DM (%)	43.7	43.7
CP (% of DM)	12.9	13.2
Crude fiber (%)	18.8	18.9
Utilizable CP (%)	14.3	14.3
NE_L (Mcal/kg)	1.65	1.65

[1]Concentrates were additionally fed according to milk yield.

[2]Composition: corn gluten 18.4%, turnips molasses chips 13.8%, wheat 10.0%, triticale 10.0%, rape cake 10.0%, maize 8.8%, malt germ 6.0%, grain distillation residual (ProtiGrain Crop Energies AG, Mannheim, Germany) 5%, rape extraction grist 5%, rumen protected rape extraction grist 5%, palm corn cake 3.3%, soy extraction grist 2.8%, sodium bicarbonate 1.0%, calcium bicarbonate 0.99%, plant oil (palm coconut) 0.40%.

[3]Composition: rape cake 20%, soy extraction grist 24%, barley 21%, wheat 18.7%, maize 10%, sodium bicarbonate 0.8%, calcium bicarbonate 1%, urea 1%.

[4]Ingredients: calcium 14%, sodium 10.0%, phosphorous 5.0%, magnesium 5.0%.

MATERIALS AND METHODS

Collection of Milk Samples

Morning milk specimens were collected at 2 research farms near Munich, Germany, from cow breeds representing different milk production levels. At the research farm Veitshof (Freising, Germany; farm 1), milk samples were collected from highly productive Brown Swiss cows with an average 305-d milk yield of 9,200 kg in early (average ± SD: 42.8 ± 25.3 DIM; n = 27), mid (143.4 ± 21.5 DIM; n = 7), and late (345.7 ± 159.2 DIM; n = 24) lactation. At the Bavarian State Research Center for Agriculture (Grub, Germany; farm 2), milk samples of Simmental cows with an average 305-d milk yield of 8,300 kg in early (DIM 25.0 ± 12.3; n = 28) and late (DIM 261.8 ± 41.3; n = 20) lactation were collected. All cows were offered ad libitum a partial TMR based on corn and grass silage. Concentrates were offered according to milk yield. Details about the different diets are given in Table 1. For technical reasons, AA composition of the ration was not determined. However, it has been shown previously that the fractional efficiencies of converting portal vein–absorbed individual essential AA into milk protein ranged from 0.42 to 0.68 (Blouin et al., 2002). Thereby, the liver changes the profile of AA available to the mammary gland relative to the amount absorbed from the gut.

One aliquot of the collected milk samples was analyzed for milk protein, fat, lactose, urea, and pH by MilkoScan FT 6000 and for SCC by Fossomatic-FC (MPR Bayern e.V., Wolnzach, Germany). The second aliquot was defatted by centrifugation for 15 min at 3,000 × g at a temperature of 4°C, and skim milk was immediately stored at −20°C until analysis.

Sample Preparation for NMR Analysis

Milk samples were thawed, thoroughly shaken, and ultra-filtered employing Amicon Ultra-4 centrifugal filter units (Millipore, Billerica, MA) with a molecular weight cutoff of 10 kDa that were spun for 60 min at 4,000 × g in a swing-bucket rotor at 22°C. Filtration was necessary to remove large biomolecules, such as proteins, from the samples. Tests without filtration showed broad background signals that severly hampered metabolite quantification. In addition, complex formation between the used NMR reference compound and proteins was observed, which resulted in a considerably diminished reference signal. The used NMR reference compound was 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (**TSP**; Sigma-Aldrich, Taufkirchen, Germany). Four hundred microliters of ultrafilrate was then mixed with 200 μL of 0.1 mol/L of phosphate buffer (pH 7.4) and 50 μL of 29.02 mmol/L of TSP in deuterium oxide as internal standard. One hundred milliliters of phosphate buffer (pH 7.4) was prepared by adding 8.02 mL of 1 mol/L of aqueous K_2HPO_4 solution to 1.98 mL of 1 mol/L of aqueous KH_2PO_4 solution and filling up to 100 mL with pure water. Finally, 30 mg of boric acid was added to prevent bacterial growth.

NMR Spectroscopy

The NMR experimental conditions and algorithms used for data analysis have been described recently (Gronwald et al., 2008). Briefly, NMR experiments were carried out on a Bruker Avance III 600 MHz spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) employing a triple-resonance (^1H, ^{13}C, ^{31}P, ^2H lock) cryogenic probe equipped with z-gradients and the SampleJet automatic sample changer. For each sample, the probe was automatically locked, tuned, matched, and shimmed. A standard-shim file optimized for milk was used as a starting point for the automated shimming procedure. All spectra were measured at 298 K (25°C) after equilibration in the magnet for 300 s. One-dimensional (**1D**) ^1H and 2-dimensional (**2D**) ^1H-^{13}C heteronuclear single quantum coherence (**HSQC**) spectra were automatically collected without spinning. One-dimensional ^1H NMR spectra were obtained using a 1D nuclear Overhauser enhancement spectroscopy (NOESY) pulse sequence. The solvent signal was suppressed by presaturation during relaxation and mix-

ing time and additional spoil gradients. A total of 128 scans were collected for each spectrum into 65,536 data points using a relaxation delay of 4 s, an acquisition time of 2.66 s, and a mixing time of 0.01 s. Throughout the paper, ppm values are used to indicate the position (chemical shift) of a signal in a spectrum. In NMR spectroscopy, chemical shifts are measured in parts per million relative to a reference resonance signal from a standard molecule. A ppm value is calculated as the difference in resonance frequency (measured in Hz) between the nucleus of interest and the reference nucleus divided by the operating frequency of the magnet (measured in Hz). Because the resulting value is rather small, it is multiplied by 1 million; therefore, the name parts per million. The spectral width was set to 20 ppm for each 1D spectrum, and 4 dummy scans were performed before each measurement. Spectra were automatically Fourier transformed and phase corrected, applying a line broadening of 0.3 Hz and zero-filling to 131,072 data points. A flat baseline was obtained by using the baseopt option of TopSpin2.1 (Bruker BioSpin GmbH), which performs a correction of the first points of the free-induction decay (FID).

Water suppression was achieved for the 2D ^{1}H-^{13}C HSQC spectra using presaturation during the relaxation delay. A total of 2,048 × 128 data points were collected for each 2D spectrum using 8 scans per increment, a relaxation delay of 3 s, an acquisition time of 0.14 s, and 16 dummy scans. These settings led to a total acquisition time of 56 min per spectrum. The spectral widths were set to 12 ppm and 165 ppm in the proton and carbon dimensions, respectively. One high-resolution 2D ^{1}H-^{13}C HSQC spectrum was acquired for initial assignment of the metabolites. These assignments were further validated using long-range proton–carbon couplings obtained from a 2D ^{1}H-^{13}C heteronuclear multiple bond correlation spectrum. Two-dimensional spectra were semiautomatically processed employing a 90° shifted squared sine-bell window function in both dimensions. In the indirect carbon dimension, increased resolution was obtained by doubling the number of data points before Fourier transform using complex forward linear prediction. All 2D spectra were manually phase corrected, and a polynomial baseline correction was applied excluding the region around the water artifact. All 1D and 2D spectra were chemical shift referenced relative to the TSP signal.

Initial NMR Metabolite Assignment

Initial metabolite assignment was performed manually as described in Gronwald et al. (2008). A summary of the obtained chemical shift data are provided in Table 2. The chemical shift information of each identified metabolite signal was stored in a so-called knowledge base to guide the quantification process of the milk metabolites. To define the regions where the individual metabolite signals in a series of measured spectra are expected, individual chemical shift ranges were specified for each metabolite signal by manually analyzing interspectra chemical shift variations in a subset of the measured spectra. Depending on the analyzed type of spectrum, individually optimized chemical shift ranges were determined for each metabolite. In addition, the knowledge base describes the individual signals of a compound in terms of multiplet patterns, coupling constants, relative intensities, and masses. Coupling values with appropriate error bounds were obtained by analyzing the pure reference compound spectra together with the experimentally measured spectra of our series. Note that multiplet patterns caused by J-couplings were observable only in the 1D spectra. Overlapping signals located in crowded regions of the real milk spectra were excluded from the quantification process; especially in the region close to the intensive lactose signals, considerable overlap was present in both 1D and 2D spectra.

Automated NMR Analysis

The next steps of the analysis were performed with the Analytical Profiler module of AMIX 3.9 (Bruker BioSpin GmbH). The 1D ^{1}H and 2D ^{1}H-^{13}C HSQC spectra corresponding to the series of milk specimens measured were automatically peak-picked. Using the information stored in the knowledge base, metabolite signals were identified and integrated. Multiplet information stored in the knowledge base was used in the integration process of 1D spectra to ensure that only the desired signals within the specified chemical shift ranges were integrated. In addition, a peak-fitting routine newly implemented in AMIX 3.9 was used to obtain accurate integrals even in regions with moderate signal overlap. Multiplet information was not applicable for 2D spectra. Here, information from the reference compound spectra database, such as the expected number of signals in a specific region, was used instead. Next, relative integrals with respect to the reference TSP signal were calculated for the individual metabolite signals. The use of relative integrals automatically corrects for machine-dependent sensitivity variations between experiments and has the additional advantage that calibration curves have to be determined only once. Finally, in-house routines were used for absolute quantification, employing individual calibration curves for each metabolite signal. Standard stock solutions were serially diluted for this purpose to yield final concentrations of 9,000, 4,500, 2,250, 1,125, 562.5, 281.3, 140.6, 70.3, 35.2, 17.6, and 8.8 μmol/L, and the correspond-

Table 2. Chemical shift values for the metabolite signals used for compound identification and quantification by high-resolution 1-dimensional ^{1}H and 2-dimensional ^{1}H-^{13}C heteronuclear single quantum coherence spectra

Compound	Assignment	^{1}H Chemical shift	^{13}C Chemical shift	Use[1]
Acetate	CH_3	1.92	25.8	q
Acetone	CH_3	2.24	32.7	q
Alanine	CH	3.79	53.4	—
Alanine	CH_3	1.48	18.9	q
Betaine	CH_2	3.90	68.7	q
Betaine	CH_3	3.26	56.0	q
Carnitine	3 * CH_3	3.21	56.1	—
Carnitine	CH_2	2.44	44.8	q
Carnitine	CH_2	3.43	72.6	—
Carnitine	CH	4.57	66.3	—
Choline	3 * CH_3	3.18	56.1	—
Choline	CH_2	4.06	58.1	q
Choline	CH_2	3.51	69.9	q
Creatinine	CH_2	4.06	59.0	q
Creatinine	CH_3	3.05	32.8	q
Ethanolamine	0-CH_2	3.83	60.3	q
Ethanolamine	N-CH_2	3.15	44.0	q
D-Galactose α	CH	4.07	72.8	q
D-Galactose α	CH	3.81	70.9	—
D-Galactose β	CH	4.57	98.8	q
D-Galactose β	CH	3.49	74.4	q
Glycerophosphocholine	CH_2	4.32	61.7	q
Glycerophosphocholine	CH_2	3.65	67.8	q
Glycine	CH_2	3.57	44.2	q
BHBA	CH_3	1.20	—	q
Lactate	CH_3	1.32	22.1	q
Lactate	CH	4.11	70.9	q
D-Lactose (total)	CH	4.45	105.0	q
D-Lactose (total)	CH	3.73	77.6	q
D-Lactose (total)	CH	3.94	71.0	q
D-Lactose (total)	CH	3.54	73.4	—
D-Lactose (total)	CH	3.67	74.9	—
D-Lactose (total)	CH_2	3.78	63.4	—
D-Lactose α	CH	5.23	94.2	q
D-Lactose α	CH_2	3.88	62.3	q
D-Lactose α	CH	3.59	73.7	—
D-Lactose α	CH	3.96	72.5	—
D-Lactose α	CH	3.66	76.8	—
D-Lactose α	CH	3.84	73.8	—
D-Lactose β	CH	4.67	98.2	q
D-Lactose β	CH	3.66	80.5	—
D-Lactose β	CH	3.29	76.2	q
D-Lactose β	1/2 CH_2	3.96	62.4	q
D-Lactose β	CH	3.60	77.3	q
D-Lactose β	1/2 CH_2	3.81	62.4	—
D-Lactose β	CH	3.84	73.8	—
3-Methylhistidine	α-CH	3.97	56.0	—
3-Methylhistidine	β-CH_2	3.30/2.25	27.5	q
3-Methylhistidine	CH_3	3.74	34.7	q
3-Methylhistidine	δ-CH	7.14	125.7	q
3-Methylhistidine	ε-CH	8.09	140.6	—
N-Acetyl-carbohydrates	CH_3	2.05	23.8	q
Phosphocholine	CH_2	4.16	60.2	q
Phosphocholine	CH_2	3.58	68.5	q
Phosphocholine	3 * CH_3	3.18	56.1	—
Phosphocreatine	CH_3	3.03	39.1	q
Phosphocreatine	CH_2	3.93	56.1	q
Taurine	S-CH_2	3.43	38.2	q
Taurine	N-CH_2	3.27	50.2	q
Trimethylamine-N-oxide	CH_3	3.27	62.1	q

[1]Groups (e.g., CH, CH_2, CH_3) used for compound identification and quantification are marked with q. Unambiguously identified groups that could not be used for quantification because of signal overlap are marked with a dash.

ing spectra were acquired. The concentration range was extended to 166 mmol/L for lactose. The number of signals that were used for compound identification is presented in Table 3 and equals the number of measured calibration curves. However, because of overlap of signals even in the 2D spectra, only a subset of these signals could be used for quantification. Because of metabolites inconsistently present in milk, a few rare cases of additional overlap were observed, leading to drastically increased integral values for certain metabolite signals. As a consequence, outlier signals that deviated from the median of the signals of a compound by more than 50% were automatically excluded from further analysis. Following absolute quantification of individual signals, the corresponding concentration ranges for each metabolite were calculated.

The limit of detection (**LOD**), which was defined as a signal:noise ratio of 3.5 for the strongest signal of a compound, was obtained from the calibration samples (Table 3). The lowest 3 points of each calibration curve were measured in triplicate, and the corresponding relative standard deviations were determined to calculate the lower limits of quantification (**LLOQ**). Following the recommendation of FDA (2001), the LLOQ was defined as the concentration value that could be determined with a relative standard deviation <20%. The LOD and LLOQ obtained were functions of the NMR acquisition time. Therefore, lower values may be obtained by increasing the acquisition time.

GC-MS of AA and of BHBA, Glucose, and Citrate Cycle Intermediates

Quantitative analysis of AA in 20-μL aliquots of skim milk was completed using GC-MS after derivatization with propyl chloroformate as described previously (Kaspar et al., 2008).

Ten-microliter aliquots of defatted milk were extracted with 50 μL of methanol containing the stable isotope labeled extraction standards [2,2,4,4-$^2H_4$]citrate, [U-^{13}C] fumarate, [U-^{13}C]glucose, [U-^{13}C]BHBA, [U-^{13}C]lactate, [2,3,3-$^2H_3$]malate, [U-^{13}C]pyruvate, [U-^2H]succinate, and [$^2H_7$]*trans*-cinnamate, and unlabeled norvaline, which had been purchased from either Cambridge Isotopes Laboratories Inc. (Andover, MA), CDN Isotopes Inc. (Quebec, Canada), or Sigma-Aldrich. The purity of all extraction standards exceeded 98%. The addition of stable isotope internal standards leads to more precise quantification, because their properties are chemically equivalent to their corresponding metabolites, but they are distinguishable from the naturally occurring metabolites by a separate mass. Samples were centrifuged at 4°C and 3,375 × *g* for 5 min, and the supernatant was transferred into a 2-mL glass vial with a 0.2-mL glass insert. Pellets were reextracted twice with 50 μL of methanol. The extracts were combined and evaporated (CombiDancer, Hettich AG, Bäch, Switzerland) to complete dryness. The vials were then closed with magnetic crimp caps for automated handling by the autosampler and placed in a cooled (5°C) tray. Prior to derivatization, 10 μL of internal standard solution containing odd-numbered, saturated straight-chain fatty acids (C9–C19; Sigma-Aldrich) at a concentration of 1 mmol/L were added to the sample residue. Then, 50 μL of 10 mg/mL of methoxylamine hydrochloride in pyridine (Sigma-Aldrich) was added and incubated at 60°C for 60 min, followed by 50 μL of *N*-methyl-N-trifluoroacetamide (Macherey-Nagel, Dueren, Germany) for 60 min at 60°C. Samples were thoroughly mixed during incubation in the agitator.

A Pegasus GC time-of-flight MS instrument (Leco Corp., St. Joseph, MI) was used for the quantitative analysis of BHBA, glucose, and intermediates of the citrate cycle. The instrument configuration comprised a model 6890 GC (Agilent Technologies, Santa Clara, CA) coupled to a fast-scanning time-of-flight mass spectrometer providing unit mass resolution, a PTV injector (Gerstel, Mülheim an der Ruhr, Germany), and a MPS-2 Prepstation sample robot (Gerstel) for automated sample derivatization and handling. The robot was equipped with 2 agitators for sample incubation and 2 syringes of different volumes. A 10-μL syringe was used for internal standard addition and sample injection, whereas a 250-μL syringe was used for adding reagents. In between the adding steps, the syringes were washed 3 times with isooctane. A RXI-5 MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness; Restek, GmbH, Bad Homburg, Germany) coupled to a RTX-1701 column (2 m × 0.1 mm i.d. × 0.1 μm film thickness; Restek) and equipped with a deactivated precolumn (5 m × 0.25 mm i.d.; Agilent) was used. The oven temperature was initially held at 50°C for 0.2 min, raised at 8°C/min to 265°C, and held for 10 min. The column flow was 1 mL of He/min. A sample volume of 1.5 μL was injected in splitless mode. The temperature of the PTV injector was set at 50°C for 0.5 min and increased at 12°C/s to 250°C. A chemically inert Siltec liner (Gerstel) was used. The mass spectra were acquired over a mass:charge ratio (m/z) of 40 to 600 at a rate of 50 spectra/s, and the solvent delay was set to 8 min.

Calibration was completed using metabolite standard solutions in methanol. In this step, 100 μL of different calibration concentrations were transferred into a glass vial with glass insert, and 10 μL of the surrogate solution containing stable isotope labeled [2,2,4,4-$^2H_4$] citrate, [U-^{13}C]fumarate, [U-^{13}C]glucose, [U-^{13}C]BHBA, [U-^{13}C]lactate, [2,3,3-$^2H_3$]malate, [U-^{13}C]pyruvate,

Table 3. Metabolites quantified by nuclear magnetic resonance (NMR) and gas chromatography-mass spectrometry (GC-MS)[1]

Metabolite	LOD[2] (μmol/L)	LLOQ[2] (μmol/L)	Signals[3] (n)	TE (mmol/L)	RSD (%)	Values above LLOQ[4] (n)	Milk concentration range (mmol/L)
α-Aminobutyric acid	0.30	0.75	—	—	4.2	99	0.001–0.010[5]
Acetate	78.00	78.00	1 (1)	0.090	—	25	0.108–0.701[6]
Acetone	1.80	1.80	1 (1)	0.002	—	106	0.012–0.661[7]
Alanine	0.08	0.75	—	—	1.5	99	0.009–0.091[5]
α-Aminoadipic acid	2.30	7.50	—	—	2.5	7	0.195–0.427[5]
Aspartate	0.75	7.50	—	—	14.1	93	0.010–0.077[5]
Betaine	20.00	45.00	2 (2)	0.564	—	24	0.459–1.410[6]
Carnitine	70.00	281.00	4 (1)	0.084	—	1	0.346[6,8]
Choline	141.00	141.00	3 (2)	0.112	—	56	0.150–0.997[6]
Citrate	0.61	4.88	—	—	7.9	104	3.026–9.854[5]
Creatinine	20.00	78.00	2 (2)	0.034	—	73	0.080–0.167[6]
Ethanolamine	78.00	312.00	2 (2)	0.026	—	1	0.323[6,8]
Fumarate	1.22	2.44	—	—	6.9	102	0.002–0.081[5]
α-D-Galactose	563.00	1,125.00	2 (1)	0.468	—	5	1.200–1.760[6]
β-D-Galactose	563.00	1,125.00	2 (2)	—	—	3	1.153–1.696[6]
D-Glucose	0.61	4.88	—	—	8.3	104	0.076–0.662[5]
Glutamate	2.25	7.50	—	—	3.3	99	0.044–0.693[5]
Glycerophosphocholine	141.00	141.00	7 (2)	0.095	—	106	0.284–1.460[6]
Glycine	0.075	7.50		—	2.2	99	0.033–1.109[5]
β-Hydroxybutyrate	2.44	4.88	—	—	8.0	103	0.010–0.531[5]
Isoleucine	0.36	2.25	—	—	2.7	88	0.002–0.013[5]
Lactate	0.61	2.44	—	—	6.7	100	0.002–3.538[5]
D-Lactose (total)	113.00	453.00	6 (3)	2.131	—	106	118.186–160.121[6]
α-D-Lactose	113.00	1,813.00	6 (2)	1.768	—	106	45.792–61.243[6]
β-D-Lactose	113.00	1,813.00	7 (4)	2.179	—	106	75.067–99.360[6]
Leucine	0.15	0.75		—	2.8	99	0.002–0.022[5]
Lysine	0.75	2.25	—	—	3.9	99	0.007–0.102[5]
Malate	2.44	4.88	—	—	7.0	104	0.008–0.441[5]
Methionine	2.25	7.50	—	—	11.8	1	0.008[5,8,9]
3-Methylhistidine	78.00	78.00	5 (3)	0.168	—	2	0.103–0.151[6]
N-Acetyl-carbohydrates	70.00	141.00	1 (1)	0.086	—	106	1.135–4.240[6]
Ornithine	0.75	2.25	—	—	4.8	96	0.002–0.015[5]
Phenylalanine	0.75	2.25		—	3.4	92	0.002–0.010[5]
Phosphocholine	141.00	141.00	3 (2)	0.082	—	56	0.143–1.355[6]
Phosphocreatinine	70.00	141.00	2 (2)	0.179	—	106	0.585–2.567[6]
Proline	0.68	0.75	—	—	3.0	99	0.011–0.047[5]
Pyruvate	2.44	4.88	—	—	7.5	101	0.002–0.188[5]
Succinate	4.88	9.76	—	—	9.9	104	0.016–0.106[5]
Taurine	20.00	312.00	2 (2)	0.076	—	3	0.327–0.621[6]
Threonine	0.45	0.75	—	—	ND[10]	99	0.001–0.046[5]
Trimethylamine-N-oxide	20.00	39.00	1 (1)	ND	—	2	0.043–0.046[6]
Tryptophan	0.08	0.75	—	—	2.8	99	0.001–0.005[5]
Tyrosine	0.75	2.25	—	—	2.5	19	0.002–0.011[5]
Valine	0.54	0.75	—	—	2.1	99	0.005–0.029[5]

[1]LOD = molar limits of detection; LLOQ = lower limits of quantification; signals (n) = number of signals used for metabolite assignment and quantification; TE = technical error estimated from duplicate measurements (NMR); RSD = relative standard deviations from calibration replicates (GC-MS).

[2]LOD and LLOQ values for the compounds quantified by NMR spectroscopy are dependent on the total acquisition time of an NMR spectrum. The corresponding acquisition times for 1-dimensional ^{1}H and 2-dimensional ^{1}H-^{13}C heteronuclear single quantum coherence NMR spectra were 15 and 56 min, respectively. Note that the different compounds were simultaneously measured in a single spectrum. Therefore, an increase in acquisition time would decrease the LOD and LLOQ values of all metabolites analyzed by NMR.

[3]Number of groups (e.g., CH, CH_2, CH_3) used for NMR-based metabolite identification and quantification (the latter in parentheses).

[4]The number of values above LLOQ varies from metabolite to metabolite because individual LLOQ were determined for each metabolite; also, the abundance of the various metabolites greatly varies. In total, 106 milk samples were analyzed, which results in a maximum number of 106 values that could be obtained for a single metabolite.

[5]Concentrations obtained from GC-MS spectra.

[6]Concentrations obtained from ^{1}H-^{13}C 2-dimensional heteronuclear single quantum coherence NMR spectra.

[7]Concentrations obtained from ^{1}H 1-dimensional NMR spectra.

[8]Only 1 value detected.

[9]Measured in colostrum milk sample obtained from farm 2.

[10]ND = not determined.

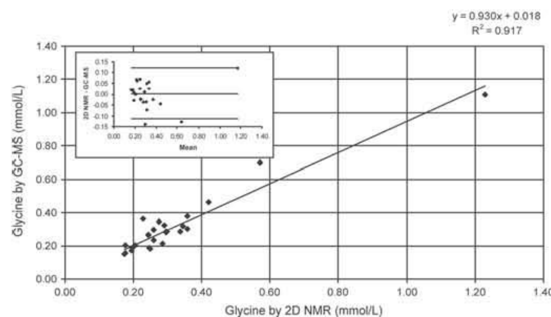

Figure 1. Linear regression and Bland-Altman plot of concentrations (mmol/L) of glycine in milk specimens measured by 2-dimensional nuclear magnetic resonance (2D NMR) and gas chromatography-mass spectrometry (GC-MS).

[U-^{2}H]succinate, [$^{2}H_7$]*trans*-cinnamate, and unlabeled norvaline (1 mmol/L each) were added. The standards were dried and derivatized as described. Over a concentration range of 0.5 to 500 µmol/L, 12 calibration points were generated.

Statistics

For calculation of technical errors, all NMR measurements were completed in duplicate and the technical errors (TE) were calculated according to the following equation:

$$\mathrm{TE} = \sqrt{\frac{\sum_{i-1}^{N}(x_{i,1} - x_{i,2})^2}{2N}},$$

where N is the number of samples that were measured in duplicate and $x_{i,1}$ and $x_{i,2}$ are the first and second measurement of a specific sample, respectively.

Pearson correlation coefficients were calculated using Excel 2007 (Microsoft, Redmond, Washington) and conservative global limits above 0.5 and below −0.5 were used to label observed correlations as significant. At the 5% level, statistical significance is generally reached with these r-values for a sample size less than 20. Although for some metabolites up to 106 measurements (Table 3) above the individual LLOQ were available, for others this number was considerably smaller. Therefore, these global limits for r-value ensured that only significant correlations were marked as such (Supplemental Figure 1; http://www.journalofdairyscience.org/).

A Bland-Altman plot (Bland and Altman, 1986) is inserted in Figure 1. In this graphical model, also known as Tukey mean difference plot, the difference y_n between data obtained by 2 methods ($y_n = x_o - y_o$) is plotted for each sample against the average x_n of the 2 methods [$x_n = (x_o + y_o)/2$]. Bland-Altman plots are well suited to analyze the agreement between 2 experimental methods.

RESULTS AND DISCUSSION

LOD and LLOQ

Molar LOD and LLOQ were determined for both NMR and MS. As described in the experimental section, LOD for the 2D ^{1}H-^{13}C HSQC and 1D ^{1}H spectra were calculated from calibration standards (Table 3). The LOD ranged for the 2D spectra from 20 to 563 µmol/L at a signal:noise level of 3.5. The lowest LOD were obtained for methyl group containing analytes, such as betaine, where the protons of the methyl group(s) give rise to a single signal in the corresponding 2D NMR spectra or for compounds that were identified from 1D spectra. One-dimensional spectra were used for quantification of acetone and BHBA; hence, considerably lower LOD of 1.8 µmol/L were obtained.

The LLOQ values for the individual compounds are given in Table 3. The lowest LLOQ values were obtained for compounds with multiple methyl groups or for compounds that were identified from 1D spectra. The LLOQ values obtained for 2D spectra of pure compounds were generally higher than those obtained for 1D spectra. However, in a previous contribution (Gronwald et al., 2008) it was shown that in real biological samples more accurate results are obtained by the use of 2D spectra. From the calibration curves, it was demonstrated that measurements remained linear up to concentrations as high as 166 mmol/L for lactose without readjusting the receiver gain.

In MS, the quantification range was determined by the LLOQ and upper limits of quantification, which were defined as the lowest and highest points, respectively, of the calibration curve, with an accuracy between 80 and 120%. This is in agreement with FDA (2001). For MS, the LOD were defined as the concentration producing a signal:noise ratio of at least 3:1. The concentrations reported in Table 3 were calculated from the analysis of 20-µL aliquots and the lowest obtained LOD amounted to 0.08 mmol/L, corresponding to an absolute injection amount of 15 fmol.

2D NMR Versus MS

An important prerequisite for the combined analysis of data from both NMR and MS is to demonstrate agreement of the 2 methods. Of the 44 milk metabolites analyzed, 23 were measured by NMR and 25 by GC-

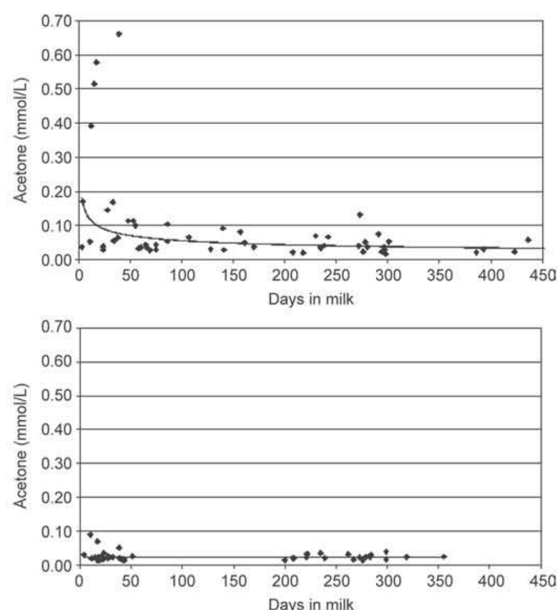

Figure 2. Correlation between lactation day and acetone concentration (mmol/L) for milk specimens from farm 1 (top) and farm 2 (bottom) as measured by 1-dimensional nuclear magnetic resonance. To clarify the general trend of the data, a potential regression line was added.

MS as either their propyl chloroformate (AA) or methoximated and trimethylsilylated derivatives (remaining analytes; Table 3). Alanine, glycine, BHBA, and lactate were analyzed by both NMR and GC-MS to check for data agreement between the methods. A comparison of the corresponding NMR and GC-MS data for Gly is shown in Figure 1. The high R^2-value of 0.92 indicates that both methods allowed the precise determination of Gly over the entire concentration range of 0.033 to 1.109 mmol/L. The slope of the linear regression curve of 0.93 and the positive y-axis intercept of 0.018 mmol/L confirmed good concordance between NMR and GC-MS data. The Bland-Altman plot shown as an insert in Figure 1 reveals an even smaller mean difference of 0.04 mmol/L between the 2D NMR and GC-MS measurements. Similar results were obtained for Ala, BHBA, and lactate (data not shown). In summary, NMR and MS data may be safely combined for statistical data analysis.

Analysis of Milk Metabolites

The observed concentration range for each of the 44 analyzed milk metabolites is given in Table 3. The ranges were calculated over all milk samples from both farms, and, because for some metabolites concentration values above their LLOQ were not available in all samples, no average values and standard deviations are given. As expected from previous literature, concentrations of lactose in milk fell within a relatively narrow concentration range of 118 to 160 mmol/L (40.4–54.8 mg/mL; Roginski et al., 2004). In contrast, metabolites such as phosphocholine showed an almost 10-fold difference between the lowest (0.143 mmol/L) and the highest (1.355 mmol/L) concentration observed. The AA in milk are absorbed from blood, but undergo extensive synthesis and degradation in the mammary gland (Bequette et al., 1998). The pathways of metabolic transformation are the same or similar as found in other tissues. In dairy ruminants producing large quantities of milk and milk protein, Met (Pinotti et al., 2002) or a combination of Met and Lys (Schwab et al., 1992) have been reported to be the first limiting AA for milk protein synthesis. Methionine is required for transmethylation reactions and milk protein synthesis and may stimulate milk and milk fat production by enhancing lipoprotein synthesis, gluconeogenesis, and provision of methyl groups for phospholipid synthesis

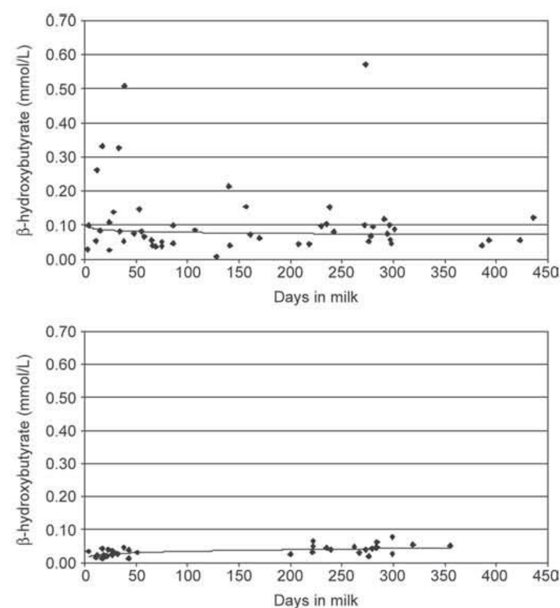

Figure 3. Correlation between lactation day and BHBA concentration (mmol/L) for milk specimens from farm 1 (top) and farm 2 (bottom) as measured by 1-dimensional nuclear magnetic resonance. To clarify the general trend of the data, a potential regression line was added.

in the liver and sulfur groups for the synthesis of Cys (Bequette et al., 1998). Methionine was detected in only a single sample of colostrum that was not included in the study. These data support the hypothesis that the total uptake of Met probably balances with its output in milk protein (Bequette et al., 1998). In regard to AA that limit milk protein synthesis, it has been recognized that they vary depending on the quality and quantity of AA in basal diets. As a result, different amounts and proportions of AA are absorbed from duodenal ingesta (Rulquin et al., 1993). High correlation between Lys and protein content (Supplemental Figure 1) throughout lactation (r = 0.57) also reflects the limiting nature of this AA for protein production.

Investigation of Metabolic Status During Early Lactation

An important objective of this study was the measurement of metabolites that might serve as indicators of a metabolic imbalance during early lactation. Known markers for the status of energy metabolism are acetone and BHBA (Enjalbert et al., 2001; Duffield et al., 2009). The top part of Figure 2 shows the correlation between lactation day and observed acetone concentration for farm 1. Some cows yielded significantly increased acetone values above threshold concentration for detection of subclinical ketosis of 0.16 mmol/L during the first days of lactation (Enjalbert et al., 2001). Drastically lower acetone values of about 0.04 mmol/L were found for other cows at a similar lactation stage. Generally, acetone values decreased during the first 70 d of lactation and remained fairly constant thereafter. The bottom part of Figure 2 shows the correlation between acetone concentration and lactation day for farm 2. Here, only slightly increased acetone concentrations of up to 0.088 mmol/L were observed for a few cows during the first 70 d of lactation. Generally, the observed acetone concentrations were lower for cows from farm 2 than for farm 1. During late lactation, average values of 0.024 mmol/L were observed for cows from farm 2 and the corresponding values for cows from farm 1 were about 0.030 mmol/L.

Similar but less pronounced effects were observed for BHBA (Figure 3). In the early lactation period, increased values above the threshold concentration of 70 μmol/L for the detection of subclinical ketosis (Enjalbert et al., 2001) were found in a subset of cows from farm 1. Note that for 1 cow, increased BHBA values were obtained at a time point in late lactation, indicating a possible health problem. In contrast to farm 1, cows from farm 2 showed constant BHBA concentrations over the entire lactation period. Acetone and BHBA showed a good correlation over all cows, with a Pearson correlation coefficient of 0.69 (Supplemental Figure 1). This is in agreement with a published correlation of milk acetone to BHBA of 0.68 (Enjalbert et al., 2001).

Baticz et al. (2002) suggested measurements of citrate as a marker of energy status in the dairy cow, being correlated with ketones in milk and de novo fatty acid synthesis. This suggestion is supported by our data with an observed Pearson correlation coefficient of 0.45 between citrate and acetone in milk and a corresponding correlation coefficient of 0.39 between citrate and BHBA. Milk citrate concentration has been found to vary widely throughout lactation (Garnsworthy et al., 2006) depending on nutrition and season (Holt and Muir, 1979). Generally, it is higher in early lactation and decreases gradually as lactation progresses (Konar et al, 1971). In the study reported here, citrate remained fairly constant throughout lactation (average ± SD: 6.270 ± 1.311 mmol/L), but concentrations that were determined by GC-MS were higher than those obtained by NMR in commercial milk in a previous study (Hu et al., 2007). In this context, it should be noted that we also determined citrate by 2D NMR. However, so-called spike in experiments where known amounts of citrate were added to milk showed that, for this metabolite, the GC-MS measurements were more accurate. One

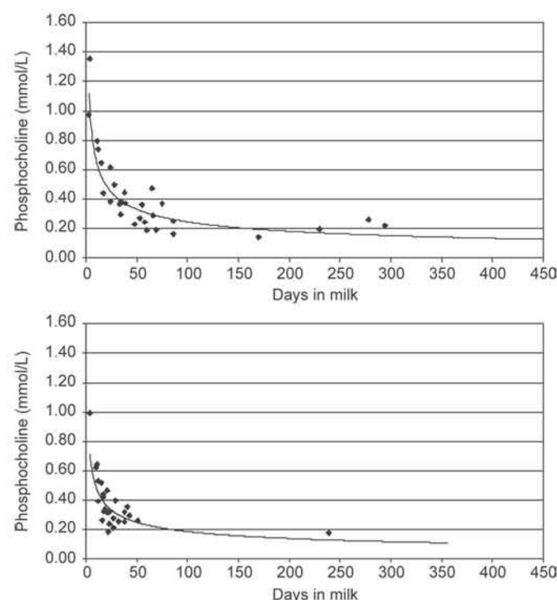

Figure 4. Correlation between lactation day and phosphocholine concentration (mmol/L) for milk specimens from farm 1 (top) and farm 2 (bottom) as measured by 2-dimensional nuclear magnetic resonance. To clarify the general trend of the data, a potential regression line was added.

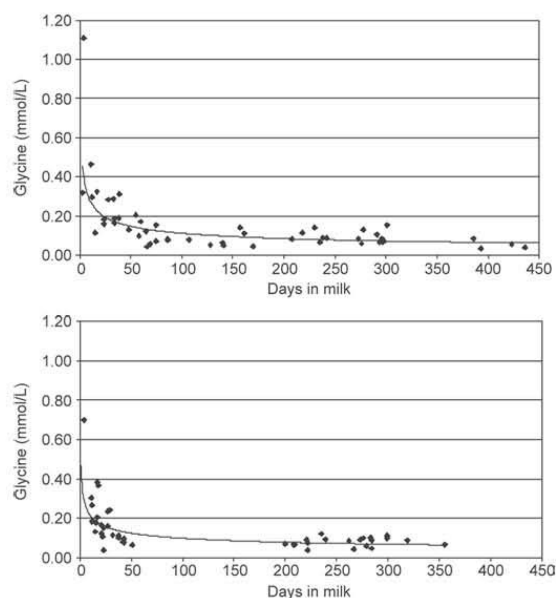

Figure 5. Correlation between lactation day and glycine concentration (mmol/L) for milk specimens from farm 1 (top) and farm 2 (bottom) as measured by gas chromatography-mass spectrometry. To clarify the general trend of the data, a potential regression line was added.

likely explanation for the observed discrepancies to the NMR measurements is that milk citrate is located in part in the colloidal phase of milk and, therefore, could not be determined by NMR.

An analyte that has been linked to udder health problems is lactate. However, the data presented here suggest no correlation between SCC and lactate. In addition, changes in pyruvate concentrations and, particularly, lactate dehydrogenase activity have been previously associated with mammary infections (Chagunda et al., 2006). However, as for lactate, no correlation can be observed between pyruvate and SCC (Supplemental Figure 1). In contrast, a high correlation (r = 0.87) between lactate and pyruvate that is its precursor in the glycolytic pathway was observed.

Significant correlation coefficients were observed between the branched-chain AA (**BCAA**) Ile, Leu, and Val. They are all catabolized by mammary cells to yield organic acids (oxo and iso acids, propionate, acetate, and citrate), carbon skeletons for synthesis of nonessential AA (Glu and Asp), and CO_2. The branched-chain keto acid dehydrogenase that is shared by all the BCAA and Met catalyzes the rate-limiting step of the BCAA catabolic pathway (Harper et al., 1984).

The analysis of specific metabolites throughout lactation showed increased phosphocholine concentrations for all cows in early lactation (Figure 4), with a maximum of 1.355 and 0.991 mmol/L for cows from farm 1 and farm 2, respectively. Around d 70 of lactation, phosphocholine concentrations had decreased to approximately 0.25 mmol/L at both farms and remained relatively constant thereafter. Note that in the last lactation third, phosphocholine concentrations had decreased below the LLOQ in some cows. Phosphocholine is a precursor of choline and serves as its storage form within the cytosol. It showed a slightly negative correlation with DIM (r = −0.42). For choline, somewhat lower values were obtained in early lactation than in the last lactation third (data not shown). These findings agree only partly with those in human milk, where a distinct decrease during lactation in the concentrations of phosphocholine and free choline was found, but only for the first 90 d and 3 wk, respectively, after birth (Holmes et al., 2000). Choline in milk can be derived by active uptake from maternal circulation (Chao et al., 1988) and from de novo synthesis within the mammary gland (Zeisel et al., 1988).

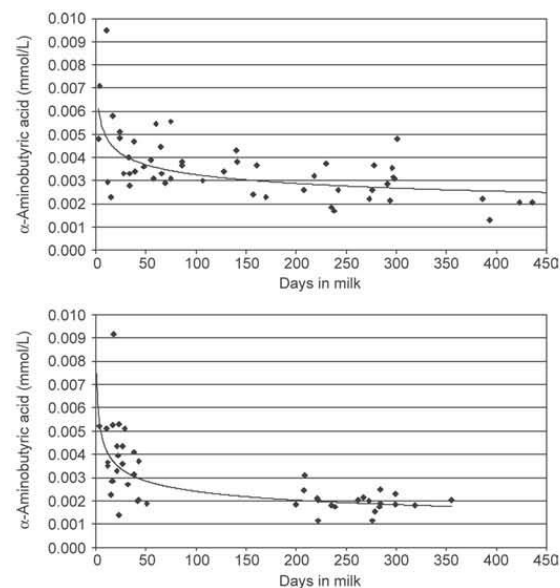

Figure 6. Correlation between lactation day and α-aminobutyric acid concentration (mmol/L) for milk specimens from farm 1 (top) and farm 2 (bottom) as measured by gas chromatography-mass spectrometry. To clarify the general trend of the data, a potential regression line was added.

Higher needs of dairy cows for the limiting AA Met for transmethylation reactions and milk protein synthesis lead to altered methyl group metabolism (Pinotti et al., 2002). Choline plays an important role in the metabolic pathways of methyl groups via the tetrahydrofolate system, which might explain the correlation between milk protein content and free choline in milk (r = 0.50). Furthermore, it has been demonstrated previously that abomasal infusion of choline and administration or feeding of rumen-protected choline increases milk protein content (Sharma and Erdman, 1988; Erdman and Sharma, 1991).

Similar to phosphocholine, a decrease in concentration over the first 70 d of lactation was observed for Gly (Figure 5). Starting at maximum concentrations of 1.109 and 0.700 mmol/L for farm 1 and farm 2, respectively, Gly concentrations dropped to approximately 0.070 mmol/L for both farms. In addition, a high Pearson correlation coefficient of 0.81 was observed between Gly and phosphocholine (Supplemental Figure 1), with the latter serving as a precursor of Gly. The increased concentrations of phosphocholine (Figure 4) and Gly (Figure 5) found in early lactation imply an increased Gly metabolism during that period. This effect is more pronounced for the higher producing animals on farm 1 (Figure 4 and 5). It has been shown that infusion of glucagon increases free Gly concentration in milk (Bobe et al., 2009). Concomitantly, above-average milk production was associated with above-average glucagon concentrations (Herbein et al., 1985).

Quite interesting was the positive Pearson correlation coefficient of 0.66 between α-aminobutyric acid and Gly. The former is a member of the α-ketobutyrate metabolism; in humans, increased plasma concentrations of α-aminobutyric acid have been observed in the early phase of prolonged starvation. Starvation of humans is somewhat similar to the negative energy balance present in the early phase of bovine lactation. Therefore, it is not unexpected that increased concentrations of α-aminobutyric acid have been observed in early lactation. In addition, α-aminobutyric acid is quite sensitive to altered systemic insulin concentrations (Felig et al., 1969). As can be seen in Figure 6, increased concentrations of up to 0.01 mmol/L were observed for α-aminobutyric acid during the first third of lactation, whereas in the second and last lactation third, average values of approx. 0.003 mmol/L were seen. This effect was evident only in animals from farm 1. No clear trends between lactation day and metabolite concentrations were observed for the remaining metabolites.

The quantification of *N*-acetyl-carbohydrates (*N*-acetyl-lactosamine and *N*-acetyl-glucosamine) by NMR was described previously and values were in a similar range as those presented here (Hu et al., 2007). *N*-Acetyllactosamine is found in many glycoproteins and glycolipids. That might explain the correlation between *N*-acetylcarbohydrates and protein content in milk (r = 0.57) and between other membrane-associated analytes such as phosphocholine (r = 0.57) and betaine (r = 0.74).

Interherd Comparison

Systematic differences between the 2 investigated herds independent of metabolic stress in early lactation were analyzed. To that end, 30 selected metabolites were selected, for which a sufficient number of measurements were available for the last lactation third. The median concentrations of most metabolites were higher in milk samples from Brown Swiss cows than from Simmental cows. A typical example was the higher concentrations of α-aminobutyric acid observed for Brown Swiss in comparison with Simmental cows (average ± SD: 2.92 ± 0.82 μmol/L vs. 1.97 ± 0.46 μmol/L). The corresponding *P*-value of $3.8E^{-4}$ shows that for α-aminobutyric acid, the 2 groups were significantly different. Because of the limited literature on α-aminobutyric acid in cows, this finding needs further elucidation in future studies. A metabolite that should remain almost constant is lactose and, as expected, almost no difference was observed between the 2 breeds. Another interesting metabolite was ornithine; in contrast with most other metabolites, significantly ($P = 4.1E^{-12}$) higher average values were obtained for Simmental cows (0.006 ± 0.002 mmol/L) than for Brown Swiss cows (0.004 ± 0.001 mmol/L). Ornithine acts as a carbon precursor of proline, a nitrogen precursor of several nonessential AA, and a precursor of polyamines (Mepham, 1982). The metabolic differences observed between the herds are most likely associated with differences in breed and feed management.

CONCLUSIONS

The analysis of numerous milk metabolites by GC-MS and NMR revealed large differences in concentrations of known biomarkers of energy metabolism such as acetone and BHBA during early lactation, indicating that individual animals cope very differently with the metabolic stress in this period. This was particularly true for high-producing animals that yielded highly discrepant values for these markers. Further metabolomic analyses might help to select cows that cope well with the metabolic stress of early lactation without a priori knowledge. Novel correlations were observed between metabolites such as α-aminobutyric acid and Gly. One avenue for gaining additional knowledge in future inves-

tigations is the combination of metabolomic analyses as performed here and genetic studies.

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