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Modification of immune defense parameters in the mammary gland and of the estrous cycle by induced energy deficiency in dairy cows

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für Mama und Papa

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Abbreviations

ACP = available crude protein

Actinγ-1 = *Actin gamma 1*

ADF = acid detergent fibre

ANOVA = analysis of variance

AUC = area under the curve

BCA = bicinchoninic acid assay

Bcl-2 = *B-cell lymphoma 2*

BCS = body condition score

BHB = β-hydroxybutyrate

Biotin-X-NHS = biotinamidohexanoic acid N-hydroxysuccinimide ester

Bp = base paires

BSA = bovines serum albumin

C = control feeding group

Casp6 = *Caspase 6*

Casp7 = *Caspase 7*

CC24 = control feeding group and untreated control cells after 24h

CC72 = control feeding group and untreated control cells after 72h

CCL26 = *Chemokine (C-C motif) ligand 26/ Eotaxin-3*

cDNA = complementary deoxyribonucleic acid

CE24 = control feeding group and *Escherichia coli* infected cells after 24h

CE72 = control feeding group and *Escherichia coli* infected cells after 72h

CMO = carboxy-methyl- oxyme

Cq = quantification cycle

CS24 = control feeding group and *Staphylococcus aureus* infected cells after 24h

CS72 = control feeding group and *Staphylococcus aureus* infected cells after 72h

CXCL5 = *Chemokine (C-X-C motif) ligand 5*

DAB = 3,3'-Diaminobenzidin

DEFβ1 = *Defensin beta 1*

DMEM/F12 Ham = Dulbeco's modified eagle medium/F12 Ham

DIM = days in milk

DM = dry matter

DMSO = dimethyl sulfoxid

DOV = delayed first ovulation

E24 = *Escherichia coli* infection for 24h

E72 = Escherichia coli infection for 72h

ECM = energy corrected milk .coli = *Escherichia coli*

e.g. = for example

EIA = enzyme immuno assay

ELISA = enzyme-linked immunosorbent assay

FAS = *FAS receptor*

FCS = fetal calf serum

GAPDH = glyceraldehyde 3-phosphate dehydrogenase

Groα = *Growth-Related Oncogene alpha*

H_2O_2 = hydrogen peroxide

HBSS = Hank's balanced salt solution

HISTON = *Histone H3*

HRP = horseradish peroxidase

Ig = immunoglobulin

IL1β = *Interleukine 1 beta*

IL6 = *Interleukine 6*

IL8 = *Interleukine 8*

ITS = insulin, transferrin, sodium selenite

LF = *Lactoferrin*

LH = luteinizing hormone

LPO = *Lactoperoxidase*

MEC = mammary epithelial cells

mRNA = messenger ribonucleic acid

MOI = multiplicity of infection

n = number of samples

NCBI = National Center for Biotechnology Information

NDF = neutral detergent fibre

NEB = negative energy balance

NFC = nonfibre carbohydrate

NEFA = nonesterified fatty acids

NEL = net energy for lactation

NOC = normal ovarian cycle

OD = optical density

P4 = progesterone

pbMEC = primary bovine mammary epithelial cells

PBS = phosphate buffered saline

PBST = phosphate buffered saline Tween

PC1 = principal component one

PC2 = principal component two

PCA = principal component analysis

POC = prolonged ovarian cycle

pp = post partum

PMR = partial mixed ration

R = restriction feeding group

RAMP = receptors and antimicrobial peptides

RC24 = restriction feeding group and untreated control cells after 24h

RC72 = restriction feeding group and untreated control cells after 72h

RE24 = restriction feeding group and *Escherichia coli* infected cells after 24h

RE72 = restriction feeding group and *Escherichia coli* infected cells after 72h

RNA = ribonucleic acid

RNB = ruminant nitrogen balance

RS72 = restriction feeding group and *Staphylococcus aureus* infected cells after 72h

RT = room temperature

RT-qPCR = reverse transcription quantitative polymerase chain reaction

RS24 = restriction feeding group and *Staphylococcus aureus* infected cells after 24h

S.aureus = *Staphylococcus aureus*

S24h = *Staphylococcus aureus* infection for 24h

S72h = *Staphylococcus aureus* infection for 72h

SCC = somatic cell count

SEM = standard error of means

SOC = shortened ovarian cycle

TLR2 = *Toll-Like-Receptor 2*

TLR4 = *Toll-Like-Receptor 4*

T_M = melt temperature

TNFα = *Tumor-Necrosis-Factor alpha*

UBQ3 = *Ubiquitin*

vs. = versus

Units and terms

celsius (with number) = C

day = d

gram = g

hour = h

joule = J

kilo = k (prefix)

kDa = kilo Dalton

liter = L

logarithm (base 10) = log10

micro = µ (prefix)

microliter = µL

milli = m (prefix)

milliliter = mL

units = U

week = wk

Abstract

In early lactation the energy demand for the establishment of milk yield cannot be compensated by energy intake and needs to be covered by mobilization of body reserves. Decreasing blood glucose concentrations are followed by extensive lipolysis and the synthesis of alternative energy forms. Blood nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) concentrations display the degree of the metabolic imbalance and predispose cows to the incidence of metabolic and reproductive diseases. The inflammation of the mammary gland is a prevailing production disease that not only affects the animal's health and productivity, but is also an economic relevant aspect for the milk producers. Mastitis and fertility problems displayed by anestrous and anovulation are closely related to the occurrence of metabolic imbalance post partum (pp). However, many but not all high yielding dairy cows are affected by the prevailing disorders in consequence of the metabolic imbalance in early lactation. To investigate the impact of energy undersupply on the innate host defense ability of the mammary gland and the establishment of the estrous cycle, an energy deficiency was provoked in mid-lactation by a dietary energy reduction of 49.2% of total energy requirements.

For this purpose 40 multiparous Red Holstein cows were assigned and kept under equal conditions during first 85 days in milk (DIM, period 1). The extent of the individual metabolic imbalance was calculated according to the individual milk yield, feed intake and body weight in period 1. The cows were grouped evenly into a control and experimental group. Exactly on day 12 of the present ovarian cycle after day 86pp, representing the peak secretory phase of the bovine estrous cycle, an energy restriction was induced for three weeks (period 2). A diet pruned in energy by straw addition, reduced in concentrate content and general feed amount was provided to the restriction group. Control cows further received the standard diet ad libitum (ad lib). Following the metabolic challenge, restricted cows were realimented to the control diet (period 3) and all cows were inseminated until day 150pp, accordingly. Throughout the experimental period estrous cycle activity was determined by rectal palpation and ultrasound scanning three times weekly supported by progesterone profiles of twice per week taken milk samples. The cows were classified anew according to estrous cycle activity in all three experimental periods, respectively: cows displaying a failed re-establishment of the ovarian cycle in period 1 by delayed first ovulation > day 45pp (DOV) were grouped into DOV whereas cycling cows were classified according to the length of the detected ovarian cycle. Cows displaying normal estrous cycle lengths (18 ≤ 24 days, NOC) were grouped compared to cows with prolonged estrous cycle (> 24 days, POC) and shortened estrous cycle (< 18 days, SOC). Intensive metabolic screening including analysis of twice weekly blood samples and weekly conduction of body condition score (BCS) was kindly provided by

the animal nutrition unit, Technische Universität München. The metabolic results were related to the estrous cycle activity.

On the last day of the energy restriction period, one liter sterile milk was taken and mammary epithelial cells (MEC) were extracted and cultured. In their third passage, primary bovine MEC (pbMEC) cultures were challenged by the most prevalent heat-inactivated mastitis pathogens *Escherichia coli* (*E.coli)* and *Staphylococcus aureus* (*S.aureus)*. Based on this simulated mastitis experiment, the host defense response of the pbMEC cultures was investigated and the suitability of the milk deprived MEC used as *in vitro* model was evaluated. On gene expression level, genes of functional classes representing receptors and antimicrobial peptides (RAMP), cytokines, chemokines and apoptosis related genes were analyzed. The final objective was the determination of one potent member of the innate host defense system: Lactoferrin (LF). The obtained LF results on gene expression and protein level were aimed to be combined with the course of milk LF throughout the experiment to point out the modulatory impact of energetic undersupply on LF.

The provoked metabolic imbalance induced a more pronounced energy deficiency of -65MJ NEL/day in the experimental cows compared to the deficit the cows experienced in early lactation (-47MJ NEL/day) (Gross et al. 2011). Nevertheless the dietary energy restriction did not affect the length of the ovarian cycle during or after the metabolic challenge and no metabolic disorders were detected. No differences were found between restriction and control group according to ovarian cycle activity and metabolic parameters. The innate defense capability of the stimulated pbMEC displayed pathogen specific regulation patterns as well as general effects in respect of the metabolic challenge *in vivo*. *E.coli* exposure evoked fast and pronounced up-regulations of innate defense genes compared to *S.aureus* stimulation. The *S.aureus* treated cells responded at a later time point and on lower expression magnitudes. The effect of the conducted energy restriction was displayed by further significant up-regulations of *E.coli* stimulated genes, whereas the combined effects of *S.aureus* stimulation and energy restriction *in vivo* led to down-regulations of innate defense genes. The amplified immune effect was further demonstrated on gene expression and on protein level of LF in cell culture supernatant, total pbMEC protein lysate and was affiliated with LF analysis in weekly milk samples throughout the experiment. Finally, treatment effects on LF gene expression were confirmed and significant higher protein concentrations were found in the energy restriction compared to the control samples.

In the conducted experiment, the reproductive performance of the experimental cows was not affected by the induced energy deficiency in mid-lactation. Under the present conditions, energy supply alone seems not the only reason for the incidence of estrous cycle dysfunctions. However, the established pbMEC culture model reflected the *in vivo* treatment

of the experimental cows. The induced energy deficit *in vivo* evoked a general innate immune stimulus on gene expression and protein level and amplifies pathogen specific immune reactions. Results indicate a memory ability of the milk deprived pbMEC even though cultivation during three passages was conducted. The present study confirms pbMEC cultures as suitable model for *in vitro* studies displaying *in vivo* stimuli and representing a non-invasive tool for mastitis research.

Zusammenfassung

In der Frühlaktation kann der energetische Aufwand für die Milchleistung nicht über die Energieaufnahme kompensiert werden, sondern wird durch Mobilisierung von Körperreserven gedeckt. Dem sinkenden Blutglukosespiegel wird durch massive Lipolyse und der daraus resultierenden Bildung von alternativen Energieformen im Organismus entgegen gewirkt. Über die Blutbahn freigesetzte nichtveresterte Fettsäuren (NEFA) und die daraus gebildeten Ketonkörper, vornehmlich β-Hydroxybutyrat (BHB), spiegeln das Ausmaß der Stoffwechselbelastung wider und werden als Indikatoren für das mögliche Auftreten von stoffwechselbedingten Erkrankungen und Fortpflanzungsproblemen herangezogen. Die Entzündung der Milchdrüse ist die häufigste Produktionserkrankung im Milchviehbereich, die nicht nur Gesundheit und Leistungsfähigkeit der Kuh betrifft, sondern auch erhebliche wirtschaftliche Folgen für den Milchproduzenten darstellt. Mastitis und Fruchtbarkeitsprobleme wie Azyklie und Ovulationsstörungen, sind eng mit dem Auftreten von Stoffwechselerkrankungen postpartum (pp) korreliert. Interessanterweise sind viele, aber nicht alle, hochleistende Milchkühe in der Hochlaktation von Stoffwechselstörungen und den daraus resultierenden Erkrankungen betroffen. Um die Auswirkung einer energetischen Unterversorgung auf die Abwehrfähigkeit des angeborenen Immunsystems der Milchdrüse sowie auf die Zyklusaktivität zu untersuchen, wurde ein Energiedefizit in der Mitte der Laktation um 49,2% des täglichen Energiebedarfs ausgelöst.

40 mehrkalbige Rotbunte Holstein Kühe wurden ausgewählt und während der ersten 85 Tage der Laktation unter gleichen Bedingungen gehalten (Periode 1). Aufgrund der individuellen Milchleistung, Futteraufnahme und des Körpergewichts während der ersten 85 Tage wurden die Energiebilanzen der Tiere berechnet und die Tiere anhand dessen gleichmäßig in eine Kontroll- und eine Restriktionsgruppe aufgeteilt. Am Tag 12 des ersten Brunstzyklus nach Gruppeneinteilung (Tag 86pp), zum Zeitpunkt der maximalen Sekretionsphase des bovinen Sexualzyklus, wurde das geplante Energiedefizit über einen Zeitraum von drei Wochen ausgelöst (Periode 2). Die Ration der restriktiv gefütterten Kühe wurde energetisch durch Heuzusatz ausgedünnt, enthielt weniger Kraftfutter und wurde in der täglichen Futtermenge reduziert. Die Kontrollgruppe erhielt weiterhin die eingesetzte Standard-Diät ad libitum (ad lib) aus Periode 1. Im Anschluss an die Phase der Energierestriktion wurden die Restriktionstiere an die Kontrollration realimentiert und bis zum Tag 150pp besamt (Periode 3). Über den gesamten Versuchszeitraum wurden drei Mal wöchentlich Zykluskontrollen durch rektale Palpation und Ultraschalldarstellung durchgeführt und die Befunde durch Progesteronbestimmungen in zwei Mal wöchentlich genommenen Milchproben bestätigt. In jeder der drei Versuchsperioden wurden die Tiere anhand ihrer Zykluslänge neu eingeteilt. In Periode 1 wurden azyklische Tiere bis > 45 Tage postpartum in

die Gruppe DOV (delayed first ovulation) eingeteilt. Zyklische Tiere wurden aufgrund der Länge des Brunstzyklus in die Gruppe mit normaler Zykluslänge (18 ≤ 24 Tage, NOC), mit verlängertem Zyklus (> 24 Tage, POC) oder verkürztem Zyklus (< 18 Tage, SOC) sortiert. Die Einteilung der Tiere anhand der Zykluslänge wurde für Periode 2 und 3 jeweils neu durchgeführt. Intensive Untersuchungen zur Stoffwechsellage wurden durch zwei Mal wöchentliche Blutanalysen und wöchentliche Body Condition Score (BCS) Bestimmungen vom Lehrstuhl für Tierernährung der Technischen Universität München durchgeführt und die Ergebnisse freundlicherweise zur Verfügung gestellt. Die Ergebnisse der Stoffwechselparameter wurden auf die Zyklusgruppen bezogen.

Am letzten Tag der Energierestriktion wurde ein Liter Milch steril gemolken und daraus die enthaltenen Euterepithelzellen (MEC) extrahiert und in Kultur genommen. In der dritten Passage wurden die primären bovinen Euterepithelzell (pbMEC) Kulturen mit den am häufigsten auftretenden Mastitiserregern *Escherichia coli* (*E.coli)* and *Staphylococcus aureus* (*S.aureus)* stimuliert. Anhand dieses *in vitro* Mastitis-Models wurde die Abwehr des angeborenen Immunsystems der pbMEC Kulturen untersucht. Auf Genexpressionsebene wurden funktionale Gengruppen wie Rezeptoren und antimikrobielle Peptide (RAMP), Zytokine, Chemokine und Apoptosegene gemessen. Schließlich wurde die Auswirkung auf ein potentes Mitglied der angeborenen Immunantwort, dem Lactoferrin (LF), untersucht. Die Ergebnisse auf Genregulations- und Proteinebene sowie LF-Messungen aus Milchproben über die gesamte Versuchsdauer sollten die Wirkung einer energetischen Unterversorgung am Verlauf von LF darstellen.

Die provozierte Stoffwechselimbalance resultierte in einem stärkeren Energiedefizit von -65MJ NEL/Tag als dem natürlichen auftretenden Energiedefizit in der Frühlaktation (-47MJ NEL/Tag) (Gross et al. 2011). Trotzdem wurde die Zykluslänge in Periode 2 und 3 nicht vonm induzierten Energiedefizit beeinträchtigt und versuchsbedingte Stoffwechselstörungen traten nicht auf. Kontroll- und Restriktionstiere unterschieden sich weder bezüglich der Zykluslänge noch konnten Unterschiede in den gemessen Stoffwechselparametern festgestellt werden. Die angeborene Abwehrfähigkeit der stimulierten pbMEC zeigte jedoch pathogenspezifisch differenziell exprimierte Gene, sowie grundsätzliche Effekte des induzierten Energiedefizits. Die Stimulierung mit *E.coli* führte zu einer schnellen und deutlichen Aufregulierung der untersuchten Gene, während die Immunantwort durch *S.aureus* später einsetzte und weniger ausgeprägt verlief. Der Einfluss der provozierten Energierestriktion zeigte sich jedoch in einer weiteren signifikanten Aufregulierung nach *E.coli* Infektion, während die Kombination aus *S.aureus* Stimulus und Energierestriktion zu einer tendenziellen Herabregulierung der untersuchten Genen führte. Der verstärkende Immuneffekt durch die Energierestriktion konnte weiterhin auf Genexpressionsebene von LF

also auch auf Proteineben des antimikrobiellen Proteins im Zellkulturüberstand und im Gesamtproteinlysat der pbMEC nachgewiesen und mit den LF-Bestimmungen aus der Milch über die gesamte Versuchsdauer hinweg dargestellt werden. Schließlich bestätigten sich die Effekte der Energieversorgung auf die Genexpression von LF durch signifikant höhere Proteinkonzentrationen in der Restriktionsgruppe gegenüber der Kontrolle.

In der vorliegenden Studie zeigte die durchgeführte Energierestriktion in der Mitte der Laktation keinen Einfluss auf die Zyklusaktivität der Versuchstiere. Unter den vorherrschenden Bedingungen scheint die Energieversorgung alleine nicht der Grund für Zyklusstörungen zu sein. Nichtsdestotrotz, spiegelten sich die Versuchsbedingungen *in vivo* in dem etablierten pbMEC Zellkulturmodell wieder. Das induzierte Energiedefizit *in vivo* ruft eine generelle Antwort des angeborenen Immunsystems auf Genexpressionsebene, aber auch auf Proteinlevel von LF hervor und verstärkt pathogenspezifische Immuneffekte. Die Ergebnisse deuten ein Erinnerungsvermögen der pbMEC an physiologische Stimuli *in vivo* an, obwohl die aus Milch extrahierten Zellen über drei Passagen kultiviert wurden. Daher ist es möglich pbMEC Kulturen als Model mit *in vivo* und *in vitro* Stimuli einzusetzen und diese nicht-invasive Methode für Untersuchungen im Bereich der Euterabwehr zu nutzen.

1 Introduction

1.1 Regulation pattern of the estrous cycle due to energetic undersupply

Parturition induces fundamental metabolic adaptations in dairy cows, which disables the provision of nutrients and energy for the enormous synthesis of milk. Especially in early lactation, energy demand cannot be covered by dietary energy intake, which in turn leads to an energy deficiency. This energetic imbalance seems to be responsible for the susceptibility of metabolic and reproductive dysfunctions and the related diseases postpartum in many but not all dairy cows.

The evoked energy deficit is compensated by the mobilization of body fat reserves. Described endocrine drivers of lipolysis are decreased insulin and increased glucagon. Further important mediators that are directed by and within control of herd management are the glucocorticoids especially hydrocortisone and the so called “fight or flight” hormones catecholamines [1]. The induced excessive lipolysis leads to an enhanced release of non-esterified fatty acids (NEFA) into the blood. Transported into the liver, NEFA are oxidized to β-hydroxybutyrate (BHB) or alternatively re-esterified to triacylglycerols [2]. Hence, the plasma increase of both NEFA and BHB are closely related to the incidence of common metabolic diseases like ketosis, fatty liver and displaced abomasum. Furthermore they are directly or indirectly, associated with an energy deficiency, responsible for the occurrence of fertility problems and even infertility. Irregular estrous cycles were implicated in states of energy deficiency and may evoke both delayed resumption of cyclicity and prolonged calving intervals due to reduced conception rate. This in turn is caused first of all by long term carryover effects of NEB through accumulation of cytotoxic NEFA and BHB in follicular fluids and thus damages oocytes [3,4]. Secondly, by a deficient development of follicles to a preovulatory size [5] and thirdly, by a decreased likelihood of ovulation [6]. Further on, conception rates are affected by energetic undersupply due to up-regulation of immune relevant gene clusters indicating a greater degree of uterine inflammation postpartum (pp) and a retarded pp repair process [7]. Nevertheless, although all dairy cows undergo the phase of early pp deficient energy accommodation by the onset of lactation, many but not all dairy cows experience obvious cycle dysregulations and fertility dysfunctions.

1.2 The innate immune capacity of the mammary gland during a nutrient energy deficiency

The peripartal time is prevailed by pregnancy and lactation stress and highest incidence for diseases. Evidence indicates that both innate and acquired immune responses are weakened and affect the systemic immunity and the immune responsiveness of the mammary gland [8]. Enhanced plasma NEFA and BHB concentrations by the means of excessive lipolysis are related to a diminished immune competence. Especially BHB is reported to have immune modulatory effects by cytotoxic properties against immune cells [9,10]. The onset of lactation and the ensuing metabolic imbalance outcome are of critical importance to the mammary gland. The consequences of an impaired immunity of the udder concern primarily its economical worth, the milk production and lead to enormous monetary losses for the dairy milk producers every year [11,12,13]. The character of this mammary inflammation is however pathogen depended and remains often undetected. A chronic-subclinical mastitis is mainly induced by gram-positive cow associated *Staphylococcus aureus (S.aureus)* [14]. Whereas acute and severe forms of this disease are developed by gram-negative environment associated *Escherichia coli (E.coli) [15]*. The severity of mastitis range from permanently increased somatic cell counts and decreased milk yield until final destruction of mammary tissue or the animal's death [16]. Therefore the mastitis directed defense mechanisms need to include factors that exhibit protective effects against a large panel of microbes as well as elements that play pivotal roles in terminating inflammatory responses and thus preventing excessive inflammation that otherwise lead to autoimmune tissue damage [17].

The mammary epithelium is protected by the acquired and the innate immune system that interact with each other [18]. First line of defense against invading pathogens that overcome the anatomical teat barriers and enter the mammary gland are cell surface associated recognition receptors and a range of antimicrobial peptides (Fig.1A) that are secreted directly by the mammary epithelial cells (MEC)[19]. These cationic peptides bind to the anionic surface of bacteria [20] and kill their target cells by membrane disruption or perturbation [21]. Simultaneously, cytokines and chemoattractants (Fig.1B) are released by activated immune cells and MEC [22].

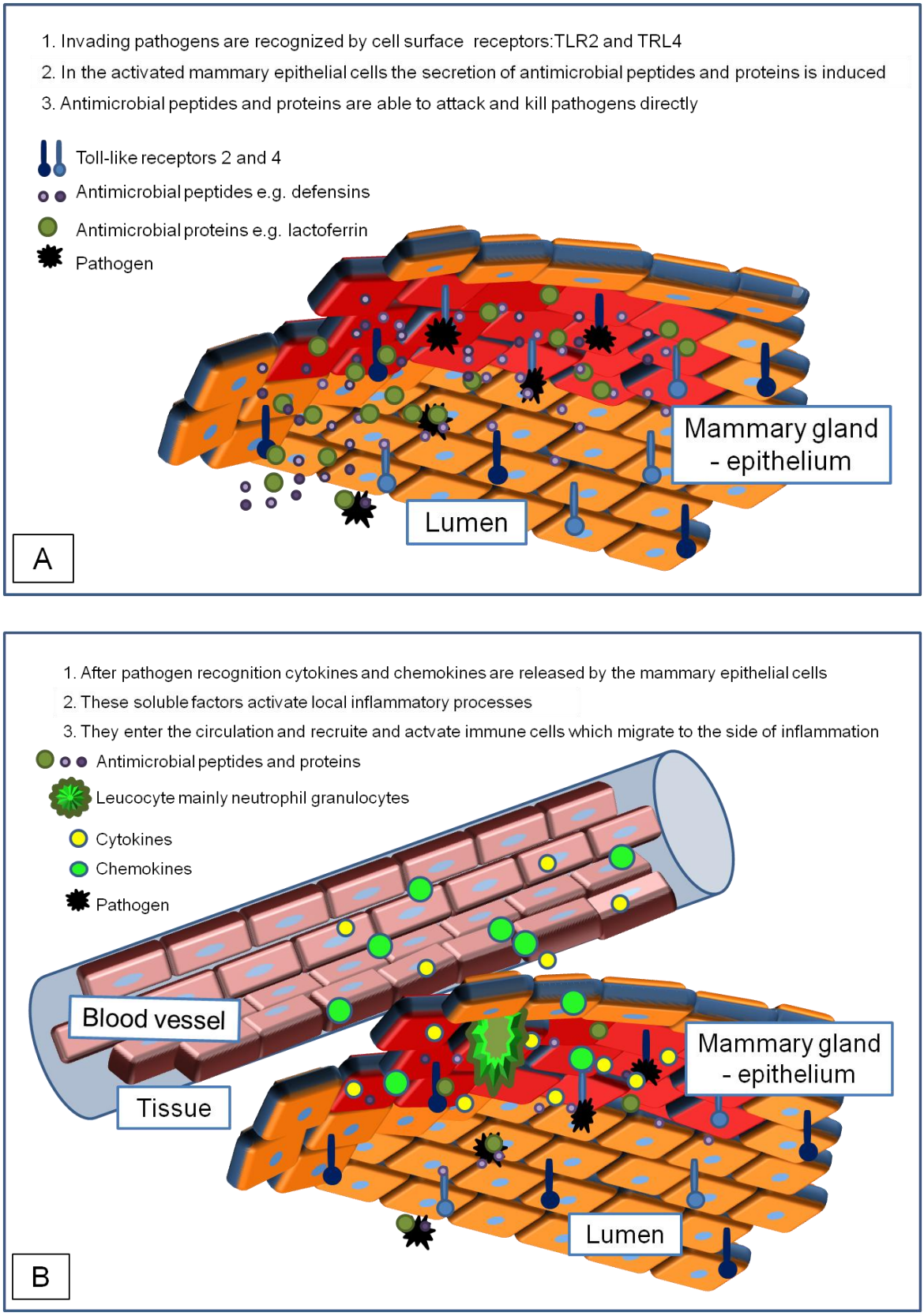

Figure 1: Pathogen recognition and the induced immune response [19, 22], own created figure.

These soluble factors induce the recruitment of resident and circulating leukocytes [12] by diapedesis illustrated in figure 2. The activation of MEC and immune cells by invading

pathogens leads to the secretion of cytokines (TNFα, IL1β and IL6), chemokines and further immune modulatory factors. Cytokines induce the expression of cellular adhesion molecules, including selectins, on the blood vessel endothelium and slow down circulating leukocytes. The immune cells are bound by the expressed selectins in marginal affinity and begin rolling over the endothel surface. Simultaneously, secreted chemokines (IL8, CCL5, CXCL5) activate the rolling leukocytes and induce the expression of integrins on the immune cell surface. Now, tight bonds are formed and cause the immobilization of the leukocytes. Vasodilatory factors like histamine and cytokines increase the vascular permeability and enable paracellular and transcellular migration of immune cells into the tissue. Chemokines, mainly IL8, form a chemotactic gradient that directs leukocytes towards the site of infection [23, 24].

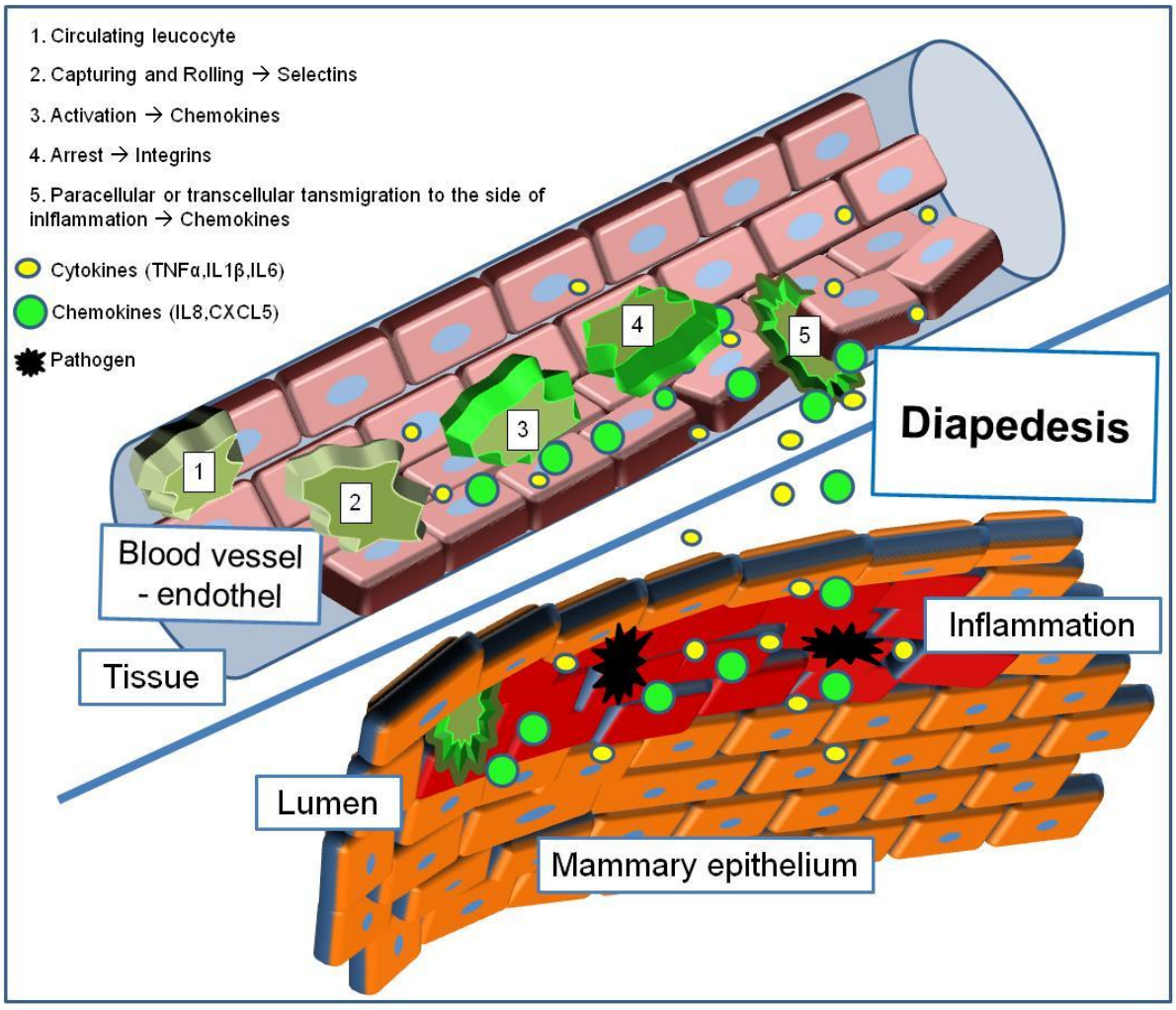

Figure 2: The principal of diapedesis. Due to pathogen contact, cytokines and chemokines are secreted by MEC and activated resident leukocytes. These factors mediate the recruitment and attraction of circulating immune cells to the side of inflammation and enable the transmigration of immune cells from the circulation into the inflamed tissue [12, 23, 24], own created figure.

The principal immune cell type found in the mammary tissue and secretions associated with bacterial infection are neutrophil granulocytes [25]. Beside their ability of phagocytosis and their bactericidal capacities, neutrophils as well as their host tissue MEC produce a potent antimicrobial peptide: lactoferrin. This 80kDa iron-binding glycoprotein is found in exocrine secretions and at mucosal surfaces of mammals, where it functions as prominent component of the first line of host defense against pathogen invasion and inflammation [26]. Furthermore it is released from the secondary granules of activated neutrophils during inflammation [27]. Beside its ability in iron homeostasis, it exerts bactericidal [28], bacteriostatic [29], antiviral [30], antifungal [31] and immune modulatory properties [32]. During phases of energy undersupply, an impairment of immune functions can be found and enables a pathogen invasion to manifest. It has been shown, that in early lactation during a state of metabolic imbalance, especially the immune ability of neutrophils including phagozytosis [33], chemotaxis [34], and their activation by respiratory burst [10] is impaired. This cell depended defense functions are mediated for the most part via LF [34,35,36], so that a modulated synthesis of LF during critical states of metabolic imbalance is suspected. Thus, special focus is laid on the defense mechanisms of the mammary epithelial cells themselves during critical phases of energetic imbalance and the role of lactoferrin during this metabolic challenge.

1.3 Aim of the study

Estrous cycle activity and mammary defense ability are critical fields affected by metabolic imbalance in early lactation. The metabolic challenge at the onset of lactation and the magnitude of accordingly occurring dysfunctions are, however, individually different pronounced.

The objective of the present study was to investigate, if a dietary energetic undersupply has effects on the estrous cycle activity and the innate immune response of the mammary epithelium. Alterations of the ovarian cycle length and the immune defense function in relation to a provoked energy deficiency should be analyzed.

Focus was also laid on the establishment of a primary bovine mammary cell (pbMEC) culture model generated by milk cell extraction and the suitability of the pbMEC cultures as non-invasive mastitis research tool. The *in vitro* approach aimed to simulate an inflammation of the mammary epithelium and hence, enabling the investigation of the immune response in relation to an energetic undersupply *in vivo*. Functional gene classes of the innate host defense system and lactoferrin should be analyzed.

2 Material and Methods

2.1 Animal trial

Feeding trial

40 Red Holstein cows were housed in a free stall barn and fed by automatical dispensers. Additional concentrate was offered at automatical concentrate stations, which were combined with scales and allowed exact body weight recording. Animals were milked twice daily and milk yield was recorded. Prior to the feeding experiment, the annual mean herd milk yield was 8,400 liters.

In mid-lactation, when metabolic stability was regained, an energy deficiency was provoked for three weeks. According to body weight, feed intake and milk yield until day 85pp (period 1), individual energy balances were calculated and cows were grouped evenly into control and restriction feeding regime. The induction of the dietary energy restriction was dependent on the stage of the ovarian cycle in order to affect physiological mechanisms mediated by steroid hormones in the following three restriction weeks. The peak secretory phase of the estrous cycle is dominated by the maximal function of the corpus luteum, the progesterone secretion. Known effects of this ovarian cycle stage on reproduction and immunity [37,38] were appointed to initiate an energy deficit and to investigate the impact of energy supply. Therefore exactly on day 12 of the first ovarian cycle after day 86pp, when metabolic instability was overcome, the experimental dietary energy restriction was induced for three weeks (period 2). Cows received an experimental diet (Tab. 1, PMR2) reduced in feed amount, concentrate amount and in energy according to straw adjustment to achieve a calculated energy deficit of at least 30% of total requirements. In the following phase of realimentation (period 3), energy deprived cows were offered the same diets (PMR1) as control cows until the end of the study on day 150pp.

Table 1: Diet composition and contents

	PMR1[1]	PMR2[1]	Concentrate[2]
Components (% in DM)			
Grass silage	33.7	21.8	
Corn silage	44.9	29.1	
Hay	6.5	39.4	
Concentrate[3]	14.9	9.7	
Nutrient values			
MJ NE_L/kg DM[4]	6.53	6.24	7.96
Crude fibre (g/kg DM)	214	251	62
Crude ash (g/kg DM)	76	75	76
Crude fat (g/kg DM)	32	28	24
Crude protein (g/kg DM)	146	138	216
ADF (g/kg DM)[5]	254	313	84.1
NDF (g/kg DM)[6]	431	529	184
NFC (g/kg DM)[4,7]	316	230	500
Available crude protein (ACP) (g/kg DM)[4]	143	137	172
Ruminant nitrogen balance (RNB) (g/kg DM)[4]	0.88	0.18	2.37

[1]Partial mixed ration
[2]Additional concentrate according to milk yield, consisting of 14.9% barley, 24.8% maize, 21.8% wheat, 20.1% soybean meal, 15.2% dried sugar beet pulp with molasses, and 3.2% vitamin-mineral-premix including limestone
[3]Concentrate: 7.9% barley, 24.7% wheat, 60.0% soybean meal, 7.3% vitamin-mineral-premix including salt and limestone.
[4]Calculated values
[5]Acid detergent fibre
[6]Neutral detergent fibre
[7]Nonfibre carbohydrates calculated by difference: 100 - (%crude protein + %NDF + %crude fat + %crude ash)

Sampling and data collection

Starting on day 20pp, two milk samples were taken weekly during morning milking until the end of the experiment on day 150pp (Fig.3). Estrous cycle activity was recorded three times weekly by rectal palpation and ultrasound scanning. Observed ovulations were confirmed by routinely milk progesterone (P4) analysis in twice weekly milk samples. On the last day of the energy restriction period, one liter milk was taken and MEC were extracted and cultured until the second passage. The conduction of the feeding trail and metabolic screening was done by the Animal Nutrition Unit, Technische Universität München and published in Gross et al. [39].

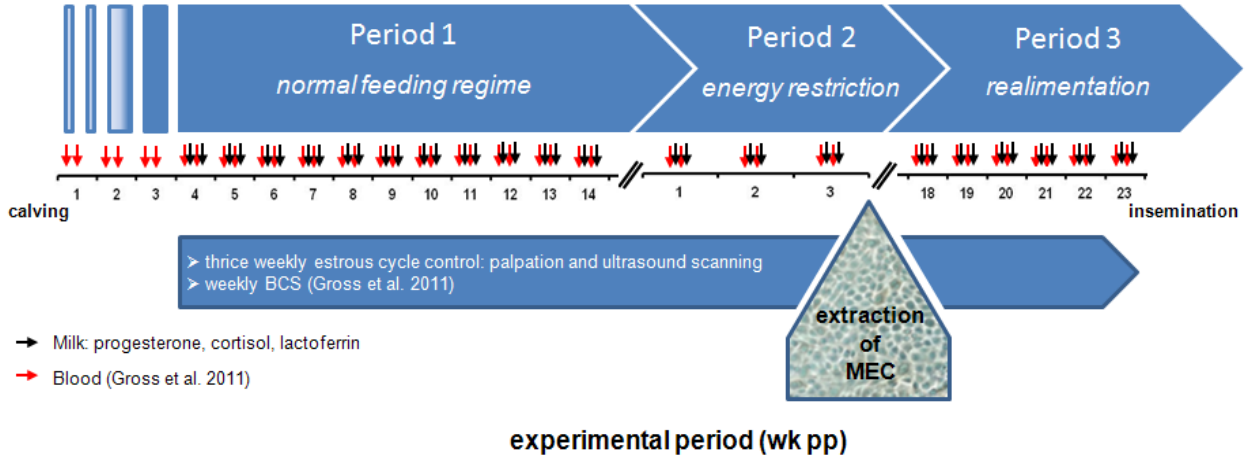

Figure 3: Study design and sampling schedule in weeks postpartum (wk pp).

2.2 Experimental grouping according to estrous cycle activity

In period 1, animals were grouped according to time of first ovulation pp and mean length of estrous cycle until day 85pp (Fig.4). Cows with no detected first ovulation until day 45pp were grouped into delayed first ovulation (DOV). Normal pp initiated cycle activity was not grouped separately, but further determined according to the lengths of observed ovarian cycles. Estrous cycle lengths of 18-24 days were considered as normal ovarian cycle (NOC). Cows showing shortened estrous cycles of <18 days or prolonged ovarian cycles of >24 days were grouped into shortened ovarian cycle (SOC) or prolonged estrous cycle (POC), respectively.

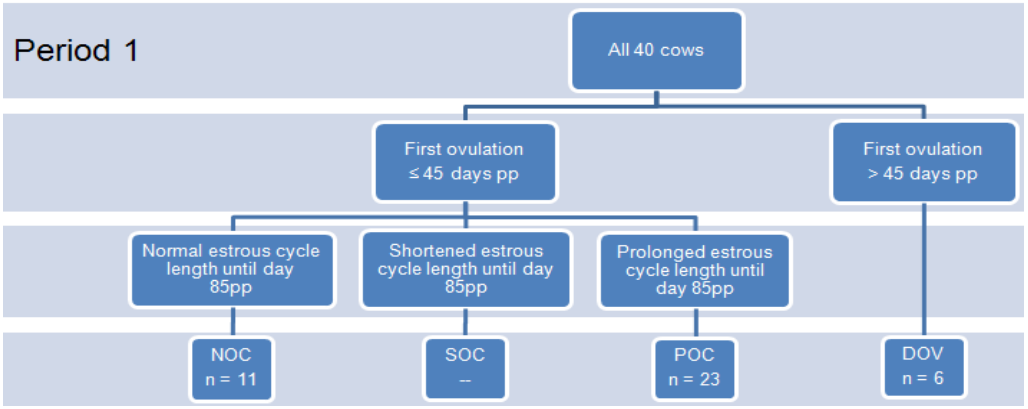

Figure 4: Classification of experimental cows according to estrous cycle activity during period 1

In period 2 (Fig. 5) all restriction and control animals were included to form new groups according to their cycle activity. According to ovulation time during restriction period, cows were grouped into normal (NOC), prolonged (POC) and shortened ovarian cycle (SOC) lengths.

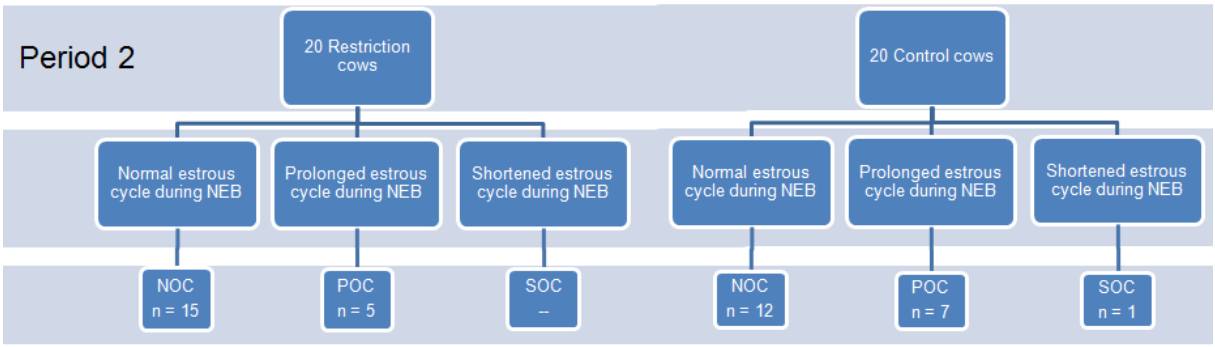

Figure 5: Classification of experimental cows according to estrous cycle activity during period 2

After three weeks restriction period, animals were realimented and received the same diet as control cows. The first ovulation during realimentation determined the cycle lengths in means of normal energy supply. All cows in the restriction and the control group were grouped anew according to the calculated cycle lengths after the induced energy deficit into NOC, POC and SOC (Fig.6).

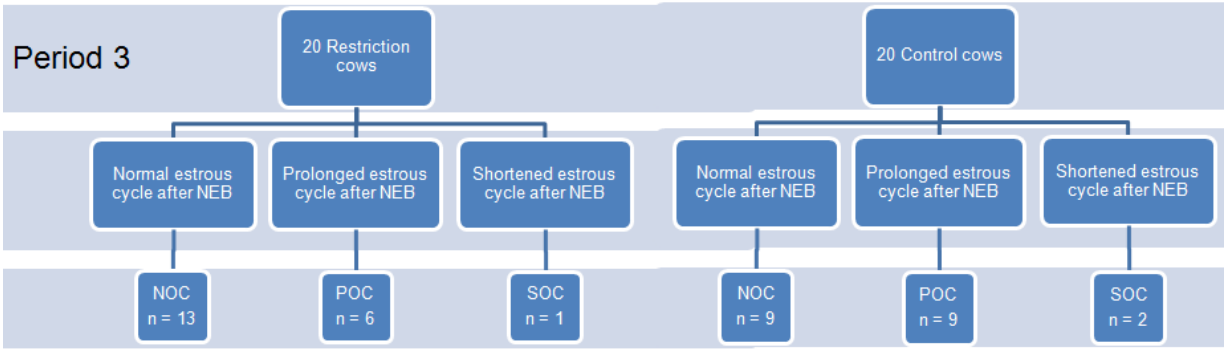

Figure 6: Classification of experimental cows according to estrous cycle activity during period 3

2.2.1 Milk progesterone and hydrocortisone

Milk progesterone enzyme-linked immunosorbent assay

Progesterone was measured twice a week in skim milk samples by an enzyme immuno assay (EIA) as described earlier [40] using the monoclonal antibody anti-progesterone clone 2H4 (1:3500) obtained from Sigma-Aldrich (München, Germany). The used label (1:3500) was progesterone-3CMO (Steraloids Inc., Rhode Island, USA.) coupled to horseradish peroxidase (HRP, Roche Applied Science Mannheim, Germany).

96 well microtiter plates (MaxiSorp®, Nunc; Thermo Fisher Scientific, Waltham, USA) were coated with 1µg/100mL affinity-cleaned goat anti-rabbit IgG (Physiology, TU München) in coating buffer (4.29g/L Na_2CO_3 x 10 H_2O, 2.93g/L $NaHCO_3$, pH 9.6) and incubated over night. The plates were satured with 280µL/well assay buffer (7.12g/L $NaHPO_4$ x 2 H_2O, 8.5g/L NaCl, 0.1% BSA (Sigma-Aldrich, Munich, Germany), pH 7.2), decanted after 45min at room temperature (RT) and stored at -20°C until use. Prior to analysis the plates were thawed and washed twice with phosphate buffered saline added with 0.05% Tween® 80, pH 7.4 (Roth, Karlsruhe, Germany) (PBST). 20 µL per well of the used standard curve points (0.2 – 12.5ng/mL in 0-plasma) and 20µL skimmed milk sample were applied in duplicates. Then 100µL progesterone-3CMO label (1:5,000 working dilution) and 100µL antibody (1:80,000 working dilution) and were added. After 2h incubation in the dark, the plates were washed 4 times with PBST and decanted. 150µL substrate (41) was added and incubated for 20min in the dark with soft shaking. The reaction was stopped using 50µL 2M H_2SO_4 and the plates were measured at 450nm by a microplate reader (Sunrise^{TM}, Tecan Trading, Switzerland). Inter- and intra-assay coefficients of variation were 13.46% and 5.69% respectively. Detectable thresholds were <0.2ng/mL and >3ng/mL in skimmed milk. Milk progesterone results were used to determine ovulation times in addition to ultrasound scanning and rectal palpations. Values >1ng/ml were assigned to preceded ovulation and a functional corpus luteum, values <0.2ng/ml indicated no ovarian activity.

Milk hydrocortisone enzyme-linked immunosorbent assay

Metabolic stress was evaluated by milk hydrocortisone and was measured twice weekly in skimmed milk. Measurement of hydrocortisone was done by competitive enzyme-linked immunoabsorbent assay (ELISA) using affinity purified goat anti-rabbit IgG (1µg/100µL, Physiology, TU München) and rabbit antihydrocortisone IgG (Physiology, TU München) as earlier developed for plasma and tissue [41]. Hydrocortisone-21-glucuronide (Steraloids Inc., Newport, Rhode Island, USA) was labeled with horseradish peroxidase (1:12,000) (Roche

Applied Science, Mannheim, Germany) as described for other steroids earlier [42]. The polyclonal antibody (C1 Pool2, 1:90,000) has been raised in rabbits against hydrocortisone-21-hemisuccinate-BSA. Its crossreactivities are: hydrocortisone 100%, cortisone 8%, corticosterone 9.5%, prednisolone 18% and dexamethasone <0.1%. Hydrocortisone standards in skimmed milk treated with activated charcoal ranged from 0.1 to 34.5nmol/l. 10µl skimmed milk in duplicates were transferred to 96 well microtiter plates (MaxiSorp®, Nunc; Thermo Fisher Scientific, Waltham, USA). Then 100µL of each enzyme-conjugated hydrocortisone (diluted 1:12,000) and hydrocortisone antiserum (diluted 1:18,000,000) were added. The plates were incubated overnight at 4°C. After incubation, the plates are washed four times with PBST and then 150µL of substrate solution, containing tetramethylbenzidine and hydrogen peroxide, was added. After incubation in the dark at RT for 40min, the reaction was stopped by 50µL 4M H_2SO_4 and the plates were photometric measured at 450nm (Sunrise^{TM}, Tecan Trading, Switzerland). For the determination of recoveries, aliquots of skimmed milk were treated with charcoal and then spiked with hydrocortisone (Sigma-Aldrich, Munich, Germany). The mean recovery was 106.48 ± 11.93%. Inter- and intra-assay coefficients of variation were 12.38% and 7.42%, respectively.

2.3 Immune challenge of primary bovine mammary epithelial cells

Generation of pbMEC cultures

On the last day of the energy restriction phase mammary epithelial cells (MEC) were extracted of one liter sterily taken milk. For that purpose the tits were cleaned and disinfected with Sterilium (Bode, Hamburg, Germany). Bacterial testing was performed to subsequently exclude cell cultures from infected quarters. 250mL each were filled into centrifuge cups and centrifuged at 1850g, 20°C for 10min. The milk was discarded and the remaining cell pellet was washed with 25mL prewarmed (37°C) washing solution containing Hank's balanced salt solution (HBSS, Invitrogen, Darmstadt, Germany), penicillin G, streptomycin, gentamycin and amphotericin B (Tab. 2). Than the resuspended cell solution was transferred into a 50mL falcon tube (Sarsted, Germany) and centrifuged again at 500g for 5min at RT. The supernatant was decanted again and the cell pellet was resuspended in further 25mL warm washing solution. Two falcon tube contents were combined and the two remaining tubes centrifuged again. The cell pellets were washed for a further third time and the solutions were filtered (Falcon Cell Strainer 100µm, BD Biosciences, Bedford, USA) into a new tube. After a last centrifugation step the cell pellet was resuspended in 37°C warm cell culture medium Dulbecco's modified eagle medium/F12 Ham (DMEM/F12 Ham) supplied with fetal calf serum (FCS), ITS supplement (5mg/mL insulin, 5mg/mL transferrin and 0.005mg/mL sodium

selenite), penicillin G, streptomycin, gentamycin and amphotericin B (Tab. 2). The cells were seeded into a 25cm^2 tissue culture flasks (Greiner Bio-one, Frickenhausen, Germany) and cultivated at 37°C, 5% CO_2 and 90% humidity.

Table 2: Contents of washing solution and cell culture growth medium

Washing Medium		***Growth medium***		
content	concentration	content	concentration	manufacturer
HBBS	10%	DMEM/F12 Ham	500mL	Sigma-Aldrich
		fetal calf serum	10%	Gibco, Invitrogen
		ITS supplement	5mL	Invitrogen
penicillin G	200U/mL	penicillin g	100U/mL	Sigma-Aldrich
streptomycin	200µg/mL	streptomycin,	100µg/mL	Sigma-Aldrich
gentamicin	200µg/mL	gentamycin	100µg/mL	Sigma-Aldrich
amphotericin B	10µg/mL	amphotericin B	5µg/mL	Sigma-Aldrich

Twice a week culture medium was replaced and cell growth was documented. Primary cell cultures are sensitive due contamination during extraction and further cultivation. Every generated culture shows individual growth and defense ability. In the early stages of cultivation, the cultures were washed with warm sterile phosphate buffered saline (PBS) (37°) in order to scavenge visible contaminations. When 80% confluency was reached, cells were passaged. Medium was removed and cells were washed with 1mL warm sterile PBS. PBS was removed and 2mL warm Accutase (37°C, PAA Laboratories GmbH**,** Pasching, Austria) were added. The flasks were returned into the incubator for 20-40min and cells were allowed to detach. The cell suspension was transferred completely into a bigger culture flask and cultured again. Cells were cultivated until the second passage, harvested and aliquoted in freezing medium consisting of DMEM/F12 HAM supplied with 20% FCS and 10% dimethyl sulfoxid (DMSO) (Roth, Karlsruhe, Germany). Aliquots were stored in liquid nitrogen until all cultures were generated.

Immunohistochemistry

Epithelial cells can be identified by immunohistochemical staining of special surface proteins, the cytokeratins. Cells were cultured on culture chamber slides (LAB-Tek, Nunc, Thermo Fisher Scientific, Waltham, USA) until confluent state. The cell culture wells were removed and cell layers were washed with PBS. The cells were fixed with ice-cold aceton-methanol mix (1:1) for 5min and dried at RT. Endogenous peroxidises were blocked by incubating the

cells with 1% H_2O_2 (Merk, Darmstadt, Germany) in phosphate buffered saline Tween buffer ph 7.4 (0.1% Tween® 80, PBST) for 30min at RT in the dark. After three washing steps in PBST for 5min, background colour was reduced. Goat serum was diluted 1:10 in PBST and 30µL were applied to each well for 30min. Goat serum was decanted except for the negative control wells, which were not treated with primary antibody in order to verify the assay. Then a primary monoclonal mouse IgG anti-pan cytokeratin antibody (F3418, Sigma-Aldrich, St-Louis, USA) was diluted 1:50 in PBST, applied to the test wells and incubated over night at 4°C. On the next day, cells were washed three times for 5min with PBST. The secondary polyclonal goat anti-mouse antibody (Immunoglobulins HRP, Dako Gostrup, Denmark) was diluted 1:400 in PBST and incubated on the cell layers for 1h at RT. Afterwards cells were washed again (three times for 5min in PBST) and enzymatic colour reaction was induced by incubation of 0.01% DAB (DAB-dihydrochloride) and 0.01% H_2O_2 in PBST for 15min in the dark. After three further washing steps in PBST for 5min, slides were dipped into aqua bidest. The cell nuclei were stained with Mayer hemalaun solution (Roth, Karlsruhe, Germany) for 15sec and colour development was obtained by rinsing the slides in tap water for 30sec. Dehydration of the histological staining was obtained by incubation of the slides in ascending alcohol sequence beginning in 50% until 100% ethanol for 2min, respectively, followed by a final incubation in xylol (2min, Sigma-Aldrich, Munich, Germany). The stained cells were covered with glass slides and fixed with EUKITT (Fluka, Sigma-Aldrich, Steinheim, Germany).

Cultivation and preparation of E.coli and S.aureus

The Immune challenge was induced by heat-inactivated *E.coli* 1303 and *S.aureus* 1027 kindly provided by Wolfram Petzl, Clinic for Ruminants, Ludwig-Maximilian-University, Munich, Germany. The cultivation of *E.coli* and *S.aureus* was done on individual agar and growth media listed in table 3. The pathogens were thawed, applied to the appropriate agar plates and cultured over night at 37°C. The following day, one colony of each pathogen was picked and transferred to 20mL of the respective growth medium. After incubation over night at 37°C, the pathogen solutions were diluted and a growth curve was performed over 4h. Every 30min the optical density (OD) of 1mL bacteria solution was determined and 5 dilution steps were seeded, respectively. After cultivation over night the developed colonies were counted. Assuming that one colony was grown out of one bacteria cell, the bacteria amount was calculated in relation to sampling time and dilution steps. Then, the growth curve was repeated. According to the first performed growth curve, bacterial growth was stopped at the optimal time point by putting the pathogen tubes on ice for 10min. The tubes were centrifuged twice and the pellet was re-suspended in 10mL PBS, respectively. After a third

centrifugation the bacteria pellets were re-suspended in 5mL PBS and were heat-inactivated in 63°C hot water for 30min. Finally, the inactivated bacteria were seeded on the respective agar plates in order to control the inactivation process. Bacterial solutions were aliquoted and stored at -80°C.

Table 3: Cultivation protocol of the applied heat-inactivated pathogens *E.coli* and *S.aureus.*

	***Escherichia coli* 1303**	***Staphylococcus aureus* 1027**
Agar	LB-agar Lennox (Serva, Heidelberg, Germany)	Blood agar (Blood Agar Base No.2, Oxoid, Cambridge, UK)
Medium	LB liquid medium (Serva, Heidelberg, Germany)	Broth liquid medium (Sigma-Aldrich, Steinheim, Germany
Cultivation	> cultivation on agar plates, over night 37°C > 1 colony was transferred to growth medium, cultivation over night 37°C	
Dilution in medium	1:1000	1:500
Growth curve	> OD at 600nm in 1mL bacteria solution every 30min for 4h > seeding of 5 and 2 dilutions steps on agar plates: 10^{-4} -> 10^{-6} and later on 10^{-9} -> 10^{-10}, over night cultivation > colony counting and calculation of bacteria amount > repetition of growth curve until optimum growth	
Bacteria harvest	> 3x centrifugation on 1850g for 10min --> final resuspension in 5ml PBS	
Heat-inactivation	> 30min in water bath at 63°C --> inactivation control by seeding on agar	
Storage	> bacteria aliquots at -80°C	

Immune challenge with heat-inactivated mastitis pathogens Escherichia coli and Staphylococcus aureus

In the third, passage pbMEC were thawed and seeded onto 48 well plates (Fig. 7), each cow and infection time on one separate plate. Concentration of 100.000 cells per well were seeded in duplicates and cultured in 1mL DMEM/F12 HAM medium supplied with ITS solely until 80% confluency was reached. Prior to infection, respective counting wells were harvested and counted. Cell count was assumed for the remaining wells and bacteria concentration for multiplicity of infection (MOI) 30 was calculated. Heat-inactivated bacteria

solutions were added to growth medium and 1mL of prepared challenge medium was added to the cells, respectively. Control cells were treated with PBS added to growth medium. Cells were stimulated for 24h and 72h on separate plates, respectively (Fig. 7). After 24h and 72h challenge, culture supernatant was removed and stored until the analysis at -80°C.

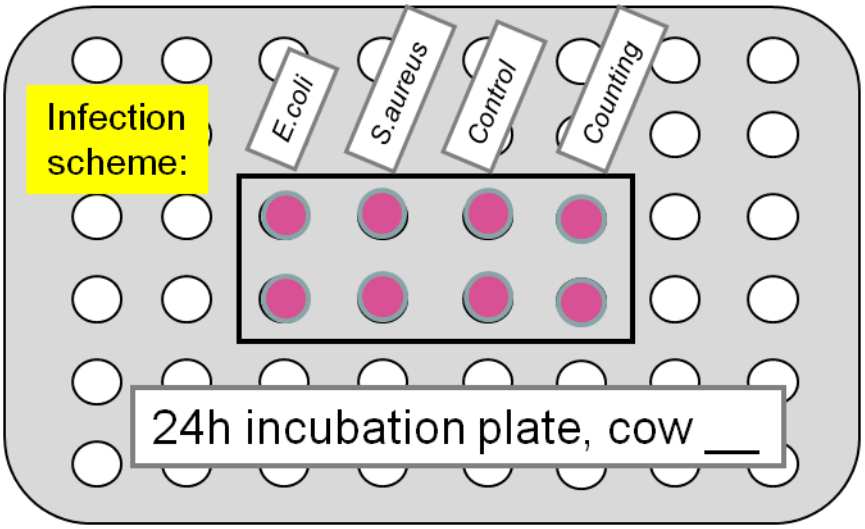

Figure 7: Infection scheme in duplicates for pbMEC from one cow and one incubation time.

2.3.1 RNA extraction and quality determination

For RNA and protein extraction the Qiagen Allprep RNA/Protein kit (Hilden, Germany) was used. Cell layers were washed twice with sterile cold PBS and lysed in 150µL of lysis buffer. The lysate was stored until RNA and protein extraction at -80°C. Extraction was done according to user's manual with additional DNAse digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany). RNA concentration in each sample was calculated by the determination of optical density (OD) using the NanoDrop 1000 (PEQLab Biotechnologies, Erlangen, Germany) and integrity of extracted RNA was measured with the Agilent Bioanalyzer 2100 and RNA6000 Nano Assays (Agilent Technologies, Waldbronn, Germany).

2.3.2 Selection of target genes and primer design

A representative gene set of the innate host defense system was chosen sectioning four functional groups: receptors and antimicrobial peptides (RAMP), cytokines, chemokines and apoptosis related genes (Tab. 4). Bovine sequences were searched via NCBI database. Primer pairs were designed with the open source software PRIMER3 (http://frodo.wi.mit.edu/primer3/input.htm) with respect to primer dimer and self-priming formation and synthesized by Eurofins (MWG GmbH, Ebersberg, Germany). All primers were

tested with pbMEC and leukocyte test cDNA in a gradient PCR determined on the iQ5 Multicolor real-time PCR detection system (Bio-Rad Laboratories GmbH, Munich, Germany). Suitable annealing temperatures were chosen according to melt curve analysis. The respective PCR product was confirmed by GelRed (Biotium inc., Hayward, USA) stained agarose gel electrophoresis.

Table 4: Selected target genes and references

Genes	Abbreviation		Sequence (5' to 3')	Size (bp)	T_M (°C)	Reference
Reference genes						
Actin gamma 1	Actinγ-1	F	aactccatcatgaagtgtgacg	233	60	NM_001033618
		R	gatccacatctgctggaagg			
Glyceraldehyde 3-Phosphate Dehydrogenase	GAPDH	F	gtcttcactaccatggagaagg	197	60	[43]
		R	tcatggatgaccttggccag			
Ubiquitin 3	UBQ3	F	agatccaggataaggaaggca t	198	60	NM174133
		R	gctccacttccagggtga t			
Target genes						
Toll-Like-Receptor 2	TLR2	F	cattccctggcaagtggattatc	202	64	NM_174197.2
		R	ggaatggccttcttgtcaatgg			
Toll-Like-Receptor 4	TLR4	F	tgctggctgcaaaaagtatg	213	64	NM_174198.6
		R	ttacggcttttgtggaaacc			
Defensin beta1	DEFβ1	F	tgctgggtcaggatttactcaagga	85	64	NM_175703.3
		R	agggcacctgatcggcacac			
Lactoferrin	LF	F	cgaagtgtggatggcaaggaa	215	64	NM_180998.2
		R	ttcaaggtggtcaagtagcgg			
Lactoperoxidase	LPO	F	ccgacaacattgacatctgg	206	64	NM_173933.2
		R	gtcacagatgaggcgtgaga			
Tumor-Necrosis-Factor alpha	TNFα	F	ccacgttgtagccgacatc	108	66	AF348421
		R	accaccagctggttgtcttc			
Interleukine 1 beta	IL1β	F	cagtgcctacgcacatgtct	209	64	NM_174093.1
		R	agaggaggtggagagccttc			
Interleukine 6	IL6	F	caccccaggcagactacttc	182	61	NM_173923.2
		R	atccgtccttttcctccatt			
Interleukine 8	IL8	F	tgctctctgcagctctgtgt	306	64	NM_173925.2
		R	cagacctcgtttccattggt			
Chemokine (C-X-C motif) ligand 5	CXCL5	F	ttgtgagagagctgcgttgt	150	66	NM_174300.2
		R	ccagacagacttcccttcca			
Chemokine (C-C motif) ligand 26 / Eotaxin-3	CCL26	F	ctcggagctgccacacgtgg	167	67	XM_002698193.1
		R	tgggcacacactttccggcc			
Growth-Related Oncogene αlpha	Groα	F	gctcggacgtgttgaagaac	116	64	U95812
		R	cctgagccagaggcggactac			
FAS	FAS	F	agaagggaaggagtacacaymga	124	61	[44]
		R	tgcacttgtattctgggtcc			
B-cell lymphoma 2	Bcl-2	F	cggaggctgggacgcctttg	116	64	NM_001166486.1

		R	tgatgcaagcgcccaccagg			
Caspase 6	Casp6	F	ggctcgcggtccaggtgaag	177	68	NM_001035419.1
		R	ctggtgccaggcctgttcgg			
Caspase 7	Casp7	F	atccaggccgactcgggacc	235	68	XM_604643.4
		R	agtgcctggccaccctgtca			

Abbreviations in the primer sequences: y = c or t, m = a or c.

2.3.3 Two-step RT-qPCR analysis

RNA reverse transcription (RT)

The extracted total RNA is very sensitive and was therefore reverse transcribed into cDNA. In this form the probes can be thawed and frozen several times without degradation.

The total RNA concentration was very low in some cell culture extracts. Therefore we reverse transcribed 300ng RNA to be able to perform at least one further RT reaction. The used protocol provided a 60µL approach per sample including 12µL 5xbuffer (Promega, Mannheim, Germany), 3µL hexamers (50µM; Invitrogen, Carlsbad, CA), 3µL dNTPs (10µM; Fermentas, St.Leon-Rot, Germany) and 1µL RT enzyme (m-MVLRT Rnase H(-) Point Mutantm, Promega, Mannheim, Germany). The reaction was conducted on an Eppendorf Thermo cycler with the following reaction steps: 10min at 21°C for extension of random primers, 50min at 48°C for second-strand synthesis, 2min at 90° and hold on 5°C for inactivation of the RT enzyme.

Reverse Transcription - quantitative PCR (RT-qPCR)

The gene expression was analyzed on 96-well plates on the the iQ5 Multicolor real-time PCR detection system (Bio-Rad Laboratories GmbH, Munich, Germany) and the MESA GREEN qPCR MasterMix Plus for SYBR® Assay w/fluorescein Kit (Eurogentec, Cologne, Germany). According to manufactor's instruction the 15µL reaction mix consisted of 1.5µL cDNA template, 1.5µL forward and 1.5µL reverse primer, 7.5µL MESA and 3µL H_2O. The employed amplification protocol was 5min on 95°C for activation of the hot start enzyme Meteor*Taq*, followed by 40 cycles: 15sec at 95°C denaturation, 20sec annealing at primer specific temperature and 40sec at 60°C for elongation. After the PCR reaction was completed a melt curve was performed. Programmed temperature gradient started at 60°C and increased on 0.5°C steps in 1sec intervals until 95°C. For gene expression quantification, an automatic threshold was set in the most linear area of the quantification curves. The crossing points (Cq values) were used for relative quantification.

2.4 Milk lactoferrin enzyme-linked immunosorbent assay

Milk LF was determined weekly by a competitive ELISA including an indirect antigen detection method and competitive epitop quantification (Fig. 8). 96-well microplates were precoated with the primary unspecific antibody goat anti-rabbit IgG (affinity cleaned, [45]) directed against all antibodies produced in rabbit, stored in coating buffer (50 mM $NaHCO_3$, pH 9.6) and incubated over night at 4°C. Plates were saturated with 280µg/well casein (SP-5020, Vector Laboratories Inc., Burlingame, CA) diluted 1:10 in PBST and incubated for 45min at RT. Afterwards the blocking solution was removed and microplates were stored at -20°C.

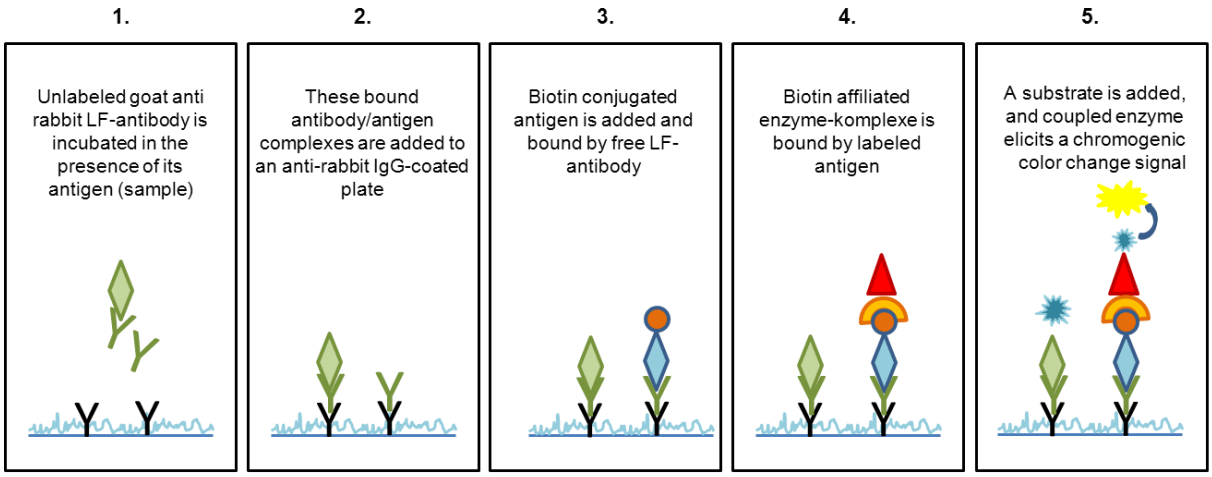

Figure 8: Principle of the LF competitive enzyme-linked immunosorbent assay (ELISA): the more antigen in the sample the less labeled antigen is retained in the well and the weaker is the determined signal, own created figure.

Prior to sample preparation the microplates were thawed at RT and washed twice with PBST. Standard curve was diluted in PBST and prepared in duplicates in 9 concentration steps: 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2ng/20µL. Four control milks differing in their SCC: I = 15,000 cells/mL, II = 310,000 cells/mL, III = 97,000 cells/mL and IV = 1,300,000 cells/mL were employed as interplate controls. Applied dilution step in PBST for milk I and III was 1:2,000 in PBST, milk II was used in 1:4,000 and milk IV 1:8,000. Skimmed milk samples were diluted 1:2,000 in PBST and 20µL/sample was applied onto the plate. The used capture antibody was produced in rabbit according to immunization with bovine LF (colostrum isolate, Sigma-Aldrich Munich, Germany). It was applied diluted 1:400,000 and incubated over night at 4°C. For detection, LF was conjugated to biotinamidohexanoic acid N-hydroxysuccinimide ester (Biotin-X-NHS, Sigma-Aldrich Munich, Germany) and precipitated with 75% saturated ammonium sulphate and stored in PBST. After overnight

incubation of sample and antibody, 3ng/100µL of biotinylated LF was applied for 2h in the dark. After 4 washing steps with PBST, enzyme streptavidin-HRP (1:20,000 in PBST) was coupled to the complex. After incubation for 15min in the dark at RT, the plates were washed again (4x in PBST) and the substrate reaction [45] was induced. After 40min at RT, in the dark the reaction was stopped with 150µL 2M H_2SO_4. Extinction was measured at 450nm (minus light absorbance at 600nm background value) using a Microplate Reader (Sunrise^{TM}, Tecan Trading, Switzerland). Mean LF concentrations were calculated against the optical density (OD) of the standard curve.

2.4.1 Lactoferrin in pbMEC

After stimulation with *E.coli* and *S.aureus*, the LF concentration in the cell culture supernatant and the cellular LF in the pbMEC-total protein extract was analyzed. The above described ELISA was used for the detection with slight modifications. To determine the total protein content in both cell culture matrixes, a bicinchoninic acid assay (BCA) was conducted and the dilution steps for total pbMEC protein and medium were evaluated. Finally, total pbMEC samples were employed in 1:3 dilutions in PBST. Cell culture medium was used undiluted. Preparation of the standard curve was done in PBST for total pbMEC protein according to the milk assay. The standard curve for supernatant analysis was prepared in DMEM/F12 Ham cell culture medium supplied with ITS. Inter- and intraassay variations for milk and pbMEC-total protein were 11.06% and 9.36%, respectively. Inter- and intraassay variation for cell culture supernatant was 10.77% and 6.06%, respectively.

2.5 Statistics and data analysis

Metabolic parameters and cycle activity

The influence of an energetic undersupply on estrous cycle activity was analyzed by SigmaPlot and the R Project for Statistical Computing (http://www.r-project.org, R Development Core Team, 2011) and the Weka Machine Learning Framework (http:sourceforge.net/projects/weka). First, area under the curve (AUC) was calculated for the metabolic parameters in period 1. For period 2 and 3 mean values were used. The effect on metabolic parameters in the three experimental groups (NOC, POC and DOV) in every week of period 1 was calculated by one-way ANOVA or by Kruskal-Wallis working on ranks for failed normality distribution. A multiple testing correction was done using sequential Holm-Sidak correction. Differences between period 2 and period 3 were analyzed by T-test or via Mann-Whitney Rank Sum test for not normally distributed data. An all-pairwise metabolic

parameter correlation matrix was computed for each period using Pearson correlation. A multiple logistic regression model with Wald test statistics was trained using the SigmaPlot Maximum Likelihood Fitting procedure on all cow parameters. The cycle length classes in period 1 (NOC POC and DOV) are treated as dependent variables and the metabolic parameters represent independent measurements. In addition, the WEKA logistic function (using a ridge penalty of 1e-8) was used to estimate the predictive power of the logistic models by setting up a leave-one-out (LOO) validation. A comparison of cycle length for period 2 and 3 between restriction and control was conducted using z-tests on the two group proportions. Additionally, z-tests were applied to detect differences in the number of services until conception and the conception rate.

Statistical analysis of gene expression

Gene expression Cq values of the immune challenge of pbMEC with heat-inactivated *E.coli* and *S.aureus* were first normalized with mean regulation values of three suitable reference genes namely Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH), Ubiquitin (UBQ3) and Actin gamma 1 (Actinγ1) selected by GenEx software 5.0.1. (MultiD Analyses AB, Göteborg, Sweden) [46]. To obtain treatment effects ΔCq values were normalized against untreated control cells and then further transposed into $2^{-\Delta\Delta Cq}$ for relative expression ratio (x-fold regulation) according to Livak and Schmittgen [47]. Outliners were identified and excluded using the GenEx function Grubbs' test.

Principal component analysis

As first statistical tool Principal Component Analysis (PCA) [48] were conducted to disclose multivariate treatment effects in pbMEC ΔCq data. The PCA is a suitable tool for multidimensional data analysis, which allows the recognition of patterns and the visualization of treatment information of a heterogeneous data set for example a gene expression experiment [49, 50]. Instead of a single parameter, analysis of the behaviour of the samples determined by a multidimensional parameter set and influenced by the conducted treatment is focused. The effects detected in a sample are reduced to two principal components (PC). These PCs are linear combinations of the initial chosen parameters of a sample in such a way that PC1 describes the largest fraction of variation whereas the following PCs account for the maximal proportions of the remaining variation [51]. With the reduction of all orthogonal PCs to only two, the data set can be plotted in a two-dimensional room. Representing one spot for each sample, the visualization of treatment effects is implemented by clustering of the sample dots. Samples revealing similar treatment effects are arranged in

sample clouds. Effects between treatment and control group lead to spatial separation of the respective samples. The more definite the effect is, the more pronounced is the separation of the groups. Beside success of treatment effects, the PCA provides an indication of variation within a data set, which in turn concerns the subsequent calculation of statistical significance. The more consistent the treatment effect is, the tighter is the arrangement within the treatment group cloud. For a clear representation of the host defense gene expression data by PCA, the data were clustered according to feeding group (■ = control, ▲ = energy restriction) and pathogen (green = *E.coli*, yellow = *S.aureus*, red = untreated control).

Stimulation of pbMEC with heat-inactivated pathogens

Effects of restriction and control feeding were determined on gene expression profile of the innate host defence gene groups. The PCA results were further confirmed by comparing the $2^{-\Delta\Delta Cq}$ arithmetic means of the control feeding versus the energy restriction feeding groups using Student's unpaired T-tests or the Mann-Whitney-Rank-Sum-Test for not normally distributed date with $P \leq 0.05$.
Comparison of means scheme: CS24 vs. RS24, CS72 vs. RS72, CE24 vs. RE24, CE72 vs. RE72, CC24 vs. RC24, CC72 vs. RC72.

Lactoferrin production in stimulated pbMEC

Arithmetic means of the restriction compared to the respective control groups in the three matrixes were calculated. The gene expression of LF was first normalized against the arithmetical mean of the reference genes as described above. To present regulation patterns also in the untreated control cells of the energy restriction group, the second normalization was done against the respective control feeding group. Significances were determined by arithmetic mean comparison using Student's unpaired T-tests or the Mann-Whitney-Rank-Sum-Test for not normally distributed data with $P \leq 0.05$.

Lactoferrin in milk

For statistical evaluation of weekly LF analysis in milk, the Mixed Model procedure in SAS 9.2 (SAS Institute Inc., Cary, USA) was used. The effects of the conducted energy restriction in period 2 as well as differences among treatment and control group were determined using REML. In the used model fixed effects of treatment (restricted feeding vs. control), week and cow individual effects were considered. The repeated measurement model further contained the factors weeks relative to calving as well as the interaction of feeding regime and weeks

relative to calving. The general test procedure compared effects of time (weeks relative to calving), treatment (restriction vs. control feeding) and time x group interactions. Differences between treatments were determined using the PDIFF option. Significant level was $P \leq 0.05$.

SAS code used for statistic analysis of LF in milk

```
libname milk "C:\Dokumente und Einstellungen\SIG\Desktop\milk";
proc import datafile="C:\Dokumente und Einstellungen\SIG\Desktop\milk\lactof.csv"
    out=milk.lactout
    dbms=csv
    replace;
    getnames=yes;
run;
%macro milk (response=);
ods rtf file ="C:\Dokumente und Einstellungen\SIG\Desktop\hmilk\statout_&response.rtf";
proc mixed data=milk.lactout;
class cow trt week;
Model &response = trt week trt*week;
repeated week / subject=cow;
lsmeans   trt week trt*week/pdiff=all;
RUN;
ods rtf close;
%mend milk;
%milk (response=lf);
```

3 Results and Discussion

3.1 Effects of the conducted dietary energy restriction on estrous cycle length

Experimental period 1

The conducted estrous cycle controls beginning on day 20pp (Fig. 9) revealed 6 cows with a delayed first ovulation until day 45pp (55 ± 3.9d pp)(DOV). Re-establishment of cyclicity was found in the remaining 34 (25 ± 1.7d pp) cows. Among them cows with normal ovarian cycle length (NOC) showed the first ovulation on mean day 28 ± 2.3pp whereas prolonged ovarian cycles (POC) were re-established on mean day 22 ± 2.2pp. Cycle activity was detected beyond 45d pp in DOV but showed normal estrous cycle lengths of mean 22 ± 1.1d, which is contrary to the findings of Samarütel et al. [6]. The delayed establishment of cyclicity was mediated in their work by a prolonged luteal activity leading to prolonged ovarian cycles.

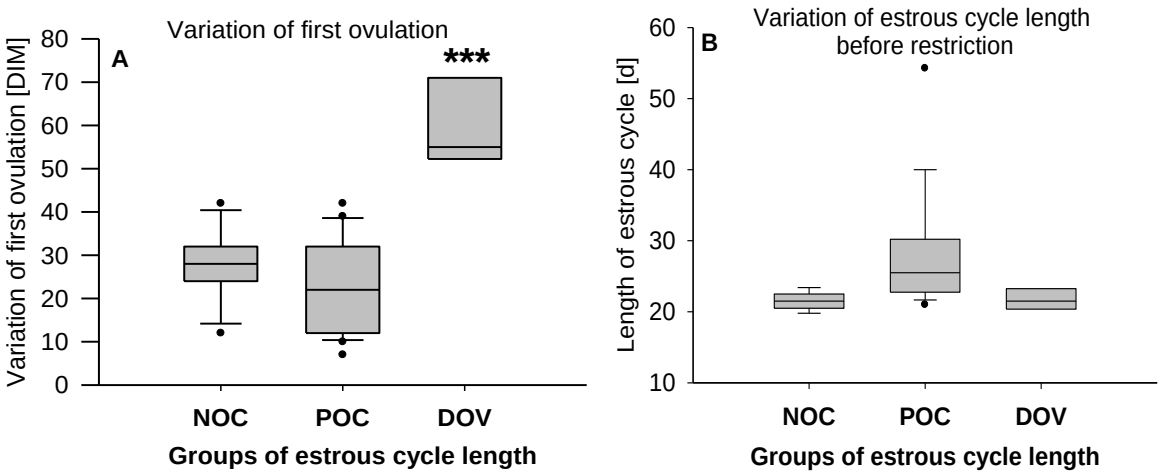

Figure 9: Onset of ovarian cycle activity by means of time of first ovulation pp in DIM (A) and the accordingly determined estrous cycle lengths (B) during period 1 in days (d).

According to the formation of estrous cycle activity into NOC, POC and DOV, the metabolic parameters are presented in figure 10. The course of the energy balance, glucose, NEFA, BHB and the body condition score (BCS) were determined beginning in the first week until day 85pp. The animals that were grouped into NOC, POC and DOV showed equal developments of the metabolic parameters throughout period 1 and represent the well-known effect on metabolic parameters of an energy imbalance forced by the onset of lactation. A calculated negative energy balance was found in all three groups and had its maximum of -50MJ NEL/day in POC and DOV in the first week pp. The energy deficit is mediated by decreased blood glucose levels in the second week pp with a minimum of 3.2mmol/L. The lack of energy is compensated by the mobilization of body fat. One physiological indicator of this mobilization process is an elevated concentration of non-esterified fatty acids (NEFA) in the blood. Body fat triglycerides are oxidized and NEFA are transported to the liver and

transformed to BHB. Used as alternative hydrophylic energy source, increased blood BHB concentrations of ≥ 1.00mmol/L predispose cows to ketosis and is used to be a second physiological parameter of a metabolic imbalance. Both blood indicators of an energy deficiency were elevated during first three weeks pp. Peak NEFA concentrations of 1.0mmol/L were measured for DOV, whereas NOC and POC showed maximum blood NEFA concentrations in the following week of 0.8mmol/L. BHB peak concentrations were reached during the third week by 1.2mmol/L in the POC group. The three estrous cycle groups, however, did not differ in the NEFA and BHB courses throughout period 1. One exception is a single sporadic significance in NEFA and BHB between the cycle groups that was found for POC and DOV on day 28pp. The metabolic imbalance ends during week 5 of the experiment when NOC reaches positive energy balance. POC and DOV enter balanced energy status one week later, but differences compared to NOC were not significant. The improvement of the calculated energy balance is also displayed in decreasing blood NEFA and BHB concentrations. According to the increasing energy balance, blood glucose concentration increases simultaneously. Concerning ovulatory activity, glucose levels can be associated with delayed ovulation due to decreased pulsatility of or an impaired ovarian responsiveness to the gonadotropin LH [2,52,53]. But in the present study no differences in glucose concentrations were found between NOC, POC and DOV.

Optimal calving BCS are quoted in the literature in the range of 3.0 to 3.25 [54]. Higher BCS scores of ≥ 3.5 are associated with metabolic disorders, whereas lower BCS than 3.0 are associated with reduced production and reproduction performance. In the present study NOC and POC show mean BCS scores of 3.35 in the first week pp. The cows with delayed first ovulation, however, have a BCS optimum of 3.2. BCS decreased slowly and on low reduction levels in our study. From the beginning until the end of period 1, a total reduction of only 0.22 BCS scale points was found for NOC and POC reaching still optimum levels at the end of period 1 of 3.15. DOV cows had significant ($P \leq 0.05$) lower BCS at the end of period 1 compared to the NOC and POC group and lost more BCS (0.55 units). Although this finding can be confirmed in other studies [6], the decrease of BCS in this study is to low as being promoted by effects of the energy deficiency in early lactation. A literature summary of Roche et al. [54] listed the published effects of BCS on reproductive performance at calving, during lactation and the amount of BCS loss. The association of calving BCS as significant contributor of reproductive success is not consistent, whereas both calving BCS and BCS nadir should be taken into account concerning the evaluation of reproductive performance. Both, calving BCS and BCS nadir are however not the reason for prolonged estrous cycle activity. Even the DOV categorized cows show optimal calving BCS, but an increased loss of BCS.

Twice weekly determined concentrations of milk hydrocortisone neither showed modified courses by time throughout the period 1 nor by differences of cycle activity. NOC, POC and DOV did not differ in the concentration of milk hydrocortisone during period 1. The calculated significance is obviously an outlier.

We evaluated the effect of the metabolic status on estrous cycle length and the resumption of estrous cycle activity after calving in period 1. The differences in ovarian cycle length and onset of estrous cycle activity after calving were not mediated by particular effects of the metabolic imbalance. The categorized cows in NOC, POC and DOV in period 1 did not differ in the courses of the determined metabolic parameters.

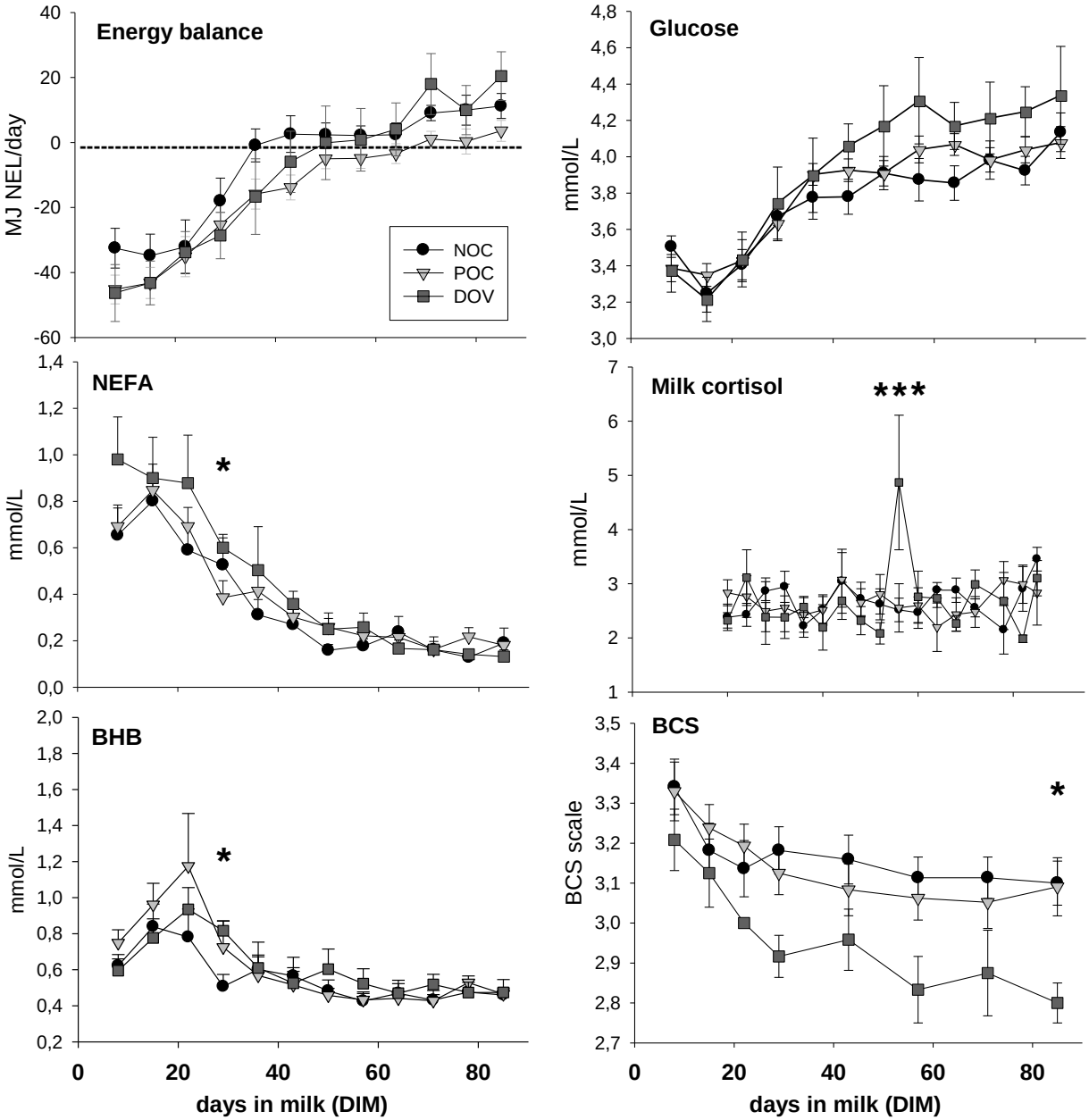

Figure 10: Development of metabolic parameters due to estrous cycle length (DOV, NOC and POC) in period 1.

Experimental period 2

Around day 100pp, but exactly on day 12 of the respective estrous cycle, the dietary energy restriction was induced. Ovarian cycle lengths influenced by the feeding regime were calculated and presented in figure 11. The mean cycle length of the control feeding group was 21 ± 1.2d, whereas the energy restricted cows had a mean cycle length of 25 ± 0.7d (Fig. 11A). The mean difference is not significant due to high variation within the control group. The prior ranking of all cows according to cycle lengths (Fig. 11B) revealed that most cows had normal cycle lengths (NOC) and most of them were energy restricted. Furthermore, most cows with prolonged estrous cycle (POC) during period 2 belong to control feeding group and only one cow with shortened ovarian cycle (SOC) was detected, which also belonged to the control feeding group. These findings are unexpected. Especially reduced energy content is considered as main reason for impaired fertility [2,52,53].

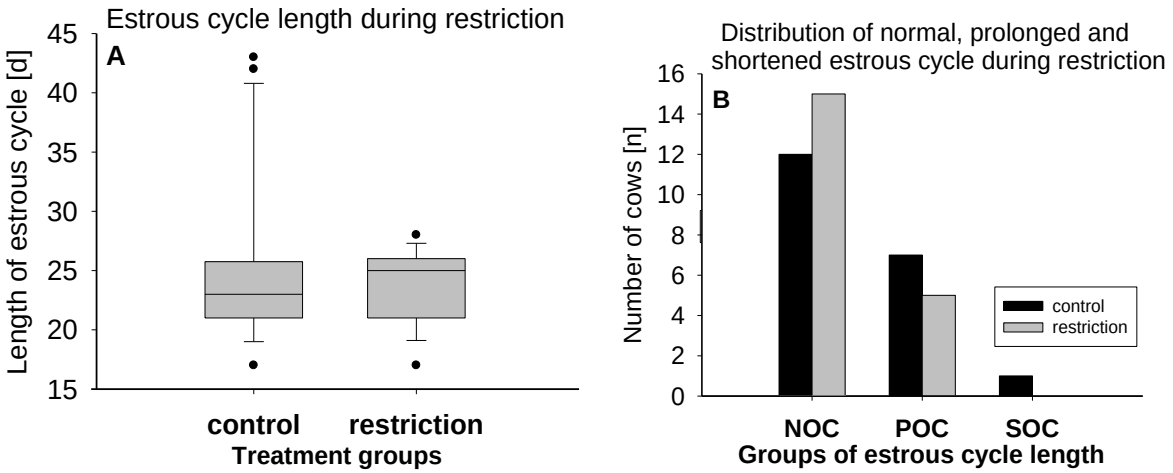

Figure 11: Variation of estrous cycle length in the control feeding and energy restriction group (A) and the distribution of cows with normal, prolonged or shortened estrous cycle during the induced energy restriction (B).

In the present study, the greater experienced energy deficit did not lead to reproductive dysfunction or was even indicated by trend in the presented results. Comparing them to literature, however, similar effects due to induced energy restriction on estrous cycle were found in Burk et al. [55]. The acute feed restriction of 44% of control group intake did not affect ovulation and conception rates. The majority of the experimental cows commenced ovulation before the feeding treatment was imposed, similar to the findings in the present study. Hence they presume that an early activated cycle activity pp may stabilize the subsequent estrous cycles and therefore reduces the susceptibility of metabolic imbalance that induces reproductive dysfunctions. Also Butler and Smith [52] point out the importance of completion of multiple ovarian cycles, especially with regard to improved insemination success. They relate fertility problems to carry over effects of energy deficiency on decreasing blood progesterone levels, which in turn affect the successful ongoing of estrous

cycles. In the present study, no blood progesterone profiles were generated, but milk progesterone level was the main tool to evaluate estrous cycle stages and is therefore included in the determination of estrous cycle lengths.

During energy restriction, no differences could be found between the extent of energy deficit between NOC and POC cows in metabolic parameters (Fig. 12). The SOC categorized cows belonged to control feeding group and showed a positive energy balance during period 2. The lack in energy supply seems not to be the reason for a shortened luteal phase. Interestingly, NEFA and BHB concentrations were also not negatively correlated with the induced energetic imbalance, although restriction cows experienced a higher energy deficit of -63MJ NEL/d than all cows during the common deficit in period 1 [39].

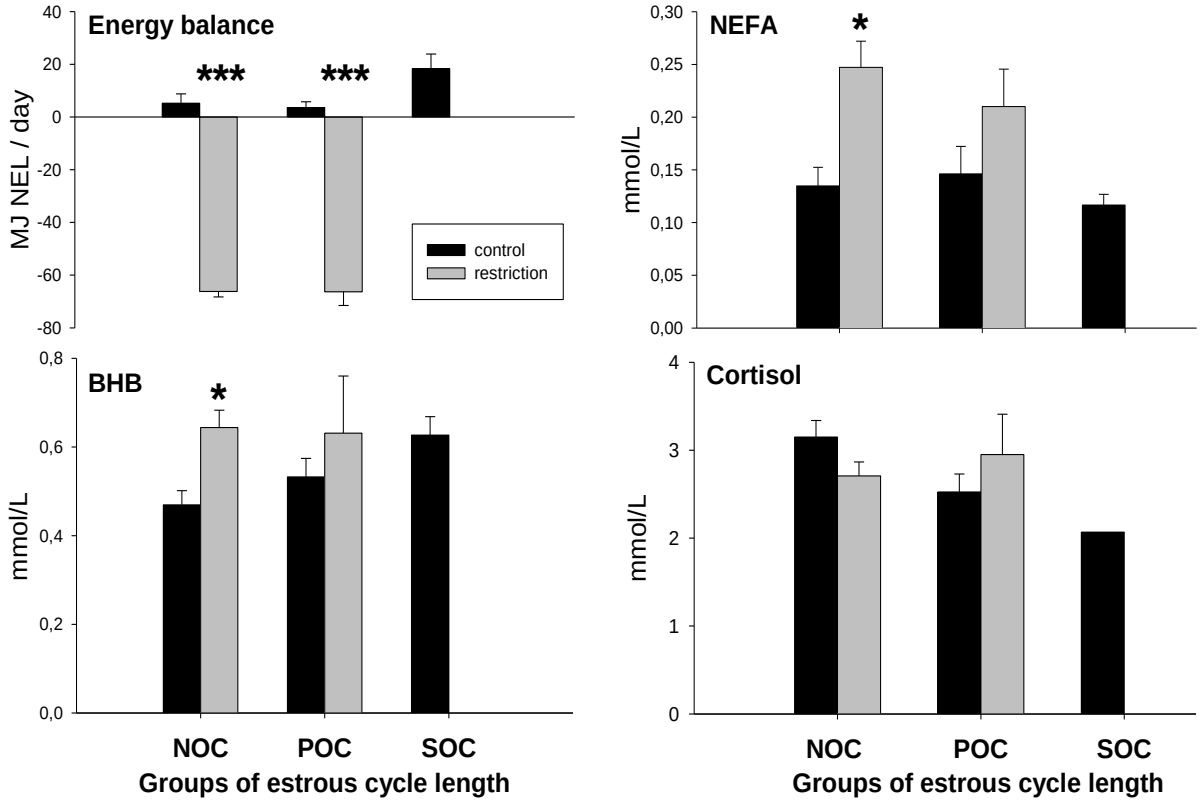

Figure 12: Metabolic parameters of estrous cycle length groups during induced energy deficiency. Significant level was $P \leq 0.05$.

Peak NEFA concentration were found in energy restricted cows with normal cycle with 0.25mmol/L. BHB concentrations were also highest in restriction group cows of NOC and POC categories with peak levels of 0.7mmol/L, respectively. The significant findings for both parameters in the NOC group indicate that although significant higher NEFA and BHB concentrations were reached in the restriction group compared to the control group, the cycle length was not influenced. The indicator for metabolic stress hydrocortisone determined in

milk showed also no effects of the induced energy deficiency on metabolic imbalance. Therefore no difference on estrous cycle length could be found due to the metabolic status. Induced energy deficiency did not influence reproductive dysfunctions.

Experimental period 3

After the energy restriction, the cows were realimented and received the same diet ad lib as the control feeding group. The cycle length showed higher variation in the restriction group, but was again shorter compared to the control feeding (23 ± 0.8d vs 25 ± 0.8d) (Fig.13A). However, no significant differences were observed. The contribution of cycle lengths during realimentation period (Fig. 13B) led to similar results shown during period 2. Most of the involved cows showed normal cycle lengths. Within this group most animals were energy restricted. In the POC group, however, most cows were control fed, whereas only 6 cows were energy restricted. In total 3 cows had shortened estrous cycles after the experimental energy restriction. Again, two of them were control cows and the remaining single cow belonged to the restriction group.

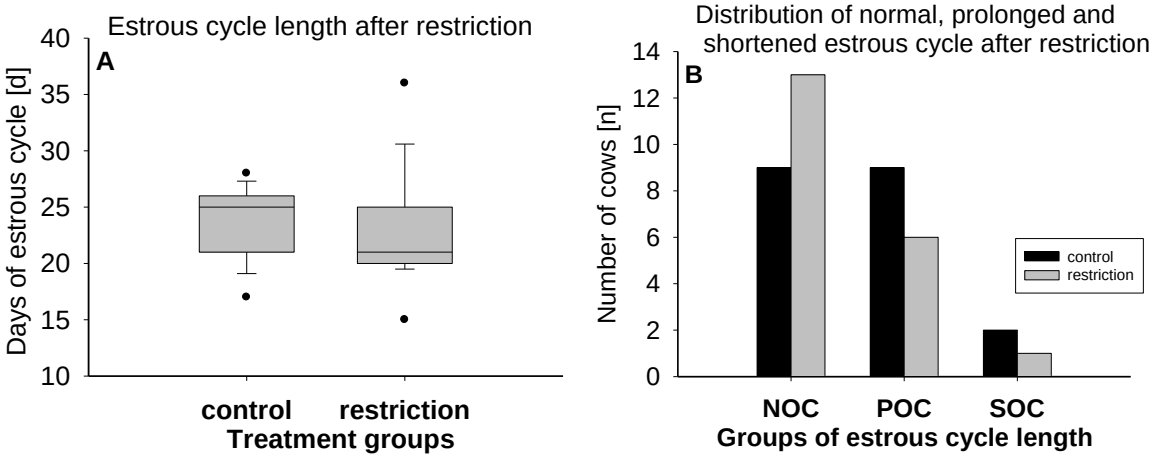

Figure 13: Estrous cycle length in the control feeding and energy restricted cows (A) and number of cows in the determined cycle length group (B) during realimentation in period 3.

According to energy balance, the energy restriction cows in NOC and POC in period 3 were still in an energy deficiency, in contrast to the SOC group (Fig. 14). Here the control group showed a negative energy balance, whereas a positive energy balance was calculated for the restricted cows. Blood NEFA concentrations showed no influence of the realimentation period. The figure presents equally determined concentrations in all groups. The BHB concentration of NOC, POC and SOC did not differ significantly. High SEM was calculated for the SOC group and a sporadic significant difference was found between control and restriction cows showing normal estrous cycle. Again milk hydrocortisone was not altered

according to the three estrous cycle groups. Even the comparison of milk hydrocortisone over period 1, 2 and 3 shows no differences. Thus, milk hydrocortisone concentration is supposed to be affected by lactation stage and is negatively correlated with milk protein [56], the detected concentrations in the present study did not exceed or decline 3mmol/L in all experimental phases.

In conclusion of period 3, no influence was found. The observed estrous cycle alterations are not related to the conducted feeding regime. The elevated energy supply in period 3 in the restriction group has no effect on estrous cycle length after the induced energy restriction.

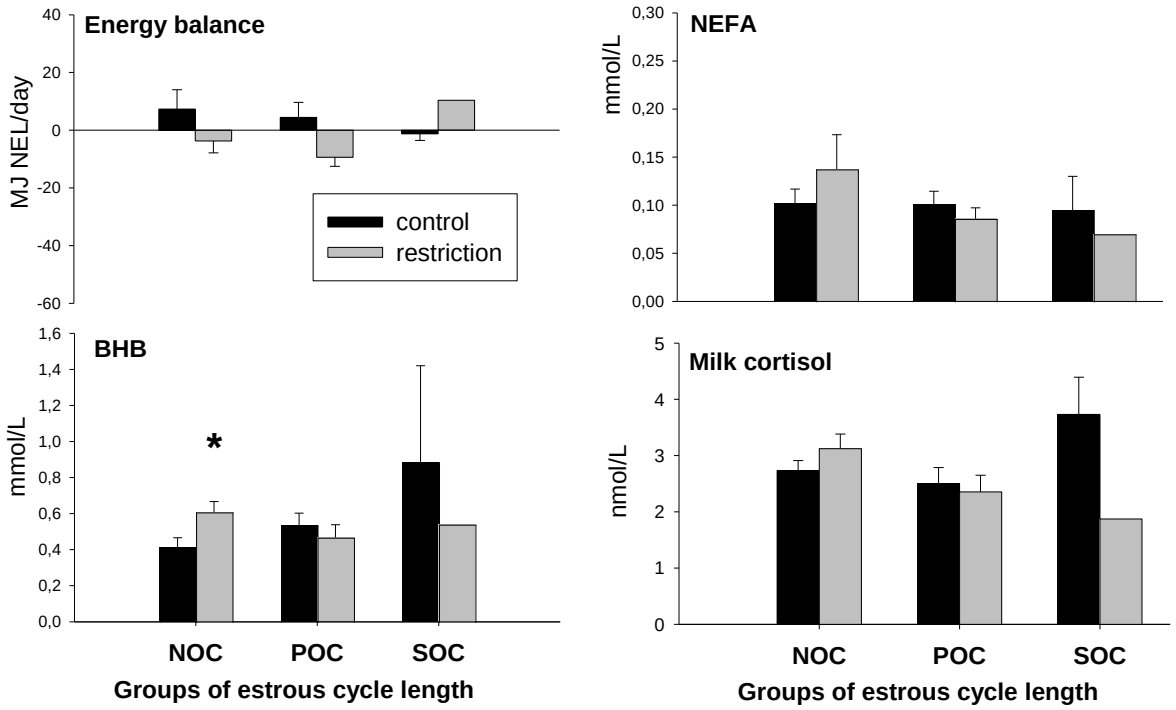

Figure 14: Metabolic parameters during realimentation. Significance level was $P \leq 0.05$.

Insemination

Beginning on day 150pp after period 3, cows were inseminated. The first four inseminations until conception of each animal were documented (Fig. 15A) and the conception rate was calculated (Fig. 15B). Energy restricted cows displayed a higher conception rate at first and second service. More control cows than restriction cows conceived at third insemination service and only members of the control feeding group needed a fourth insemination to conceive. The total conception rate within the first four conducted inseminations for the restriction and the control feeding group was identically. However, the results cannot be

related to the conducted feeding experiment. Due to administrative order, the cows had to pass one estrous cycle before insemination was conducted.

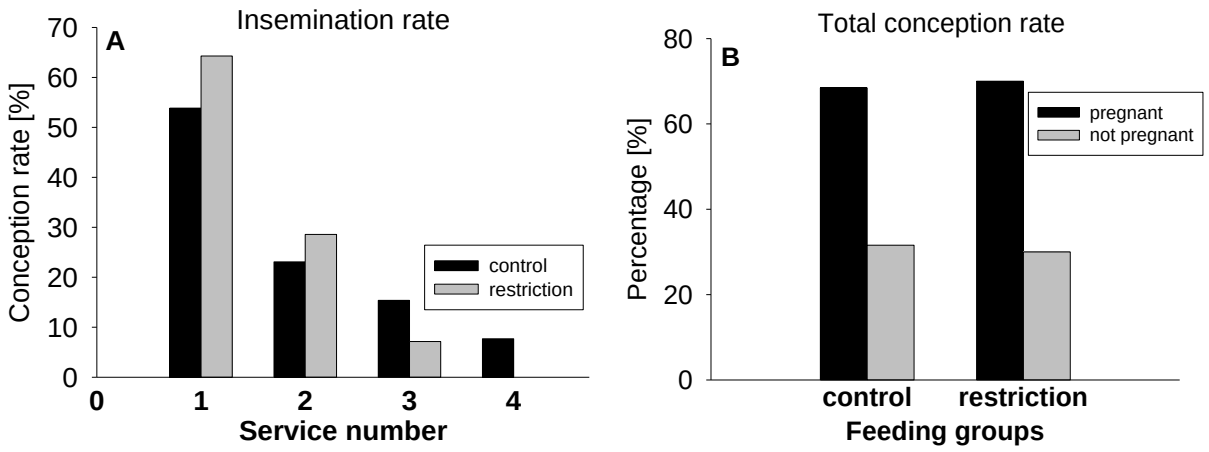

Figure 15: Service until conception rate (%) (A) and total conception rate of the control and energy restricted group (B) within first four documented inseminations.

3.2 Innate immune competence of pbMEC affected by restricted energy supply *in vivo*

Primary bovine mammary epithelial cells

20 control and 20 energy restricted cows were involved in the conducted feeding experiment. From every cow pbMEC were extracted on the last day of the energy restriction period. After one week first small cultures were visible on the culture flask bottom (Fig. 16).

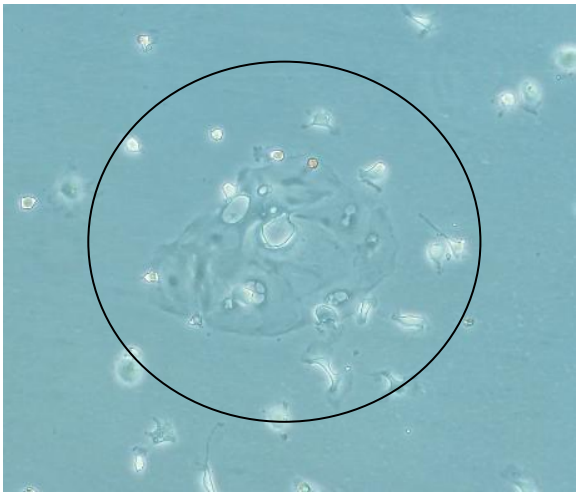

Figure 16: First small pbMEC colonies are visible after one week of cultivation.

In the following days and weeks they connected to bigger cell plates (Fig. 17A) and finally form a confluent monolayer (Fig.17B). In the present study pbMEC were passaged when cells reached 80% confluent state.

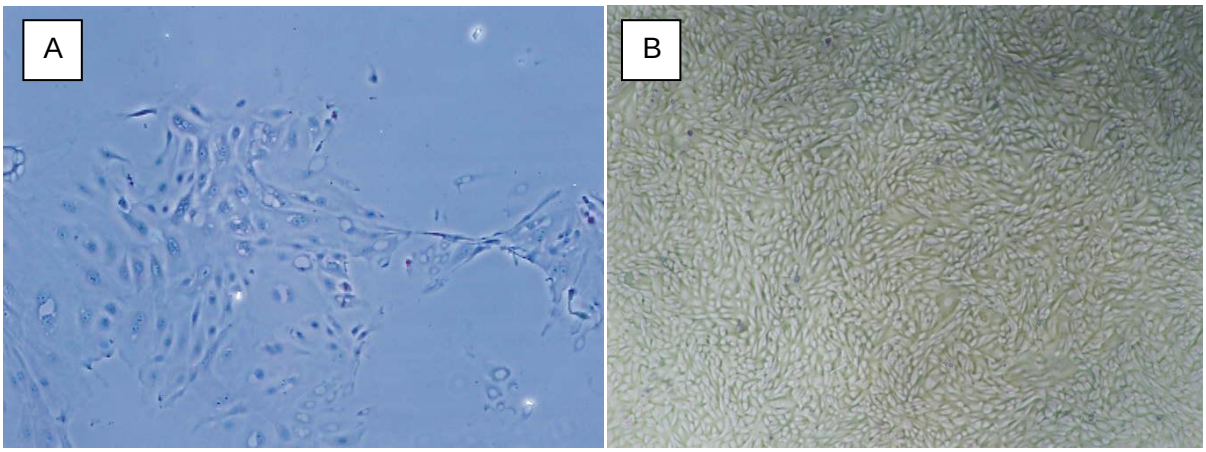

Figure 17: After two weeks several colonies have developed (A) and finally form a connected monolayer when confluent state is reached (B).

Contaminations in primary cell cultures are a serious problem so that many cultures had to be excluded from the experiment. Already during the extraction process contaminations and existing mammary infections were indicated by red blood cells in the cell pellet or by extra huge cell pellets after centrifugation. After re-suspension of the cell pellet and final seeding fat, cell and pathogen contaminations were visible. During the first days of cultivation, the young cultures were rinsed with warm PBS to remove cell contaminations, cell debris or fat globules. Bacterial or fungal contaminations showed up at early cultivation stages and were also treated with PBS washing. Interestingly, cow individual growth performance was found of the respective cell cultures. In fact, every pbMEC culture behaves individually and shows individual defence ability in case of microbial contamination. Beside infections some cultures showed atypical growth as presented in figure 18.

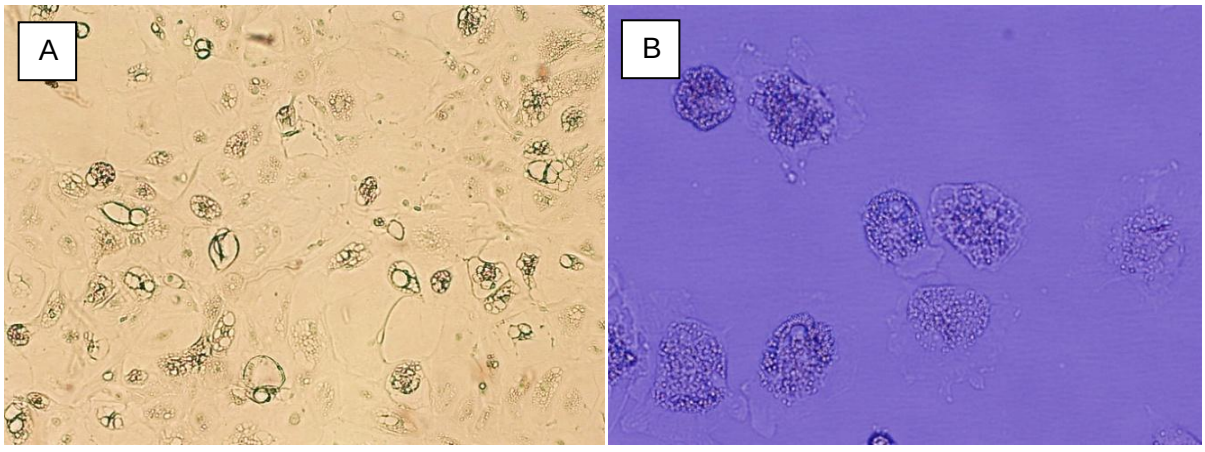

Figure 18: Vacuole forming within the pbMEC appears during unsuitable culture conditions (A). Granulocytes are often co-cultured, but will disappear after washing steps and passaging (B).

These cultures grew slowly and developed pores or vacuoles (Fig. 18A) and were not able to overcome bacterial contamination. Contaminated cell cultures with fungi, bacteria and morphological atypical growing cells were removed (Fig. 18B). Therefore, we were able to cultivate 8 pbMEC cultures from energy restricted and 7 cultures from control feeding cows.

Immunohistochemisty

Primary bovine mammary epithelial cell cultures of 8 restriction and 7 control feeding group cows were successfully generated. Their identity as epithelial cells was confirmed by immunohistological staining of cytokeratins. The determination and calculation of brown stained cells revealed a purity of 97% epithelial cells. The results are presented in figure 19.

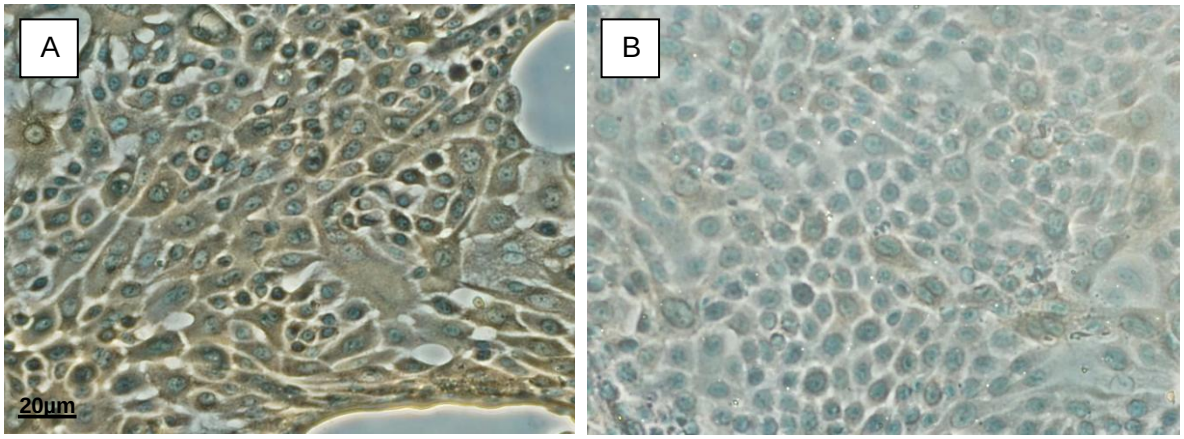

Figure 19: Positive cytokeratin staining in pbMEC (A) and negative control (B).

RNA integrity

The integrity of the extracted pbMEC RNA was determined using the Agilent Bioanalyzer 2100 and RNA6000 Nano Assays and is presented as RNA Integrity Numbers (RIN). The mean RIN value in the presented experiment was 7.9 ± 0.2 SEM.

RT-qPCR

Receptors and Antimicrobial Peptides (RAMP)

As first statistical tool, a PCA was conducted to visualize the gene expression results of the stimulated pbMEC. Figure 20 presents the PCA of the first gene group, the pattern

recognition receptors and antimicrobial peptides. One clear treatment effect in this descriptive method is the separation of the green sports representing the stimulation with *E.coli*, whereas the yellow *S.aureus* infected and the red untreated control cells form a tight cluster. No further separation between control feeding (squares) and energy restriction feeding (triangles) group is visible. Moreover, the more space is occupied by a sample cloud the higher is the variation within the data set. Thus, there is evidence to suggest that high SEM will be calculated for the *E.coli* groups.

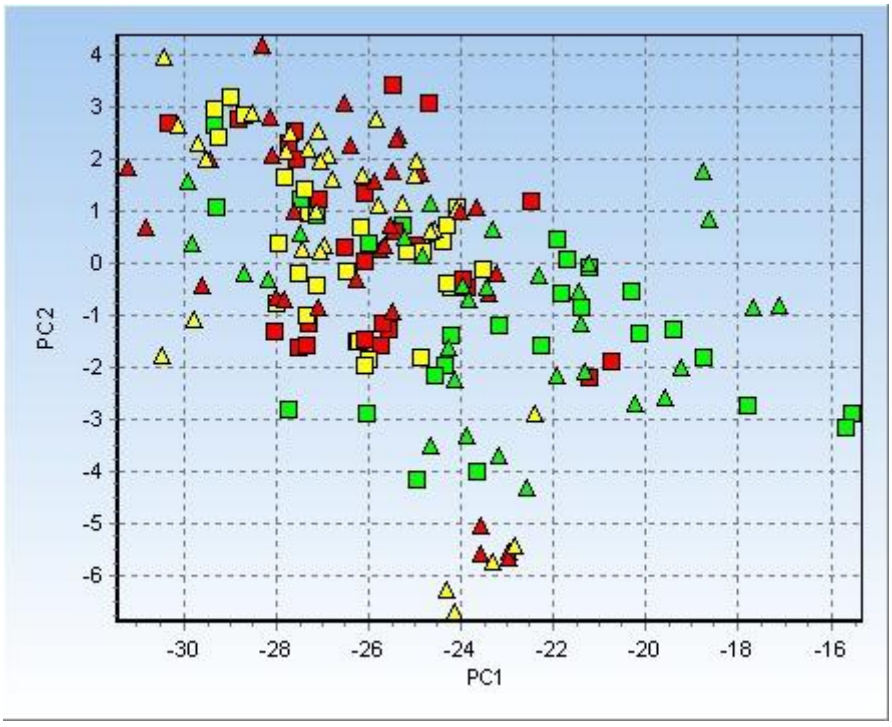

Figure 20: Principal component analysis (PCA) of host defence genes: **receptors and antimicrobial peptides**, ■ =control, ▲ = energy restriction, green = *E.coli*, yellow = *S.aureus*, red = untreated control.

To confirm PCA results, a comparison of means was conducted. The bar chart for RAMP is presented in figure 21. Highest treatment effects were found due to *E.coli* exposition, without incubation time effects. As first instance of defence the TLR2 reacts upon the simulated invasion at once, whereas TLR4 is not activated. After pathogen recognition by TLR2, defence factors DEFβ1 and LPO are activated after 24h and further increase after 72h to react upon the simulated *E.coli* invasion. Most impressive regulations were found in those cells that were exposed to the provoked energy deficiency *in vivo* compared to the control feeding group. DEFβ1 and LPO displayed 24 ± 14.2 SEM -fold and 46 ± 22.4 SEM -fold up-regulations in the restriction group after *E.coli* 72h compared to 13 ± 6.06 SEM -fold and 2 ± 0.71 SEM -fold up-regulations in the control group, respectively. But again high calculated

SEM prevented significant differences as already indicated by the PCA. However, those cells that experienced the induced energy deficiency *in vivo* showed a significant down-regulation ($P \leq 0.005$) for TLR2, due to *S.aureus* exposure after 72h, accompanied by a down-regulation of DEFb1 by trend ($P = 0.1$). The presence of *S.aureus* is recognised initially 72h post infection by TLR2. This behaviour promotes the induction of a chronic subclinical infection as described in other studies [14,15], since the defence mechanisms neglect to inhibit the pathogen invasion in an early stage of infection. This finding supports the impaired immune situation under an energy deficiency, which seems to be initiated at an early stage of the immune cascade. It seems that the energetic imbalance assaults the immune ability in its weakest point indicated by the repressed defence genes and supports the manifestation of *S.aureus* induced mastitis. Of course, this is only a small finding in the huge area of innate defence. Nevertheless, the importance of activation of TLR2 and its further effect on pro-inflammatory factors, to initiate local and systemic immune responses, is discussed and described in other studies [57,58].

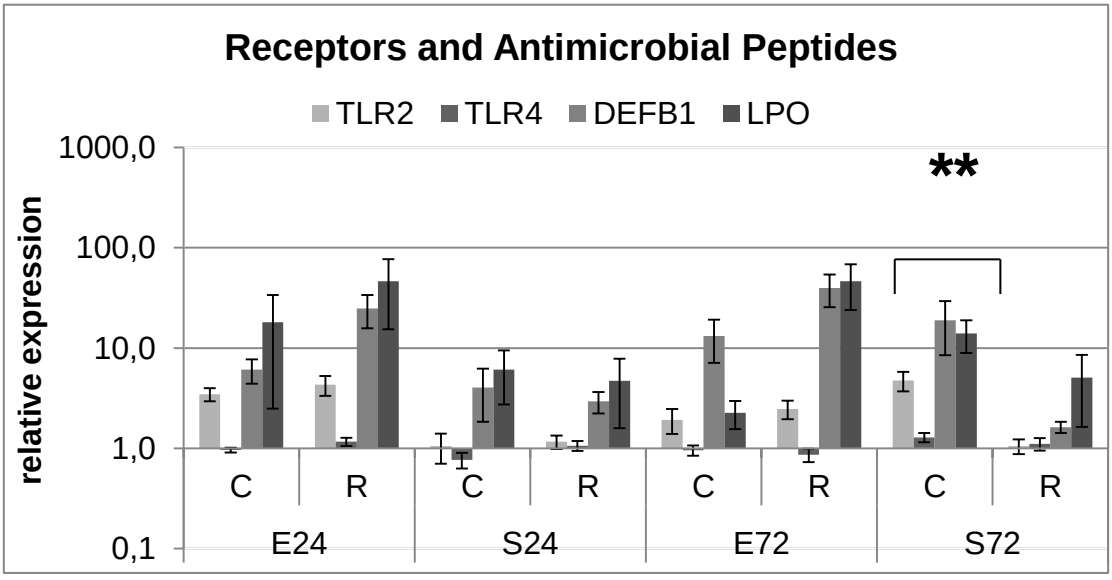

Figure 21: Comparison of gene expression of receptors and antimicrobial peptides between control feeding and energy restricted feeding group and the effect of exposition time to mastitis pathogens. Data are presented as mean $2^{-\Delta\Delta Cq}$ ± SEM on log 10 scale. The bracket with ** indicate significant difference between control and restriction group of the same infection group (P≤ 0.01); E = *E.coli*; S = *S.aureus*; 24, 72 = incubation time with pathogen in hours; C = control feeding, R = energy restriction feeding group.

Cytokines

The cytokine gene set seems more influenced by the *E.coli* treatment compared to *S.aureus* and the untreated controls as seen before. However, focusing square and triangles a grouping can be presumed within the red-yellow cluster (Fig. 22).

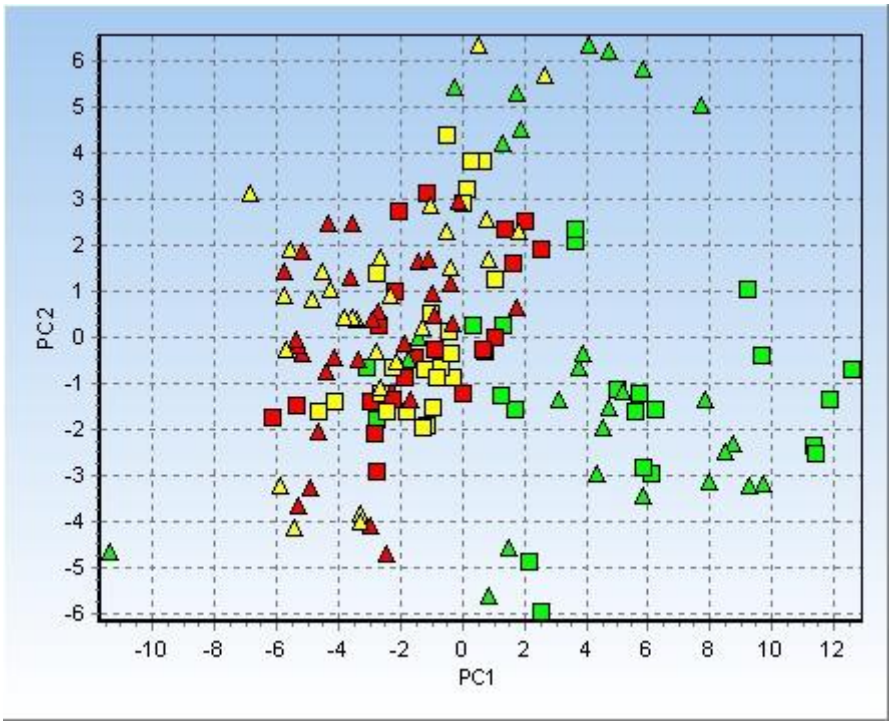

Figure 22: Principal component analysis (PCA) of host defence genes: **cytokines**, ■ =control, ▲ = energy restriction, green = *E.coli*, yellow = *S.aureus*, red = untreated control.

The comparison of $2^{-\Delta\Delta Cq}$ mean values is presented in figure 23. Here the dominant effect of *E.coli* infection compared to *S.aureus*, is even more visible than in the RAMP gene expression. The remarkable 411-fold up-regulation of IL1β and 147-fold of TNFα indicate a massive inflammatory reaction right after 24h in the presence of the gram-negative *E.coli* as reported in Günther et al. [59]. Especially IL1β displays higher expression levels, which can be explained by its promoting effects on TNFα and IL6 [15]. Furthermore, in energy deprived cells the expression of IL1β and TNFα is even more increased (IL1β $P \leq 0.05$) after 24h than in the control feeding group. After 72h, pathogen exposition shows higher expression levels in the restriction group compared to the control feeding cells with a significant difference in TNFα ($P \leq 0.05$), whereas IL1β has already reached peak levels in both feeding groups. The pronounced immune response is confirmed by Buitenhuis et al. [60]. They found that after *E.coli* infection, most genes involved in the immune response were up-regulated and further

affected genes were involved in pathways related to pro-inflammatory action. In contrast, minor effects of *S.aureus* on cytokine expression were found. After 24h mild signs of inflammation are determined [59]. Nevertheless, IL6 is significant up-regulated in the energy restricted group ($P \leq 0.05$). However, by far more interesting is the reaction of the pbMEC after 72h exposure to *S.aureus* compared to 24h. The same effect as described above for the expression of TLR2 and DEFβ1 is visible: the inflammatory response against the detected *S.aureus* seems to be impaired by the restricted energy supply *in vivo*. TNFα was significantly down-regulated in the restriction group. As indicated in the PCA, high SEM was determined within the groups and prevented further significant results.

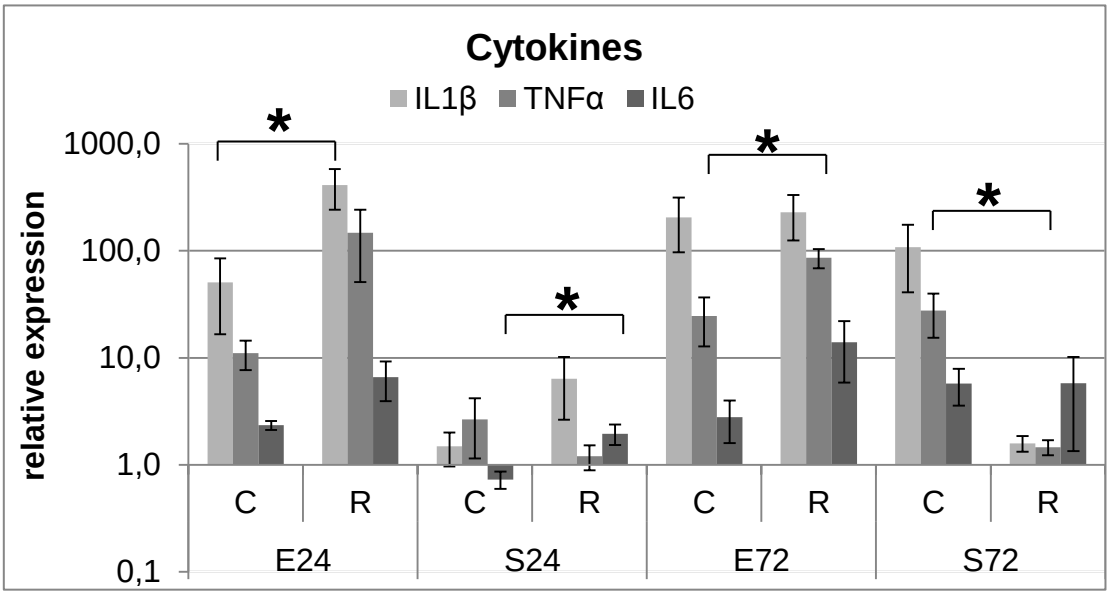

Figure 23: Cytokine expression profiles of pbMEC related to pathogen and incubation time between control feeding and energy restriction feeding regime *in vivo.* Results are presented as mean $2^{-\Delta\Delta Cq}$ ± SEM on log 10 scale. The Brackets with * indicate significant differences between control and restriction group within the same infection scheme ($P \leq 0.05$); E = *E.coli*; S = *S.aureus*; 24, 72 = incubation time with pathogen in hours; C = control feeding, R = energy restriction feeding group.

Chemokines

The dominant effect of the stimulation with heat inactivated *E.coli* on the modulation of chemokine expression is displayed in the PCA in figure 24 which presents a wide arrangement of the spots.

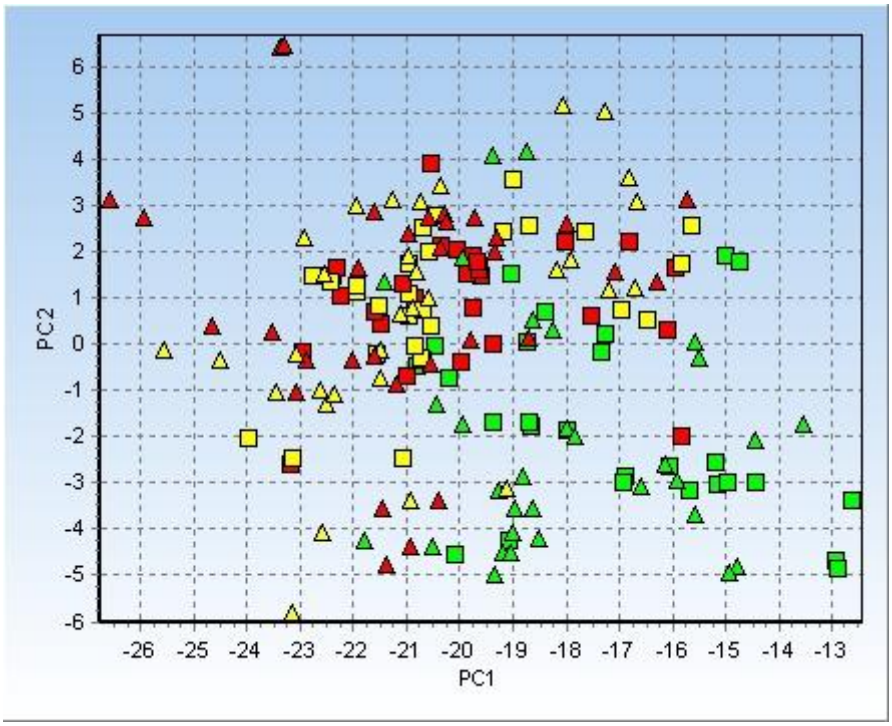

Figure 24: Principal component analysis (PCA) of host defence genes: **chemokines**, ■ =control, ▲ = energy restriction, green = *E.coli*, yellow = *S.aureus*, red = untreated control.

The determined chemoattractans (Fig. 25) are also most pronounced up-regulated in the presence of *E.coli*. IL8 exerts long-lasting potent chemotactic signals, preferentially recruiting neutrophils and enhancing their function. Its expression is induced by pro-inflammatory stimuli and is increased during *E.coli* mastitis [15]. Therefore the present high expression levels of IL8 can be explained by the expression profiles of IL1β and TNFα. In the chemokine group IL8 and CXCL5 are both most affected genes. The *E.coli* induced stimulus is more pronounced by energy supply *in vivo* than by increasing incubation time. Higher expression levels by trend were found in the energy restricted cells compared to control feeding cells. The known effect of 72h *S.aureus* challenge in energy deprived cells is also visible here: the responsiveness of immune factors, here the chemoattractans IL8 and CXCL5, in energy restricted cells after 72h *S.aureus* infection seems to be impaired. Visible, but not significant down-regulations, due to high SEM, were found in restriction compared to control feeding cells. Further significant regulations were found for the up-regulation of CXCL5 ($P \leq 0.05$) in *S.aureus* infected cells after 24h in the restriction feeding group, an up-regulation of Groα (*P*

≤ 0.05) in *E.coli* after 72h also in the energy restriction group and a further up-regulation of Groα ($P \leq 0.05$) in *S.aureus* infected restriction cells after 72h. The increased Groα levels can be related to neutrophil-mobilizing chemokine signaling upon bacterial stimulation [61] as described for IL8. However, an earlier increase of Groα gene expression is found in literature within the first 3h of infection. In the present study earliest sampling time was 24h. Thus, we missed the time point of highest activity, which may explain the low determined expression levels.

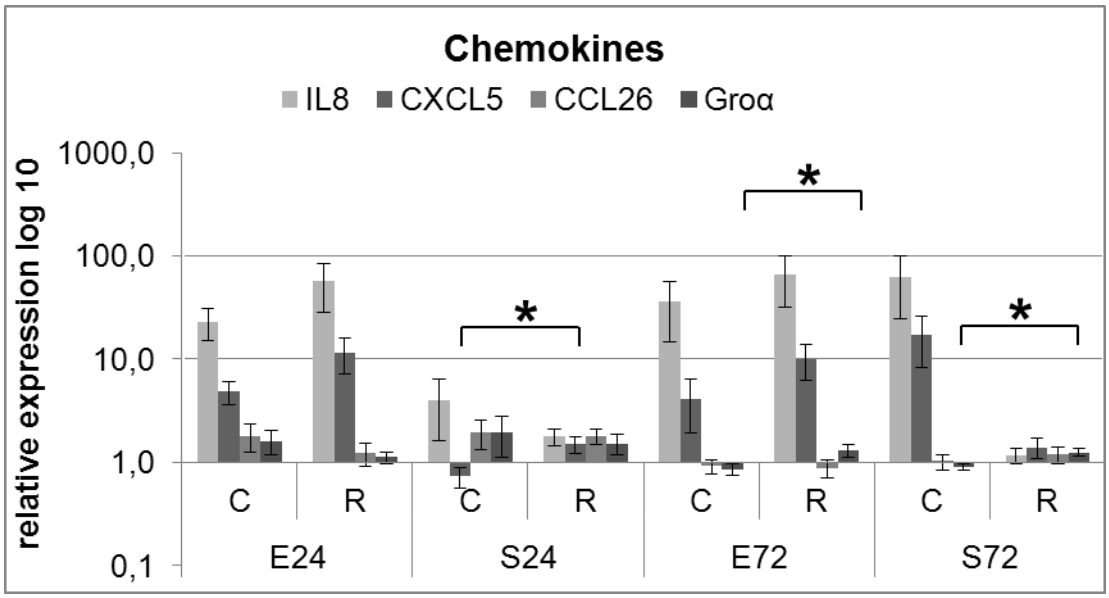

Figure 25: Chemokine gene expression profile according to infection and energy supply *in vivo*. Data are presented as $2^{-\Delta\Delta Cq}$ ± SEM on log 10 scale. Brackets with * indicate significant differences between control and restriction group within the same infection scheme ($P \leq 0.05$); E = *E.coli*; S = *S.aureus*; 24, 72 = incubation time with pathogen in hours; C = control feeding, R = energy restriction feeding group.

Apoptosis related genes

In contrast to the above presented PCAs of receptors and antimicrobial peptides, cytokines and chemokines no separating effect of the applied pathogen type is found in the PCA of apoptosis related genes (Fig. 26). The stimulation with heat-inactivated *E.coli* and *S.aureus* does not affect the regulation of apoptosis related genes displayed by green or yellow color. No signs of induced apoptosis were found in the PCA, even though the stimuli were strong enough to induce high regulation changes in the immune response related gene classes, presented above. Although more triangles of the energy restriction feeding group, can be presumed in the middle area of the graph, no clear separation due to feeding regime can be found by this descriptive statistical method.

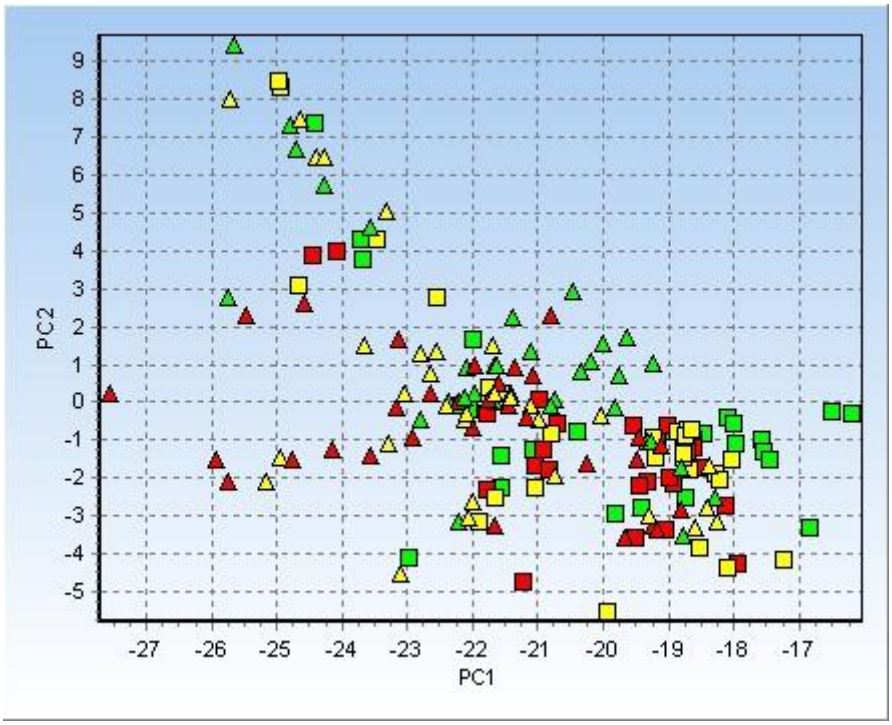

Figure 26: Principal component analysis (PCA) of host defence genes: **apoptosis related genes**, ■ =control, ▲ = energy restriction, green = *E.coli*, yellow = *S.aureus*, red = untreated control.

The PCA results can be confirmed in the presented bar chart (Fig. 27). Minor effects of the conducted energy restriction *in vivo* and the immune challenge on gene expression of the four presented genes were found. Focusing the effect of pathogen type on apoptosis, neither of the two heat inactivated pathogens is supposed to induce cell death directly. In the gram-negative *E.coli,* most potent inducers of apoptosis and tissue damage are the released endotoxins. Therefore applied LPS is discussed to not induce tissue damage to mammary epithelial cells [16]. The authors further suppose that *S.aureus* may induce apoptosis *in vivo* directly, which still must be proven. Under *in vitro* situation, cell death can have different reasons and may be induced indirectly by the applied pathogen or particle due to mediation of a substantial cytokine reaction. Beside the pro-inflammatory abilities of IL1β and TNFα, dysregulations of their expression can have deleterious effects to the host [15]. Although high cytokine expression levels were reached (411-fold and 147-fold, respectively) due to the stimulation with heat-inactivated pathogens, no reproducible effect in apoptosis related genes was found. High cytokine release may have minor effects in cell culture compared to the effects on tissue and the complete organism with regard to fever reaction and even shock.

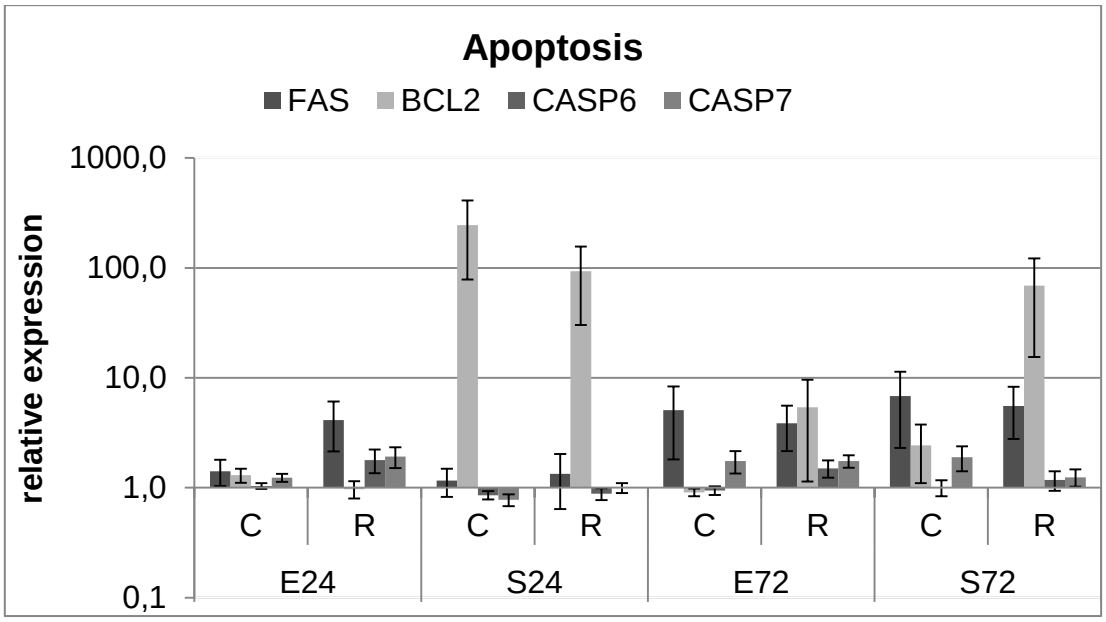

Figure 27: Regulation profile of apoptosis related genes affected by immune (pathogen and inoculation time) and metabolic (control vs. energy restriction feeding *in vivo*) challenge. Data presented as mean $2^{-\Delta\Delta Cq}$ ± SEM on log 10 scale. E = *E.coli*; S = *S.aureus*; 24, 72 = incubation time with pathogen in hours; C = control feeding, R = energy restriction feeding group.

3.3 Modulation of lactoferrin in milk and the *de novo* synthesis in pbMEC

Lactoferrin in milk

An enzyme-linked immunosorbent assay was developed to analyze LF in milk samples of control feeding and energy restricted cows. The measured LF concentrations were similar to other findings [62,63] and ranged between 27µg/mL and 418µg/mL. Inter- and intraassay variations for milk were 11.06% and 9.36%, respectively.

The LF concentrations in milk change during lactation. In general, colostrum contains higher concentration of LF compared to milk. Furthermore, bovine milk LF is correlated with the lactation stage [27] and negative correlated with milk yield [62]. During the progress of lactation and the decreasing milk yield, the concentrations of LF increase and reach peak values during dry period and mammary gland involution. According to its further correlation to the somatic cell count (SCC) and therefore the immune status, LF can be used as promising mastitis indicator [64]. These findings are confirmed by the present LF analysis (Fig. 28). Lowest LF concentrations are found at the beginning of the experiment and increase continuously until the end of period 1 in week 16pp. Within the following three weeks a dietary energy deficiency was induced in the restriction group compared to control fed cows (period 2). In the first week of period 2, the energy restricted cows display a 41% reduction of LF. Furthermore, significant lower LF concentrations ($P \leq 0.005$) were found compared to

control feeding cows. In the second restriction week the difference compared to control cows remained, but significant level was not reached any more (P = 0.07).

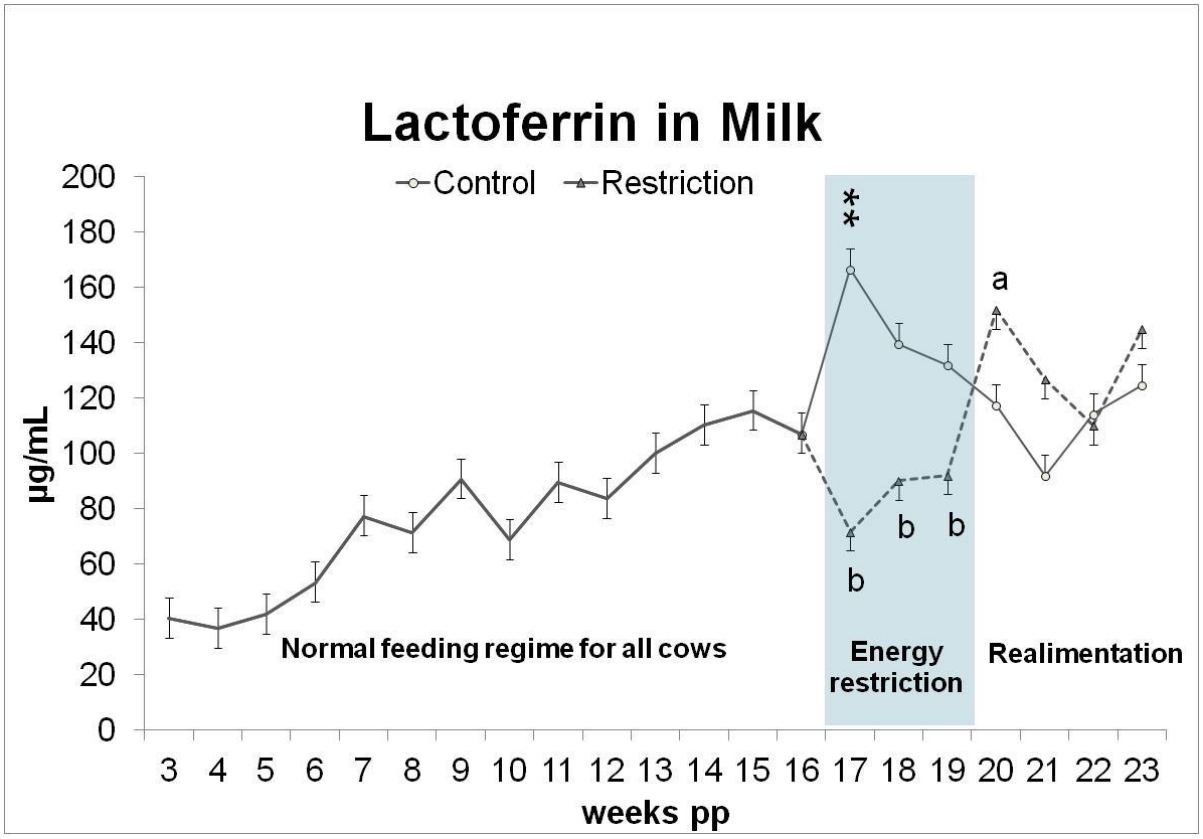

Figure 28: Lactoferrin concentration in twice weekly taken milk samples from control and energy restricted cows in µg/mL. Milk samples were taken from week 3pp until week 23pp. Different superscript letters indicate significant differences ($P \leq 0.05$) within the restriction feeding group. ** indicate significant differences with $P \leq 0.005$ in the control vs restriction feeding group.

In the last week of energy restriction the difference between control (132.0µg/mL) and restriction group (92.26µg/mL) was also beyond significance (P = 0.14). However, the conducted energy deficiency was more pronounced compared to the common energy deficit in early lactation. Therefore an impaired immune status was assumed. Some may suppose that the reduced LF concentration may be due to reduced milk yield solely. But in fact, during the three weeks of energy restriction of 49% of total requirements the mean milk yield reduction in the experimental cows was only three liters per day [39]. Therefore it is questionable, if the milk yield reduction of only three liters is responsible for the 41% reduction of milk LF in the energy restriction group.

As a metabolic imbalance is correlated with impaired immunity [65], the reduced LF concentrations may display the impaired effect of the induced energy deficit on the host

defense of the mammary gland, hence the LF reduction could be seen as indicator for the affected immune status as discussed previously [36,66]. After the induced energy deficiency, restricted cows were realimented and received the same diet ad lib as the control cows. Right after energy and feed adjustment, LF concentrations increase significantly ($P \leq 0.05$) compared to the three weeks of restriction and reach over the levels of control feeding cows. In the work of Gross et al. [39], milk yield increases in the second week of the realimentation period and is henceforward increased by trend compared to the yield of control feeding cows until the end of the experiment. The same tendency can be found in the course of protein yield.

Lactoferrin gene expression and protein level in pbMEC

The results of LF gene expression, synthesis and secretion due to energy supply *in vivo* and the immune challenge *in vitro* of pbMEC are presented in detail in figure 29. The graph shows highest regulations on $2^{-\Delta Cq}$ level due to stimulation with *E.coli* after 24h and 72h. The effect of energy restriction on LF gene expression is presented in the small graph. Here ΔCq values were further normalized against the respective control feeding group to display the effect of energy supply on LF expression level even in the untreated control cells. Values were further transposed into $2^{-\Delta\Delta Cq}$ to present x-fold regulations. Although a visible tendency for higher expression in energy restriction cells compared to control feeding cells is present in all groups, the only significant difference can be found in untreated control cells after 24h ($P \leq 0.05$). On x-fold regulation levels, presented in the insert graph, higher mean up-regulations are found in restriction compared to the control feeding group cells accompanied by extreme high SEM, which prevent significant differences. The inserted graph indicates an effect of the feeding treatment, but this is negligible due to unfavourable high SEM. However, an interesting effect can be found in the insert graph. In contrast to the *E.coli* challenged cells, which led to an up-regulation of LF at both time points, LF was down-regulated after 24h exposure to *S.aureus*. After 72h gene regulation was nearly suspended and no treatment effect was found. These findings are confirmed in several others studies [67,68]. Signs of mild inflammation are detected within first hours after invasion, but only on a low magnitude [59]. The pathogen's character is to persist undetected and often lifelong, while inducing a chronic-subclinical disease pattern. The strategies are to control and, most suitably, silence host defence mechanisms. Hence, the mechanisms of precluding the host defence in order to manifest chronic diseases are still not understood.

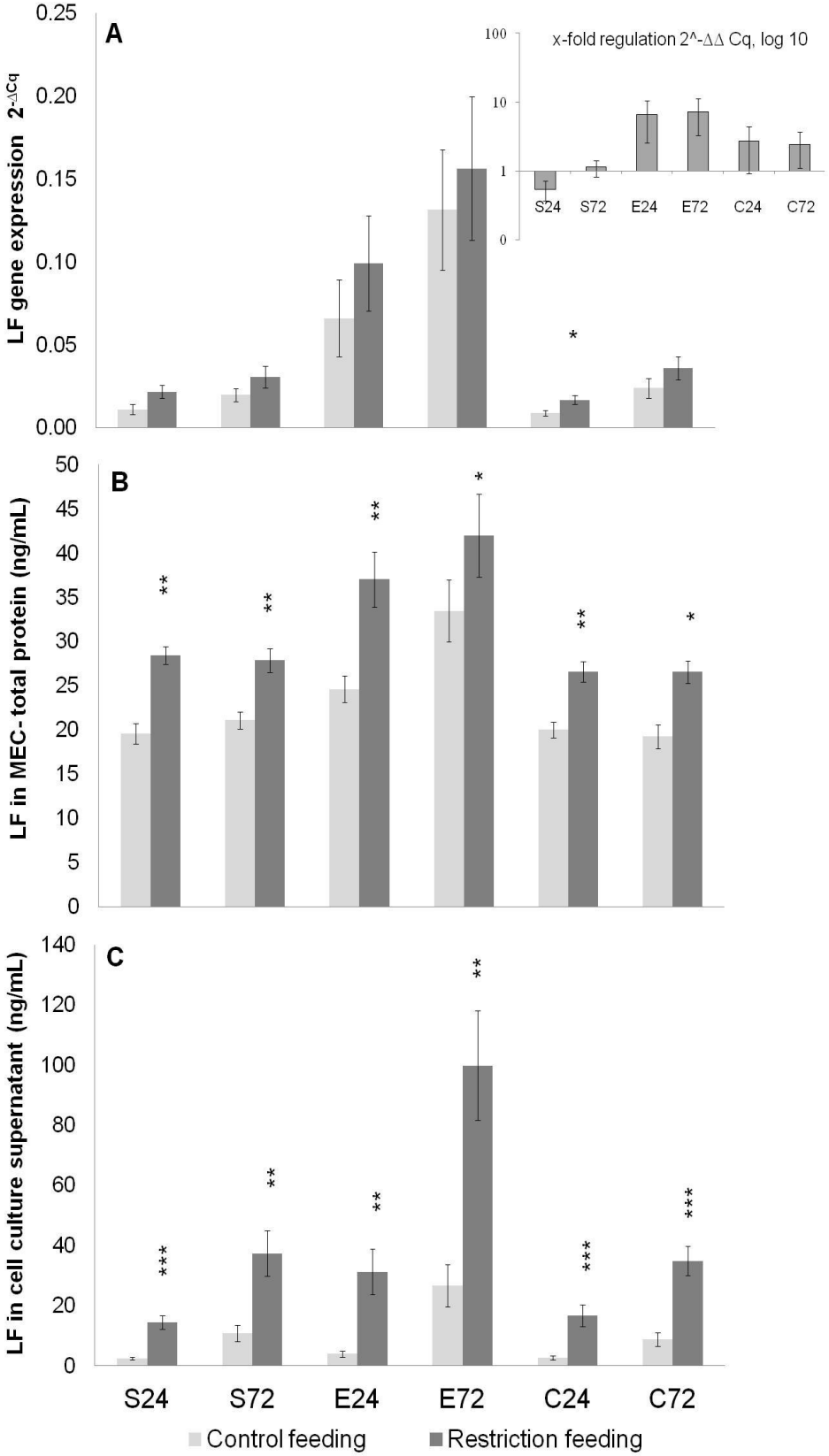

Figure 29: Lactoferrin gene expression ($2^{-\Delta Cq}$) (A), cellular lactoferrin concentration in pbMEC-total protein lysate (ng/mL) (B) and LF concentration in cell culture supernatant (ng/mL) (C) of pbMEC during immune challenge with *E.coli* (E), *S.aureus* (S) after 24h (24) and 72h (72) and control (C) cells without stimulation according to an induced energy deficiency and control feeding *in vivo*. The inserted graph presents gene expression data on $2^{-\Delta\Delta Cq}$ level after further normalisation against the respective control feeding group. Significant levels were * ($P \leq 0.05$), ** ($P \leq 0.005$), *** ($P \leq 0.001$) between control and restriction group.

The first indications provided by the gene regulation assay are further confirmed on protein level by ELISA. The first matrix to measure LF concentration was the total-pbMEC protein, which was extracted simultaneously by the employed RNA extraction. The generated data represent the synthesized LF concentration, which was not secreted yet. The actual analyzed cellular protein is presented in figure 29B. The most noticeable difference between gene regulation of LF and its cellular concentration is the lower SEM displaying consistent effects within the treatment groups. Furthermore, *E.coli* challenge still evokes the highest effect upon LF after 72h. The main effect is, however, the different concentration of LF according to the induced energy restriction. Those cells that experienced the induced energy deficit *in vivo* produced significant more LF compared to the control feeding group in all six treatment groups. Throughout these results, highest significant differences ($P \leq 0.001$) were found in the *S.aureus* challenged cells after 24h.

The absolute synthesized and secreted protein was measured in the cell culture supernatant after 24h and 72h of pathogen contact. The detected concentrations are not a status quo report like the gene expression or the cellular LF, but comparable with the concentrations in milk samples. Milk is also an accumulation of LF, secreted by pbMEC and milk leukocytes over the 12h milking interval. Although the synthesis time of the pbMEC in cell culture was longer (24h and 72h), the distribution of effects in milk LF and cell culture supernatant might, however, be comparable, unless the influence of the unknown LF contribution of the mammary immune cells. Figure 29C presents these results. Generally, most LF in the *E.coli*, *S.aureus* and untreated control cells was produced and secreted during 72h, which is a clear time effect. However, the comparison between treatments reveals once again, that *E.coli* seems to be the stronger stimulus compared to *S.aureus* and untreated control (99.9 ± 18.2 µg/mL, 37.3 ± 7.6µg/mL and 34.9 ± 4.9 µg/mL, respectively). Moreover, these are the results in the energy restriction group. The cells of the control feeding group *in vivo* produced significant fewer LF in all 6 treatment groups. The most pronounced effects were measured in *S.aureus* after 24h ($P \leq 0.001$) and interestingly, in the untreated control cells after 24h and 72h (both $P \leq 0.001$). The synthesis difference in *S.aureus* after 24h is conflicting, because it is also found in the cellular LF measurements, but not on gene expression level. The $2^{-\Delta\Delta Cq}$ transposed data display 0.5-fold level expression, which indicates a down-regulation of 50% of the LF gene affected by the induced energy restriction. Inhibitory effects on genes of the innate host defence after stimulation with *S.aureus* for 24h were found and discussed on the previous chapters. But, the actual synthesis rate of LF on both protein levels is, however, completely contrary to the findings on gene expression level in this treatment group. The significant difference of LF in the two untreated control cell samples represents the general higher level of LF produced by the pbMEC without an immune stimulus. As discussed above, these samples might be comparable to situations in milk, of

course without the influence of side contribution of LF secreted by immune cells. The milk course in figure 28 presents the LF concentrations in milk before, during and after the energy restriction. The related situation of the pbMEC affected by the induced energy deficiency *in vivo* is now, however, being cultivated under normal energy and nutrient supply, accomplished by the standard cell culture medium comparable to the cows during realimentation. The milk LF course during realimentation gives only subtle hints in this direction. The course of total milk yield and protein strengthens this speculation by trend, because energy restricted cows produced more milk and more milk protein during the realimentation period until the end of the experiment.

Finally, the trend indicated on gene expression level and the LF determination, actually reveals a phenomenon that is difficult to explain and needs further research. Especially the impact of leukocytes that contribute to the milk LF content as well as other factors such as the acquired immune system are not considered in this study and might shift the total LF production due to effect of energy deficiency into a different direction. Although it is discussed that the innate and the acquired immune system complement each other during pathogen invasion (20), the present results might indicate a modulatory effect of the induced energy restriction on the two immune defence systems: displayed by significantly elevated LF synthesis of the pbMEC compared to minor effects of milk LF concentrations. On the other hand metabolic factors that are supposed to be indicators of energy imbalance, did not display immense effects due to the conducted energy restriction. Therefore, tremendous effects on altered immune functions and disconnection of collaborations cannot be assumed.

3.4 pbMEC culture model

A remarkable finding of this work is the performance of the pbMEC. The establishment of a primary cell culture model to study the immune competence of the mammary gland is often avoided and replaced by experimental procedures that are critical concerning animal welfare. The inoculation of the mammary gland or single quarters is a critical point in an animal trial affecting directly the health and wellbeing of the experimental animals. The target tissue is gained after slaughter or by mammary biopsy [58,68,69,70]. High blood flow in the udder and the additional impact of dirt in the stables, promote the establishment of infections and increase the need for aftercare and the application of antibiotics. Besides the risk of infection by biopsy procedure, contamination of the pbMEC cultures with fibroblasts [57,67,71,72] can be crucial. This fast growing mammary stroma cells are able to manifest in a cell culture, overgrow and displace the initial target cells or adulterate determined results, especially in studies that focus the mammary immune function, which is not a competence of stroma cells

[73]. The potential of milk derived mammary epithelial cells was published in Boutinaud et al. 2002 [74] and others [75,76,77,78]. After centrifugation, pbMEC were separated out of the formed pellet by magnetic beads bound to cytokeratin antibodies and total RNA was extracted. However, they did not culture the cells. The employed cell cultures in the present study were also extracted out of milk and further cultured to increase the cell amount for further experiments. Expressed numerically, a small proportion of 2% of the SCC of milk are epithelial cells [74] that were extracted and successfully cultured. Furthermore, the cells displayed an impressive memory ability concerning the metabolic challenge *in vivo*. These results show, that cells from energy restricted cows react different upon an immune stimulus compared to the control feeding group. They produced significant higher levels of LF on gene expression and particularly on protein level and substantiate their suitability as an *in vitro* model for mastitis research.

4 Conclusions and Perspective

A dietary energy deficiency was induced in mid-lactation in 40 Red Holstein cows to evoke a metabolic situation comparable to the postpartum energy imbalance. The effects of energetic undersupply on the estrous cycle activity and on factors of the innate immune system of the mammary gland *in vitro* were investigated in the present thesis.

Although the induced energy deficit was more severe compared to the postpartum imbalance, the induced dietary energy deficit of 49% of total energy requirements did not affect the estrous cycle activity. The cows showed normal estrous cycle activity during and after the restricted feeding period and did not differ from control feeding cows neither on metabolic stability nor on estrous cycle length or reproductive health. To elucidate the role of energy deficiency on ovarian cycle activity, further research should be done with selected high yielding cows, which are affected or unaffected by the metabolic instability. The experimental cows in the present study displayed an annual milk yield of 8,400 liters. The milk production might not be high enough to evoke metabolic dysfunctions.

A cell culture model was generated of milk extracted mammary epithelial cells on the last day of energy restriction. The conducted stimulation with the most prevalent heat-inactivated mastitis pathogens *E.coli* and *S.aureus* revealed tremendous gene up-regulations of *E.coli* exposed cells, whereas *S.aureus* infected samples displayed only moderate gene expression profiles and even down-regulations of the focused gene classes. Significant differences between control and energy restricted cells were visible. The energy deficit intensified the up-regulation due to *E.coli* and the repressive impact on the defense system due to *S.aureus* exposure.

Furthermore, the focus was led on a prominent and potent member of the innate host defense system, lactoferrin (LF). In weekly milk samples throughout the animal trial, the course of lactoferrin was analyzed by a developed LF ELISA. Additionally, lactoferrin was determined in three sample matrixes of the cell culture experiment, the medium supernatant, total pbMEC protein and on gene expression level according to the metabolic challenge *in vivo* and the conducted immune challenge *in vitro*. While high inter-cow variations indicated only tendencies for an effect of energy supply *in vivo* on LF gene expression *in vitro,* significant differences were found on protein level. In all treatment groups of the cellular LF fraction significant differences between control and restriction group were found. In cell culture supernatant the differences were even more distinct.

The pbMEC cultures displayed an impressive memory ability of the conducted metabolic challenge *in vivo* in the present thesis. The effects of the conducted feeding experiment on

the mammary epithelium were transferred to *in vitro* conditions and were determined in a following experimental approach. It seems to be a promising cell culture model to reduce the number of animal used in *in vivo* mastitis experiments, but a synergetic research approach is needed to confirm the suitability of milk extracted primary mammary cell culture models as replacement for *in vivo* mastitis experiments.

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transcription-polymerase chain reaction. Journal of Pharmacology and Experimental Therapeutics 303: 487-496.

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7 Scientific Communications

Original research papers

Innate defense capability of challenged primary bovine mammary epithelial cells after an induced negative energy balance *in vivo*. K. Danowski, D. Sorg, J.J. Gross, H.H.D. Meyer, H. Kliem. Czech Journal of Animal Sciences. Accepted. December 2011.

Effects of induced energy deficiency on Lactoferrin concentration in milk and the lactoferrin reaction of derived primary bovine mammary epithelial cells in vitro. K. Danowski, J.J. Gross, H.H.D. Meyer, H. Kliem. Journal of Animal Physiology and Animal Nutrition. Accepted. February 2012.

Dairy cow reproduction: Does a natural or induced energy deficiency influence oestrous cycle activity? K. Danowski, J.J. Gross, K. Gellrich, T. Petri, H.A. Van Dorland, R.M. Bruckmaier, H.D. Reichenbach, R. Zimmer, H.H.D Meyer, F.J. Schwarz and H. Kliem. International Journal of Livestock Production. Under Review.

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8 Curriculum vitae

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9 Appendix

Appendix I

Innate defense capability of challenged primary bovine mammary epithelial cells after an induced negative energy balance *in vivo*.

Appendix ll

Effects of induced energy deficiency on Lactoferrin concentration in milk and the lactoferrin reaction of derived primary bovine mammary epithelial cells *in vitro*.

Appendix lll

Dairy cow reproduction: Does a natural or induced energy deficiency influence oestrous cycle activity?

Innate defense capability of challenged primary bovine mammary epithelial cells after an induced negative energy balance *in vivo*

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Abstract

Negative energy balance (NEB), if followed by metabolic imbalance, is a common problem in high yielding dairy cows and highly associated with inflammation of the mammary gland. After entering the teat canal the mammary epithelium is the first line of defense against a pathogen invasion. To investigate the effect of NEB on the innate host defense of the mammary epithelium primary bovine mammary epithelial cell (pbMEC) cultures were generated by cell extraction of milk derived from energy restricted and control feeding cows. pbMEC were obtained from 8 high yielding dairy cows affected by an induced NEB in mid-lactation due to a reduction until 51 ± 2% of total energy requirement (restriction group) and from 7 control cows (control group). They were exposed to heat inactivated *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (*S.aureus*) for 24h and 72h to investigate the influence of NEB on gene expression profiles of cytokines, chemokines, genes associated with apoptosis and antimicrobial peptides plus their receptors (AMPR) of the innate immune response. The immune challenge of pbMEC demonstrated an effect of immune capacity and NEB in 15 differential expressed genes. NEB induced a substantial up-regulation in restriction compared to control cells by trend in *E.coli* and a down-regulation in *S.aureus* exposed cells. Our investigations showed that the dietary induced NEB *in vivo* influenced the immune response of pbMEC *in vitro* and altered the expression of immunological relevant genes due to a difference in energy supply. These results demonstrate that pbMEC are a suitable model for mastitis research, in which even effects of feeding regimes can be displayed.

Introduction

Mastitis is the most cost intensive production disease in dairy industry. Medical treatment, reduced fertility, extra labour and reduced milk yield cause considerable financial burden.

Calculations of annual losses due to mastitis revealed an amount of 10% of total value of farm milk sales, and two third are due to reduced milk yield caused by subclinical udder inflammation (Schroeder, 2010). During early lactation high energy requirements for milk production cannot be adjusted by increasing feed-intake and result in a negative energy balance (NEB) often followed by metabolic imbalance. Energy deficit leads to extensive mobilization of body fat reserves and may result in increased blood none-esterifies fatty acid (NEFA) and β-hydroxy butyrate (BHB) concentrations. Elevated NEFA and BHB levels are considered to have inhibiting effects on immune cells (Suriyasathaporn et al., 2000) and to assist the state of impaired immune system (Loor et al., 2007; Roche et al., 2009). Inflammation of the mammary gland is induced by gram-negative and gram-positive pathogens that cause different appearances of mastitis. The most prevalent gram-negative bacteria *Escherichia coli* (*E. coli*) is a typical environment associated pathogen and leads to an acute and severe systemic mastitis. In contrast, *Staphylococcus aureus* (*S. aureus*) is among the most prevalent gram-positive bacteria causing a chronical and subclinical form of mastitis (Wellnitz et al., 2006; Tesfaye et al., 2009). Under practical conditions most mastitis incidences are disposed subclinically and remain unnoticed in dairy livestock. Besides their milk secretory function mammary epithelial cells (MEC) participate in the first line of defense against invading pathogens (Vorbach et al., 2006) and operate together with immune cells during pathogen invasion. Cell culture studies with MEC revealed the expression of host defense mechanisms for example pathogen recognition receptors as well as antimicrobial peptide (Petzl et al., 2008; Griesbeck-Zilch et al., 2009), which enable them to react on pathogen invasion before the acquired immune defense factors intervene. They are also responsible for immune modulatory effects in the udder due to secretion of chemokines (Bournazou et al., 2009) which enables the interaction with immune cells to defend against pathogen invasion.

Most investigated receptors are the transmembrane toll-like receptors (TLR) that mediate pathogen recognition via the pathogen-associated molecule pattern (PAMP) such as lipopolysaccharides (LPS) from *E.coli* and lipoteichoic acid (LTA) of *S.auerus*. In cattle, currently 10 different TLR are described and characterized (Werling et al., 2006). Petzl et al. (2008) demonstrated previously, that TLR2 and TLR4 are selectively up-regulated in case of clinical mastitis, whereas TLR9 was not affected. Beside receptor based defense, mammary epithelial cells secret a wide range of antimicrobial peptides (AMP) (Zasloff, 2002; Lutzow et al., 2008; Molenaar et al., 2009; Roosen et al., 2004). These proteins and peptides react upon all invading pathogens and exhibit strategies of killing. Antiviral, antifungal and antibiotic mechanisms include membrane disruption, thus perturbing bacterial permeability as well as metabolic inhibition (Almeida and Pokorny, 2009; Bocchinfuso et al., 2009). Additionally, in contrast to the therapeutical problems of increasing antibiotical resistance of pathogens, interest on those potent peptides increases due to minimal resistance development of the pathogens (Kraus und Peschel, 2006). Acute symptoms of mammary infection most often associated by *E.coli* mastitis lead to increasing inflammation parameters. First of all Tumor-necrosis-factor alpha (TNFα) and Interleukin 1 beta (IL1β) are mentioned here. In the acute phase of cytokine release they mediate both local and systemic inflammatory responses. They are most potent endogenous inducers of fever and have both beneficial and injurious properties (Sordillo and Streicher, 2002). Furthermore, TNFα is one of the factors to induce apoptosis in the mammary gland (Bannerman, 2009). During mammary inflammation epithelial cells take part in chemotaxis to recruit immune cells by the release of chemoattractants (Haston and Shileds, 1985). In case of acute mastitis 90% of milk-derived cells are neutrophiles (Mehrzad et al. 2005), which are also supposed to be the first cells to arrive at inflammation due to secretion of growth related oncogen alpha (Groα) and Interleukin 8 (IL8). Sever mastitis leads to mammary tissue damage and cell death by either apoptosis or necrosis, which is contributed by both bacteria and host defense factors (Zhao

and Lacasse 2008). Apoptosis initiating and regulatory factors are the FAS receptor, the anti-apoptotic B-cell lymphoma 2 (Bcl-2) family members involved in mitochondrial death cascade, and up streamed initiator and down streamed effector cysteine proteases called caspases activated by the death receptor and the mitochondrial cascade (Nunez et al., 1998).

However, in most above cited works analysis was done in milk or the established cell culture models were generated by mammary biopsy or slaughter after intra mammary infection (Griesbeck-Zilch et al., 2008; Wellnitz and Kerr, 2004; Petzl et al., 2008). Beside its invasive character concerning animal welfare and animal's life, the main disadvantage of mammary biopsy is the high risk of contamination with fibroblasts. This fast growing stroma cells may overgrow the target epithelial cells and might tamper with the results. According to the advice of Boutinaud and Jammes (2002) the establishment of a cell culture model of milk derived cells was implemented and focus was led on the immune defense capability of primary bovine mammary epithelial cells (pbMEC) affected by an induced *in vivo* NEB. We wanted to investigate with this study, if the induced NEB *in vivo* also influences the immune capacity of MEC, since it is know that it has an inhibiting effect on immune cells (Suriyasathaporn et al., 2000). Therefore cell cultures of pbMEC of energy restricted and control fed cows were generated and an immune challenge was conducted. A set of 15 comprehensive genes involved in the different areas of the innate host defense was selected and the immune response was determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Material and Methods

Animals and dietary induced NEB

A detailed description of the experimental design and the conduction of the feeding experiment were published in Gross et al. (2011). In brief, Red Holstein cows were housed in a free-stall barn and were evenly assigned to control and restriction feeding according to milk yield, calculated energy balance and feed intake during first 85 day post partum (pp). After re-establishment of metabolic stability and a positive energy balance on day 100pp, a dietary energy deficit until 51 ± 2% of total energy requirements was individually induced for 3 weeks, followed by a re-alimentation period.

Cell culture of primary bovine mammary epithelial cells

Milk samples were taken at the last day of the energy restriction period. A one litre milk sample of each animal was taken and a bacterial milk test for each quarter was conducted to exclude bacterial infection prior to the experiment. Only milk free of bacteria was used to extract pbMEC. The milk was dispersed evenly to four centrifuge cups (250ml each). The four cups were centrifuged at 3100g, 20°C for 10 min. Milk was decanted and each cell pellet was re-suspended in 25ml pre-warmed (37°C) washing medium (HBSS; Sigma-Aldrich, Munich, Germany) containing 200µg/mL penicillin G, 200µg/mL of streptomycin, 200µg/mL gentamicin, and 10µg/mL amphotericin B (Sigma-Aldrich, Munich, Germany). Two cell solutions were combined into a 50ml falcon tube, washed by gentle mixing and centrifuged at 500g for 5min at room temperature (RT). The pellets were re-suspended in 25ml HBSS-solution and filtered (Falcon Cell Strainer 100µm, BD Biosciences, Bedford, USA) into one falcon tube. After centrifugation for 5min at 500g, the pellet was re-suspended in warm growth medium consisting of DMEM/F12 Ham (Sigma-Aldrich, Munich, Germany), 10% fetal calf serum (FCS) (Gibco, Invitrogen, Carlsbed, CA), ITS supplement (5mg/mL insulin, 5mg/mL transferrin and 0.005mg/mL sodium selenite; Invitrogen, Carlsbed, CA), 100µg/mL penicillin, 100µg/mL streptomycin, 100µg/mL gentamycin and 5µg/mL amphotericin B. The

cells were seeded into a 25cm^2 tissue culture flasks (Greiner Bio-one, Frickenhausen, Germany) and cultivated at 37°C, 5% CO_2 and 90% humidity. Cells were allowed to attach for 24h. Unattached cells were removed by gentile washing with warm phosphate buffered saline (PBS) ph 7.4 and medium was exchanged. Growth medium was changed twice weekly and growth of primary cells was documented until reaching 80% confluence. Due to higher sensibility and higher contamination risk in primary cells compared to cell lines, infected cultures were eliminated at first appearance of bacterial contamination. Additionally, only morphological healthy cultures were further cultivated and selected for the experiment. Cells were harvested at 80% confluence state in second passage and stored in DMEM/F12 HAM with 20% FCS and 10% Dimethyl sulfoxid (DMSO) (Roth, Karlsruhe, Germany) in liquid nitrogen until all samples were taken. Finally, primary mammary epithelial cell cultures of 8 restriction and 7 control cows were successfully generated.

Immunohistochemistry

Epithelial identity was confirmed by immuno-histological staining of cytokeratins 4, 5, 6, 8, 10, 13 and 18. Concurrently to the seeding of the 48-wells challenge plates pbMEC were seeded on culture chamber slides (LAB-Tek, Nunc, Germany) in four times approach. After reaching confluent state, medium was removed and pbMEC were washed twice with PBS. Chambers were removed and attached cells were fixed with ice-cold aceton-methanol mix (1:1) for 5min. Slides were dried at RT. Wells were incubated with 1% H_2O_2 (Merk, Darmstadt, Germany) in PBS-Tween (PBST) for 30min at RT in the dark to block endogenous peroxidases. After washing 3 times with PBST for 5min, respectively, the slides were incubated with goat serum (Dako, Glostrup, Denkmark) diluted 1:10 in PBST for 30min at RT. A primary monoclonal mouse IgG anti-pan cytokeratin antibody (F3418, Sigma-Aldrich, St-Louis, USA) was diluted 1:50 in PBST, applied to the wells and incubated over

night at 4°C. Goat serum remained on negative controls and was not replaced by primary antibody. On the next day the slides were washed 3 times with PBST for 5min, respectively, and secondary polyclonal goat anti-mouse antibody (1:400; Immunoglobulins HRP, Dako Gostrup, Denmark) was applied. After one hour incubation at RT cells were washed 3 times with PBST for 5min, respectively, and peroxidase was visualized by incubating the wells with 0.01% DAB-dihydrochloride (D-5905, Sigma-Aldrich, Germany) and 0.01% H_2O_2 in PBST for 15min at RT in the dark. Afterwards slides were washed 3 times with PBST for 5min, respectively, and were dipped in aqua bidest. The cell nuclei were stained with Mayer hemalaun solution (Roth, Karlsruhe, Germany) for 15sec and colour development was obtained by dipping the slides into tap water. Slides were dehydrated in increasing alcohol (50-100%) for 2min, respectively followed by 2min incubation in xylol (Sigma-Adlrich, Germany). Cover glasses were fixed with EUKITT (Fluka, Sigma-Aldrich, Steinheim, Germany). Results are shown in figure 1.

Cultivation of *E. coli* and *S. aureus*

S. aureus 1027 and *E. coli* 1303 (Petzl et al. 2008) were donated from Wolfram Petzl (Clinic for Ruminants, Ludwig-Maximilians-University, Munich, Germany). The gram negative pathogen *E.coli* was cultured in lysogeny *broth* (LB) liquid medium and on LB-agar Lennox (SERVA, Heidelberg, Germany) plates. The cultivation of the gram positive *S.auerus* was conducted in casein-soy-peptone (CASO) broth liquid medium (Fluka, Sigma-Aldrich, Steinheim, Germany) and on blood agar (Blood Agar Base No.2, Oxoid, Cambridge, UK) plates. The pathogens were thawed and applied to the appropriate agar plates and incubated over night at 37°C. One colony of each pathogen was picked and applied to 20ml growth mediums. After overnight incubation at 37°C *E.coli* was diluted 1:1000 and *S.auerus* 1:500 into fresh growth medium. Optical density (OD) of 1mL bacteria solution was measured at 600nm every

30min for 4h to generate a growth curve. Simultaneously to each OD measurement, 5 dilution steps of the pathogens were seeded on respective agar plates and incubated at 37°C. At the beginning 10^{-4} until 10^{-6} dilution steps and with increasing time and pathogen growth 10^{-9} -10^{-10} dilution steps were used. On the following day colonies were counted. According to assumption that one colony was grown out of one bacterium within the dilution steps the amount of bacteria was calculated. The growth curve was repeated and according to the optimal harvest time growth was stopped by putting the pathogen tubes on ice for 10min. The tubes were centrifuged twice for 10min on 1850g and re-suspended in 50mL PBS. After third centrifugation step, the pellet was re-suspended in 5mL PBS and was put into the water bath at 63°C for 30min to inactivate the pathogens. To control the inactivation respective agar plates were inoculated with the pathogens. Bacteria solutions were aliquoted and stored at -80°C.

Immune challenge of pbMEC with heat inactivated *E.coli* and *S.aureus*

Cells were thawed in the third passage and seeded into 48 well plates with a concentration of 100.000 cells/well. Two wells were seeded for *E.coli*, *S.auerus* and untreated control cells, respectively. Additional two wells served as counting wells. Those wells were detached prior to treatment and counted twice. The determined mean cell count was assumed for the treatment and the control cell wells to calculate the concentration of applied pathogen. Until 80% confluency was obtained growth medium was replaced by 1mL DMEM/F12 Ham supplied with ITS solely (challenge medium). Cells in counting wells were detached, counted and pathogen concentrations for multiplicity of infection (MOI) 30 were calculated. Challenge medium was replaced and wells were infected with MOI 30 of respective heat inactivated bacteria solution. Control wells were treated with PBS. A double approach was conducted.

quantitative reverse-transcription PCR (qRT-PCR) for mRNA quantification

After 24h and 72h cells were harvested, challenge medium supernatant was removed and stored at -80C. Total RNA was extracted with the Qiagen Allprep RNA/Protein kit (Hilden, Germany) as described in the manufacturer's instructions and an additional DNAse digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was conducted. RNA integrity was determined with the Agilent Bioanalyzer 2100 and RNA6000 Nano Assays (Agilent Technologies, Waldbronn, Germany). The reverse transcription was conducted on Eppendorf Mastercycler Gradient (Hamburg, Germany). For converting the RNA template into cDNA 300ng of RNA was reverse transcribed with 1µL of M-MLV reverse transcription RNase (H−; Promega, Mannheim, Germany) using 3µL random primers (Invitrogen, Karlsruhe, Germany) and 3µL dNTP (Fermentas, St. Leon-Rot, Germany). The protocol started with 10min at 21°C for optimized primer annealing, followed by 50min at 48°C for transcription and 2min at 90°C for inactivation of the enzyme and separation of generated cDNA and RNA template, and a final hold at 5°C. A negative control was added without enzyme for excluding genomic DNA contamination. Primers (Tab. 1) were designed using open source primer design software Primer3 and synthesized by Eurofins (MWG GmbH, Ebersberg, Germany). qRT-PCR and primer testing were conducted on the iQ5 Multicolor real-time PCR detection system (Bio-Rad Laboratories GmbH, Munich, Germany) using twin.tec PCR Plate 96 formats (Eppendorf, Hamburg, Germany). For qRT-PCR reaction 1.5µL of cDNA equivalent to 7.25ng of total RNA was amplified in 13.5µL reaction volume with the MESAgreen qPCR Mastermix Plus for SYBR assay with fluorescein (Eurogentec Deutschland GmbH, Köln, Germany). 1.5µL forward and reversed primers were added. The used protocol started with 5min polymerase activation at 95°C, followed by 40 cycles: denaturation for 15sec at 95°C, primer specific annealing for 20sec, and the elongation for 40sec at 60°C. A melt curve

starting from 60°C until 95°C was performed in 10sec with 0.5°C steps per cycle. The size of the PCR products was confirmed by agarose gel electrophoresis after GelRed (Biotium inc., Hayward, USA) staining.

Data analysis and statistics

Statistical description of the generated gene expression data set was analysed by GenEx software 5.0.1. (MultiD Analyses AB, Göteborg, Sweden). The Cq values were normalized with the arithmetic means of reference genes. The three suitable reference genes Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH), Ubiquitin (UBQ3) and Actin gamma 1 (Actinγ1) were selected using GenEx software. To calculate the effects of treatment versus control ΔΔCq method according to Livak and Schmittgen (2001) was used and the data transformation with $2^{-\Delta\Delta Cq}$ into relative expression ratio (x-fold regulation) was conducted. Target gene expression is represented as x-fold up-regulation for $x > 1.00$ and down-regulation is represented in values $x < 1.00$ with standard error of means (SEM), respectively. Outliners were identified and excluded using the GenEx function Grubbs' test.

A principal component analysis (PCA) was conducted for ΔCq values to disclose multivariate treatment effects. The PCA is a suitable tool for multidimensional data analysis, which allows the recognition of patterns and the visualization of treatment information of a heterogeneous data set. The calculation of the two principal components out of the measured data for every sample leads to the reduction of dimensions and enables the plotting of samples each as one spot in a two-dimensional room. Therefore, treatment effects can be visualized according to formation of clusters and separation of the samples represented by one spot per sample (Kubista et al., 2006; Riedmaier et al., 2009). The PCA results were further confirmed by comparing the $2^{-\Delta\Delta Cq}$ arithmetic means in a one-way ANOVA (analysis of means) on ranks

and subsequent Kruskal-Wallis-Test using SPSS (IBM SPSS Statistics 19.0). *P*-values ≤ 0.05 were considered as significance level.

Results

Immunohistochemisty

The immune-histological staining of cytokeratins is presented in figure 1. Positive brown staining illustrates the purity of the generated cell cultures and identifies the used cells as pbMEC without contamination of fibroblasts. The calculation revealed 97% positive stained cells. The proof of quality is provided in the negative control without primary antibody presented in the insert of figure 1. Unstained cells had an elongated cytoplasm with an oval nucleus and were excluded from the calculation of epithelial cells characterized by typical anti-cytokeratin staining. According to their morphological appearance they might be fibroblast, which do not stain for cytokeratins (data not shown).

RNA integrity

The integrity of RNA was determined using the Agilent Bioanalyzer 2100 and RNA6000 Nano Assays and presented as RNA Integrity Numbers (RIN). Mean RIN value was 7.9 ± 0.2 SEM.

qRT-PCR

Antimicrobial peptides and receptors (AMPR)

As first applied statistical tool the PCA presented in figure 2A revealed an emigration of *E.coli* treated samples out of the general sample cloud. *S.auerus* and control samples are

evenly spread and therefore indicate no effect of the *S.aureus* treatment versus control. Differential expressed genes of AMPR (Fig.3A) were influenced by trend by NEB. Significant effects were measured for TLR2 and TLR4, which were significantly up-regulated in *E. coli* infected control cells after 24h compared to 72h ($P \leq 0.05$). Mean expression levels of TLR4 were low in all treatment groups. Expression levels were highest in restriction cells exposed to *E.coli* (25-40-fold for Defensin-beta1 (DEFβ1)) and 46-fold for Lactoperoxidase (LPO). *S.aureus* induced an up-regulation from 24h until 72h within control fed group, but showed down-regulated expression profiles by trend in the energy restriction group.

Cytokines

The comparison between *E. coli* and *S. aureus* for control feeding and restriction in the PCA analysis for cytokines revealed a separation of *E.coli* samples and a slight emigration to the left of restriction samples out of the central cloud (Fig.2B). *E.coli* treatment showed a more pronounced transcript increase, especially in IL1β than *S.aureus* (Fig.3B). The combination of *E.coli* and energy restriction induced generally higher expression levels compared to control fed group, but without significance due to high SEM. Expression of all three genes increased from 24h to 72h under *S.aureus* influence in the control group, which was not seen in the restriction group. TNFα transcripts decreased significantly ($P \leq 0.05$) in restriction cells after 24h compared to control cells after exposure to *S.aureus*. *E.coli* induced a higher expression compared to *S.aureus* in the restriction group after 24h ($P \leq 0.001$). That effect could not be found in the control group. The same regulation pattern but lower expression levels without significance were found for Interleukin 6 (IL6).

Chemokines

E.coli provoked an increased chemokine responses in pbMEC compared to *S.aureus* in the PCA (Fig.2C), which was even higher in the restriction cells (Fig.3C). Highest up-regulation

of 125-fold was found in IL8 due to restriction feeding and *E. coli* exposure. A significant difference was found between *E.coli* and *S*.aureus for 72h in the restriction group ($P \leq 0.05$). Gene expressions of chemokine (C-C motif) ligand 26 (CCL26) and Groα in the control group were up-regulated after 24h for both pathogen stimulations and down-regulated after 72h. However, low expressions were found in the restriction group. Furthermore a remarkable effect of the *S.aureus* stimulation was determined in the restriction group compared to the control group. All genes in this group were down-regulated after 24h as well 72h, compared to control feeding group. But high SEM prevented the calculation of significant differences.

Apoptosis

In contrast to the PCAs of the above mentioned gene classes, no clear clustering of apoptosis genes due to pathogen type could be found (Fig.2D). However we could assess tendencies for tight clusters of restriction samples. Control feeding samples were arranged in a wide variety indicating a high variation within the data set. Further analysis revealed high SEM and low significant differences. Among apoptosis related genes (Fig.3D) most pronounced up-regulation was found for the death receptor FAS and Bcl-2. A significant up-regulation was induced by *S.aureus* treatment for anti-apoptotic Bcl-2 compared to *E.coli* infected restriction cells after 24h. FAS and Bcl-2 were also influenced by NEB and were up-regulated in the restriction group compared to the control feeding group after 24h for *E.coli* by trend.

Discussion

The accomplished PCAs on ΔCq-level according to the functional gene groups showed a clear separation of *E.coli* infection compared to *S.aureus* and control cells (Fig.2). High variation within the data set is also displayed due to wide arrangement and increased distances of the *E.coli* sample clouds compared to *S.aureus* and control cell arrangements. This is also

confirmed by high SEM within the presented bar charts (Fig.3A-D). *S.aureus* samples are arranged around the tight clustering of control samples in the PCA, which was most pronounced in the cytokine and chemokine group. This visualization cluster indicates the lower effect of *S.aureus* treatment compared to *E.coli*. However, the wide spread *S.aureus* sample dots indicate high variance and therefore high SEM were calculated, leading to few significant results especially within the AMPR and the apoptosis group (Fig.2A and D). Therefore the calculation of significant differences of infection and feeding confirm the PCA findings and clearly point out that PCA is a suitable tool for first step statistical analysis to describe treatment effects within the presented heterogeneous data set.

Antimicrobial peptides and receptors were influenced by both pathogens. Furthermore, the restriction additionally increased *E.coli* affected gene expression but decreased the expression due to *S.aureus* infection, which could be explained by impaired immune capability caused by the NEB. Cytokine responses were highest among the analyzed functional gene groups. IL1β followed by TNFα showed a rapid up-regulation within 24h indicating the activation of inflammatory action (Fig.3B). In contrast to Wellnitz and Kerr (2004) *E.coli* and not *S.aureus* induced the intensified up-regulations of IL1β and TNFα in our experiment, especially in the energy restriction group. The energy restriction reduced the expression level of TNFα after 24h in *S.aureus* treated cells and even more but without significance after 72h. Buitenhuis et al. (2011) goes in line with our findings. They report up-regulated transcripts of pro-inflammatory genes due to *E.coli* treatment after 24h. Lower expression of cytokines and other inflammatory mediators after *S.aureus* challenge in our study are also reported in Bannerman (2009) and Griesbeck-Zilch et al. (2008). The latter found higher regulation patterns of pro-inflammatory cytokine induced by *S.aureus* after 1h by trend. The early responses after *S.aureus* infection may be due to the disease pattern induced by the gram positive pathogen. Although an earlier sampling time than 24h was not conducted in our

experiment, the high magnitude of cytokine expression hypothesizes a rapid establishment of cytokine release and showed even further increase of the immune responds until 72h post infection. This is characteristic for the innate immune system as it is poised to react as first line defense against invading pathogens in the udder. IL1β and TNFα are most reactive in case of inflammation and most potent to induce systemic immune reaction as far as shock, vascular leakage and multiorgan failure (Bannerman, 2009). In the control fed group the expression of those cytokines rises up until 72h seen in both bacteria, but is considerably decreased in the restriction group after 72h for *S.aureus* only. This could indicate an effect of the conducted energy restriction on *S.aureus* infected cells. The measured down-regulation might demonstrate an impaired immune function and therefore may support the manifestation of a chronicle and subclinical *S.aureus* induced mastitis. The reaction of IL1β and TNFα further indicate the potential of our heat inactivated *E.coli* 1303 used in MOI 30 to simulate an acute mammary infection as well as the defense capacity of the generated pbMEC towards *E.coli* infection (Gunther et al., 2009).

Immune challenge also activated the chemotaxis pathway in pbMEC. Highest expressions for IL8 and chemokine (X-C motif) ligand 5 (CXCL5) were found in the present work and confirm the findings of Pareek et al. (2005) using microarray technology on LPS stimulated bMEC, even though RANTES (regulated upon activation, normal T-cell expressed and secreted) was measured but not expressed in our experiment. Results by trend show a down-regulation of those chemokines by energy restriction of the *S.aureus* stimulated cells. GROα showed only low regulation changes due to treatments. This is contrary to Lahouassa et al. (2007) who reported a 30-fold up-regulation of GROα after 24h *E.coli* infection. Again as found in the cytokine group, a further up-regulation was found in the pbMEC of energy restricted cows compared to control fed cows due to *E.coli* infection whereas a down-

regulation of the chemokine expression was found due to *S.aureus* infection. The differences were not significant though because of a high SEM.

The comparatively small effects of the dietary induced energy deficit could also be explained by the metabolic screening results published in Gross et al. (2011). Cows were able to overcome induced NEB without suffering from metabolic instability and metabolic disorders even though only 51 ±2% of total energy requirement was covered. This might be a reason for the existing but low reaction of the pbMEC upon the feeding regime. However, our results by trend indicate an effect of the conducted dietary energy restriction. In the present study *E.coli* exposed an immune stimulus and lead to up-regulations of 15 innate immune system genes from 24h to 72h and additional increase in the restriction group. *S.aureus* also induced effects on target genes with mostly increasing gene expressions from 24h to 72h. In the restriction group, however, expression decreased considerably at both time points which might indicate a delayed immune function against *S.aureus* due to energy restriction. These findings are also reported in other studies. By means of the induced clinical signs of *S.aureus* caused mastitis, which remains subclinical and even chronicle, the activation of the immune responds occurs within the very first hours postinfection (Griesbeck-Zilch et al., 2009; Lahouassa et al., 2007) but remains generally on low levels. This strategy enables *S.aureus* strains to persist concealed by the immune system und develop lifelong infections. In our study no earlier time points than 24h were sampled but the reaction due to *S.aureus* penetration was on lower levels than *E.coli*. Ongoing infection activated the immune responds against *S.aureus* and led to higher expression than *E.coli* at 72h in the control fed group (Fig.3A, C). This late immune function seems to be blocked and decreased in the situation of the induced NEB, which might enable a *S.aureus* induced mastitis to establish and persist. Concomitantly, anti-apoptotic Bcl-2 (Akbar et al, 1996) was considerably up-regulated by additional low regulation levels of the death receptor FAS for *S.aureus* infected cells at 24h. The up-regulation of Bcl-2 might be a

reaction on the restraining impact of NEB in order to overcome and protect the cells. By this assumption, the impact of the conducted dietary energy restriction could be indirectly confirmed.

Conclusion

In the present work the immune challenge of *E.coli* and *S.aureus* induced expression changes of the determined AMPR, cytokine, chemokines and apoptotic genes by time. Moreover, the accomplished energy restriction until 51 ±2% of total energy requirement influenced the immune capacity of the generated cell cultures visibly, but with marginal significances. The immune responses in *E.coli* infected cells increased in the restriction compared to the control feeding group, whereas *S.aureus* infected cells seemed to be immune impaired by the induced NEB which led to down-regulations of the determined target genes.

Furthermore, our results approve the capability of MEC as model for mastitis research. Physiological effects of metabolic challenges conducted to the animals seem to be transmitted into cell culture situation and even measurable in the immune responds of primary cell cultures in the third passage. Additionally, we approve the capability of the principal component analysis (PCA) for visualization of treatment related differences within a heterogonous data set.

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Tables and Figures

Figure 1: Immuno-histological identification of pbMEC by cytokeratine staining. Positive brown staining of cytokeratines 4, 5, 6, 8, 10, 13 and 18. The insert shows the negative control.

Figure 2: Principal component analysis (PCA) of four different immune functional gene groups presented on ΔCq level: (A = antimicrobial peptides and receptors (AMPR), B = cytokines, C = chemokines, D = apoptosis). Data sets are arranged according to feeding regime (control=square, restriction=circles) and treatment (*E.coli* = green, light green; *S.aureus* = red, pink; control =black, grey) and infection time (24h=dark colours, 72h=light colours).

Figure 3: Relative gene expression of means presented as $2^{-\Delta\Delta Cq}$ in log 10 scales ± SEM; A) antimicrobial peptides and receptors (AMPR), B) cytokines, C) chemokines, D) apoptosis related genes. (**S24**, **S72**: *S.aureus* infection for 24h and 72h; **E24**, **E72**: *E.coli* infection for 24h and 72h). Significant difference within control or restriction group (E24 vs. E72, S24 vs. S72) are presented in different lowercased letters; significant differences between control and restriction group (E24 vs. E24, E72 vs. E72, S24 vs. S24, S72 vs. S72) are presented as different capitals; significant level $P \leq 0.05$.

Table 1: Primer sequences, PCR product lengths (bp) and sequence references for reference genes and differential expressed target genes (F: forward; R: reverse).

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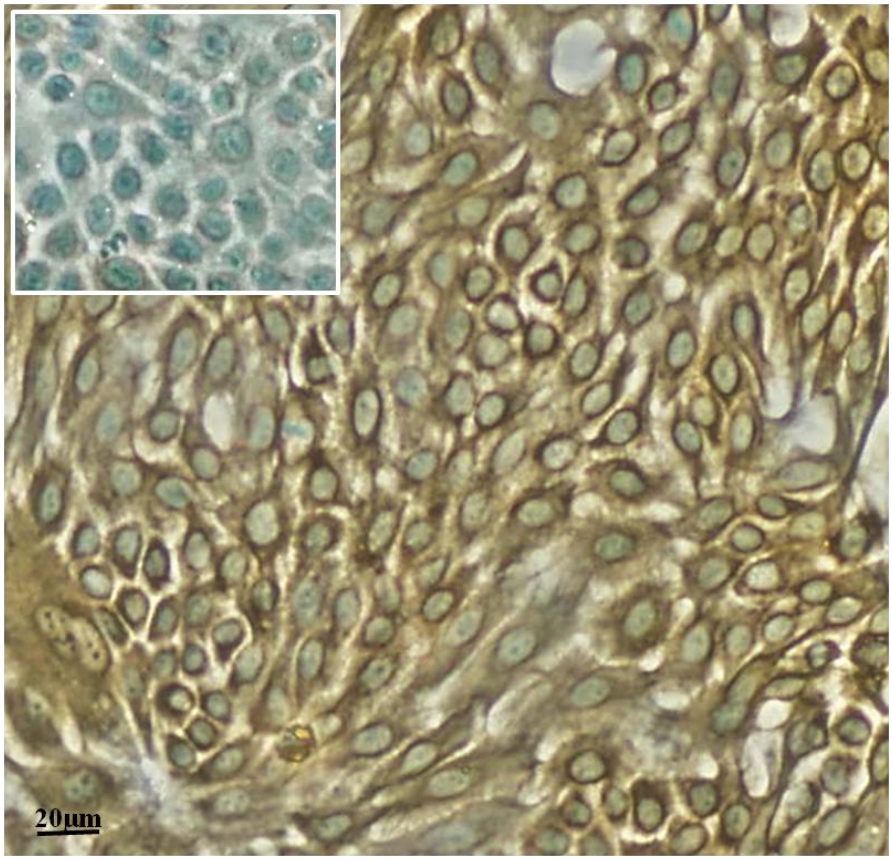

Figure 1

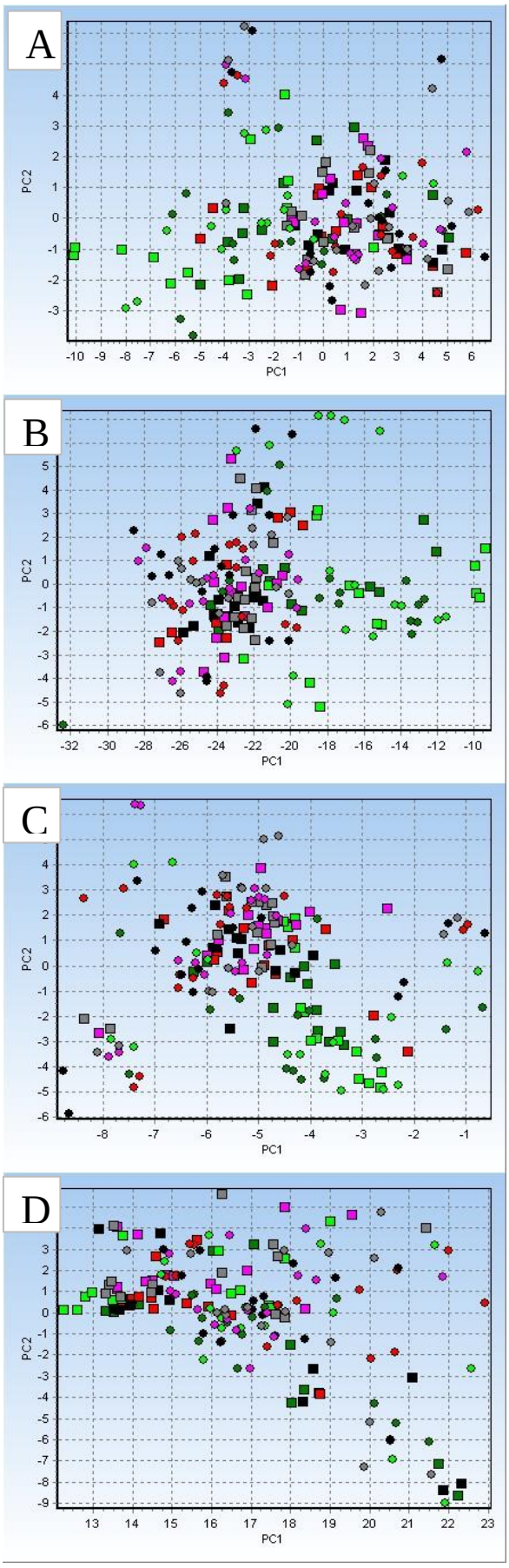

Figure 2

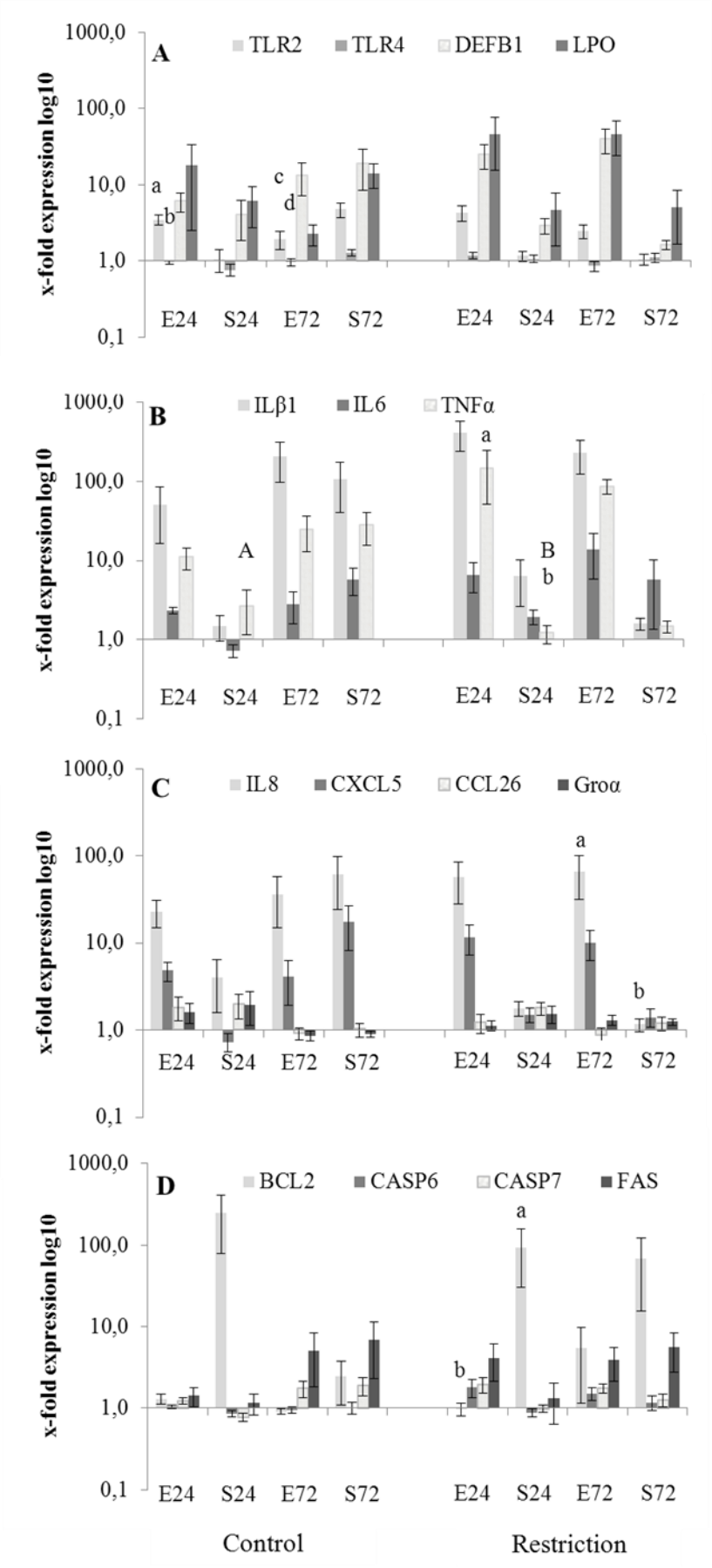
A
TLR2
TLR4
DEFB1
LPO
B
ILβ1
IL6
TNFα
C
IL8
CXCL5
CCL26
Groα
D
BCL2
CASP6
CASP7
FAS
x-fold expression log10
1000,0
100,0
10,0
1,0
0,1
E24
S24
E72
S72
Control
Restriction

Figure 3

Table 1

Genes	Abbreviation	Primer	Sequence (5' to 3')	Size (bp)	Reference
Reference genes					
Actin gamma 1	Actinγ-1	F	aactccatcatgaagtgtgacg	233	NM_001033618
		R	gatccacatctgctggaagg		
Glyceraldehyde 3-Phosphate Dehydrogenase	GAPDH	F	gtcttcactaccatggagaagg	197	Berisha et al. 2002
		R	tcatggatgaccttggccag		
Histone H3	HISTON	F	actgctacaaaagccgctc	233	AF469469
		R	acttgcctcctgcaaagcac		
Ubiquitin 3	UBQ3	F	agatccaggataaggaaggcat	198	NM174133
		R	gctccacttccagggtgat		
Target genes					
Toll-Like-Receptor 2	TLR2	F	cattccctg gcaagtggattatc	202	NM_174197.2
		R	ggaatggccttcttgtcaatgg		
Toll-Like-Receptor 4	TLR4	F	tgctggctgcaaaaagtatg	213	NM_174198.6
		R	ttacggcttttgtggaaacc		
Lactoperoxidase	LPO	F	ccgacaacattgacatctgg	206	NM_173933.2
		R	gtcacagatgaggcgtgaga		
Defensin beta1	DEFβ1	F	tgctgggtcaggatttactcaagga	85	NM_175703.3
		R	agggcacctgatcggcacac		
Interleukin 1 beta	IL1β	F	cagtgcctacgcacatgtct	209	NM_174093.1
		R	aga gga ggtggagagccttc		
Tumor-Necrosis-Factor alpha	TNFα	F	ccacgttgtagccgacatc	108	AF348421
		R	accaccagctggttgtcttc		
Interleukin 6	IL6	F	caccccaggcagactacttc	182	NM_173923.2
		R	atccgtccttttcctccatt		
Chemokine (C-C motif) ligand 26 / Eotaxin3	CCL26 /Eotaxin3	F	ctcggagctgccacacgtgg	167	XM_002698193.1
		R	tgggcacacactttccggcc		
Growth-Related Oncogene αlpha	Groα	F	gctcggacgtgttgaagaac	116	U95812

		R	cctgagccagaggcggactac		
Chemokine (C-X-C motif) ligand 5	CXCL5	F	ttgtgagagagctgcgttgt	150	NM_174300.2
		R	ccagacagacttcccttcca		
Interleukin 8	IL8	F	tgctctctgcagctctgtgt	306	NM_173925.2
		R	cagacctcgtttccattggt		
FAS	FAS	F	agaagggaaggagtacacaga	124	NM_000043
		R	tgcacttgtattctgggtcc		
B-cell lymphoma 2	Bcl-2	F	cggaggctgggacgcctttg	116	NM_001166486.1
		R	tgatgcaagcgcccaccagg		
Caspase 6	Casp6	F	ggctcgcggtccaggtgaag	177	NM_001035419.1
		R	ctggtgccaggcctgttcgg		
Caspase 7	Casp7	F	atccaggccgactcgggacc	235	XM_604643.4
		R	agtgcctggccaccctgtca		

Running head: Energy restriction and the lactoferrin reaction of pbMEC

Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells *in vitro*

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Summary

A dietary energy restriction to 49% of total energy requirements was conducted with Red Holstein cows for three weeks in mid-lactation. At the last day of the restriction phase primary bovine mammary epithelial cells (pbMEC) of 8 restriction and 7 control cows were extracted out of one liter milk and cultured. In their third passage an immune challenge with the most prevalent, heat inactivated mastitis pathogens *Escherichia coli* (*E.coli*) and *Staphylococcus aureus* (*S.aureus*) was conducted. Lactoferrin (LF) was determined on gene expression and protein level. An enzyme-linked immunosorbent assay (ELISA) was developed to determine LF in milk samples taken twice weekly throughout the animal trial, beginning on day 20pp (post partum) until day 150pp, in cell culture total protein and in cell culture supernatant. Milk LF increased throughout the lactation and decreased significantly during the induced energy deficiency in the restriction group. At the beginning of realimentation LF concentration in the restriction group increased immediately and reached higher levels than before the induced deficit and followed the upward trend seen in controls. Cell culture data revealed higher levels (up to 7 fold up-regulation in gene expression) and significant higher LF protein concentration in the restriction compared to the control feeding group cells. A further emphasized effect was found in *E.coli* compared to *S.aureus* exposed cells. The general elevated LF levels in the restriction pbMEC group and the further increase due to the immune challenge indicate an unexpected memory ability of milk-extracted mammary cells that were transposed into *in vitro* conditions and displayed even in the third passage of cultivation. The study confirms the suitability of the noninvasive milk extracted pbMEC culture model to demonstrate the influence of feeding experiments on immunological situations *in vivo.*

Keywords: lactoferrin, negative energy balance, primary bovine mammary epithelial cell, *E.coli*

Introduction

Besides its ability to bind iron LF has important immune regulatory functions and plays an active role in the host defense system (Levay and Viljoen, 1995). In the bovine mammary gland it is secreted by mammary epithelial cells and emigrated neutrophils during pathogen invasion, and it is involved in the modulation of the innate immune responses against invading pathogens. Besides its bacteriostatic action due to binding iron and being essential for bacterial growth, LF and especially its reactive peptide Lactoferricin-B exhibit strong bactericidal properties. Interaction with the bacterial cell membrane changes its permeability and causes cellular damage (Flores-Villasenor et al., 2010).

In early lactation bovine milk contains relatively low concentration of LF, which increases during lactation. Highest levels were reached in dry period during udder involution representing a 100-fold increase in LF concentration compared to normal milk (Welty et al., 1976). In early lactation, however, the immune response is low due to energetic imbalance and metabolic instability. Mobilized body fat reserves result in increasing blood non-esterified fatty acids (NEFA) and β-hydroxybutyrate (BHB) concentrations. NEFA and BHB amongst others appear to reduce the responsiveness of neutrophils against invading bacteria (Sartorelli et al., 1999), which in turn leads to decreasing LF concentrations and therefore may reflect the impaired immune response in the critical state of a negative energy balance. However, LF belongs to the host defense and is secreted as an unspecific, but potent antimicrobial peptide and is most potent as inhibitor of *E.coli* (Kutila et al., 2003). Most importantly, it is produced in many epithelia that are confronted with invading pathogens as first step of defense before the acquired immune system is activated. The present study was conducted to investigate the innate immune competence of the mammary gland by means of LF in the state of an induced energy deficiency. Therefore, primary bovine mammary epithelial cell (pbMEC) cultures of energy restricted and control fed Red Holstein cows were generated at the last day of a 3

weeks lasting energy restriction. Cultures were established of milk extracted mammary epithelial cells. Compared to mammary biopsies the milk extraction is a non-invasive method without negative side effects for the animal. Furthermore it diminishes the contamination with fibroblasts, which is a serious problem in primary cell cultures gained from mammary tissue. After an immune challenge with the most prevalent heat inactivated mastitis pathogen *E.coli* and *S.aureus* the immune response was determined according to the secretion and gene expression of LF. A competitive enzyme-linked immunosorbent assay (ELISA) was established to analyze LF in twice weekly taken milk samples throughout the experimental phase, in the cell culture supernatant and in the total cellular pbMEC protein. Gene expression level of LF was determined in the challenged pbMEC by qRT-PCR.

Material and Methods

The animal trial had been approved by the responsible committee of the government of Upper Bavaria and was carried out on the research farm Hirschau of the Technische Universität München.

Feeding trial

A feeding trial with 50 multiparous Red Holstein cows was conducted to investigate the influence of the common metabolic imbalance postpartum (pp) as well as an induced energy deficiency in mid-lactation on metabolic (Gross et al. 2011).

From the day of calving a partial mixed ratio (PMR1) with additional 1.3kg dry matter per day (DM/d) concentrate (CONC, Tab.1) was offered for 5 days. In the following 30 days concentrate content was increased from 1.8kg until a maximum of 8.9kg DM/d. Thereafter

PMR1 was calculated for 650kg body mass, an assumed daily feed intake of 16kg DM/d and a daily milk production of 21kg. Concentrate intake was adjusted according to individual milk yield and offered at transponder based automatic feeding stations. According to their energy balance within the first 85 days of lactation animals were grouped evenly into control and restrictive feeding group (n=25 control and n=25 restriction). A dietary energy deficiency of finally 49% of total energy requirements was conducted for 3 weeks starting on approximate day 100pp, but exactly on day 12 of the oestrous cycle. The restriction cows received PMR2 (Tab. 1) reduced in energy by hay addition, reduced in concentrate content and reduced in feed amount (Gross *et al*., 2011). During the experimental period of energy restriction control cows were fed PMR1 ad libitum. After three weeks restriction time the restriction cows were realimented ad libitum with PMR1 (Gross *et al*., 2011).

Blood samples

Blood samples were taken once weekly and metabolic parameters like NEFA, BHB and glucose were measured by Gross et al. 2011.

Milk samples

Two milk samples were taken weekly beginning on day 20pp until day 150pp. At the last day of the restriction period one liter milk was taken from each cow and mammary epithelial cells were extracted and taken into cell culture cultivation.

Primary bovine mammary epithelial (pbMEC) cell cultures and immune challenge

On the last day of restriction period one liter milk from each cow was sterile milked and immediately taken to the lab. The pbMEC were extracted and cultivated until the second passage.

In detail, the milk was distributed to four centrifuge cups and centrifuged at 20°C, 1850 x g for 10min. Milk was decanted and each cell pellet was re-suspended and washed three times in 25ml pre-warmed (37°C) washing medium (HBSS; Sigma-Aldrich, Munich, Germany) containing 200µg/mL penicillin G, 200µg/mL of streptomycin, 200µg/mL gentamicin and 10µg/mL amphotericin B (Sigma-Aldrich, Munich, Germany). pbMEC were seeded into a 25cm^2 tissue culture flasks (Greiner Bio-one, Frickenhausen, Germany) and cultivated in DMEM/F12 Ham (Sigma-Aldrich, Munich, Germany), 10% fetal calf serum (FCS) (Gibco, Invitrogen, Carlsbed, CA), ITS supplement (5mg/mL insulin, 5mg/mL transferrin and 0.005mg/mL sodium selenite; Invitrogen, Carlsbed, CA), 100µg/mL penicillin, 100µg/mL streptomycin, 100µg/mL gentamycin and 5µg/mL amphotericin B at 37°C, 5% CO_2 and 90% humidity. In the second passage at 80% confluency the cells were harvested and stored in DMEM/F12 HAM with 20% FCS and 10% dimethyl sulfoxid (DMSO) (Roth, Karlsruhe, Germany) in liquid nitrogen until all cultures from the experimental cows were generated. In the third passage an immune challenge with the most prevalent mastitis pathogens namely, *Escherichia coli* 1303 (*E.coli*) and *Staphylococcus aureus* 1027 (*S.aureus*) (Petzl et al., 2008) was conducted with an MOI 30 (multiplicity of infection), respectively. Bacteria were cultured and heat inactivated. Their potential to evoke an immune response was tested prior to immune challenge of pbMEC. The stimulation of pbMEC was conducted in double approach. After 24h and 72h exposure to the pathogens cell culture supernatants consisting of 1mL DMEM/F12 Ham supplied with ITS solely were removed and stored at -80°C. Cell layers were washed twice with PBS and lyses buffer for extraction of RNA and protein was added according to the manufacturer's instructions (Qiagen Allprep RNA/Protein kit, Hilden,

Germany). Epithelial identity was confirmed by immuno-histological staining of cytokeratins 4, 5, 6, 8, 10, 13 (mouse IgG anti-pan cytokeratin antibody F3418, Sigma-Aldrich, St-Louis, USA) and presented in figure 1. The calculation revealed 97% positive stained cells and unstained fibroblasts.

Lactoferrin enzyme linked immunosorbent assay (ELISA) in milk

The analysis of LF was conducted using a competitive ELISA on 96 well format microplates. The microplates (MaxiSorp®, Nunc; Thermo Fisher Scientific) were pre-coated with 1µg/100µl affinity purified goat anti-rabbit-IgG (Prakash et al., 1987) in coating buffer (50 mM $NaHCO_3$, pH 9.6) and incubated over night at 4°C. Plates were saturated with 280µg/well casein (SP-5020, Vector Laboratories, Inc. Burlingame, CA) diluted 1:10 in phosphate buffered saline-Tween-buffer pH 7.4 (0.1%Tween, PBST) and incubated for 45min at room temperature (RT). Afterwards the blocking solution was removed and microplates were stored at -20°C. The bovine LF antibody (Ak8836, BE 08.09.2009) was obtained by immunization of rabbits with bovine LF (colostrum isolate, Sigma-Aldrich Munich, Germany) in our own lab. The bovine LF was conjugated to biotinamidohexanoic acid N-hydroxysuccinimide ester (Biotin-X-NHS, Sigma-Aldrich Munich, Germany) and precipitated with 75% satured ammonium sulphate.

Prior to sample preparation microplates were thawed at RT and washed twice (Microplate Washer HydroFlex^{TM}, Tecan Trading, Switzerland) with PBST. For quantification lactoferrin standard curve (colostrum isolate, Sigma-Aldrich Munich, Germany) was prepared in PBST in glas cups (Witeg, Wertheim, Germany) in the range of 3.1ng/20µL to 0.0125ng/20µL. Additional four different control milks characterized by different somatic cell counts (SSC; I = 15,000 cells/mL, II = 310.000 cells/mL, III = 97,000 cells/mL and IV = 1,300,000 cells/mL) were used as external control. Milk samples and controls I and III were diluted 1:2,000

whereas control milk II was diluted 1:4,000 and control IV with highest SCC was diluted 1:8,000 in PBST.

Twenty µL of diluted standards, control milks and samples, respectively, were applied in duplicates to the microplate, 100µL LF antibody (Ak8836, BE 08.09.2009) diluted 1:400,000 in PBST was added and the plate was incubated over night at 4°C with slight shaking.

On the following day 1.5ng/100µL LF- biotin was added and incubated for further 2h shaking at RT in the dark. Then the microplate was washed four times with PBST, 100µL streptavidin coupled to horse-radish-peroxidase (Roche Applied Science Mannheim, Germany) (1:20,000 in PBST) was added and incubated for 15min at RT in the dark. After four washing steps with PBST the substrate reaction (Prakash et al., 1987) was started at RT in the dark. After 40min the reaction was stopped with 150µL 2M H_2SO_4. The absorbance of each well was read at 450nm (minus light absorbance at 600nm background value) using a Microplate Reader (Sunrise^{TM}, Tecan Trading, Switzerland). Mean LF concentration were calculated against the optical density (OD) of the standard curve. Inter- and intraassay variations for milk were 11.06% and 9.36%, respectively.

LF in pbMEC-total protein and pbMEC-supernatant

For the analysis of LF in the pbMEC-total protein and pbMEC-supernatant, the same ELISA as described above was used with slight modifications. Total protein content of pbMEC-total protein and cell culture supernatants were determined by bicinchoninic acid assay (BCA). pbMEC-total protein samples were diluted 1:3 in PBST and standards were prepared in PBST. Cell culture supernatants were used undiluted for the assay and standards were prepared in DMEM/F12 Ham (Sigma-Aldrich, Munich, Germany) supplied with ITS. Inter- and intraassay variations for pbMEC-total protein were 11.06% and 9.36%, respectively.

Inter- and intraassay variation for cell culture supernatant was 10.77% and 6.06% respectively.

LF Enzyme-linked immunosorbent Assay

The developed ELISA was able to detect LF in the three different matrixes milk, pbMEC-total protein and cell culture supernatant with slight modification. The detection limit was found to be 0.014ng/20µL according to calculation of Chen and Mao (2004). Reliable measurements within the linear range of the standard curve for milk were between the 20% and 80% intercepts of 2.03ng/20µL and 0.25ng/20µL, respectively, and for the standard curve diluted in DMEM/F12 Ham supplied with ITS 0.72ng/20µL and 0.11ng/20µL, respectively. The determined LF concentrations range according to the published ELISA data from Chen and Mao (2004) and Hiss et al. (2009) and where not biased by the supplemented ITS in the assay for cell culture supernatant.

Quantitative reverse-transcription PCR (qRT-PCR) for mRNA quantification

Total RNA and protein was extracted using the Qiagen Allprep RNA/Protein kit (Hilden, Germany) as described in the manufacturer's instructions and an additional DNAse digestion (RNase-Free DNase Set, Qiagen, Hilden, Germany) was conducted. Samples were stored at –80°C. RNA integrity was determined using the Agilent Bioanalyzer 2100 on RNA 6000 Nano Assays (Agilent Technologies, Waldbronn, Germany). RNA was reversed transcribed into cDNA on an Eppendorf Mastercycler Gradient (Hamburg, Germany) using a standard protocol. LF primers (Tab.2) were designed using the open source primer design software Primer3 and synthesized by Eurofins (Eurofins MWG GmbH, Ebersberg, Germany). A gradient PCR was conducted on the iQ5 Multicolor Realtime PCR detection system (Bio-Rad Laboratories GmbH, München, Germany) on twin.tec PCR Plate 96 formats (Eppendorf,

Hamburg, Germany) to determine optimal annealing temperature. The size of the PCR products was confirmed by agarose gel electrophoresis after GelRed (Biotium inc., Hayward, USA) staining. Gene expression of LF was measured with MESAgreen qPCR Mastermix Plus for SYBR Assay with fluorescein (Eurogentec Deutschland GmbH, Köln, Germany) on the iQ5 Multicolor Realtime PCR detection system. The used protocol contained 5min polymerase activation at 95°C, followed by 40 cycles: denaturation for 15sec at 95°C, primer specific annealing for 20sec and the elongation for 40sec at 60°C. A melt curve starting from 60°C until 95°C was performed in 10sec with 0.5°C steps per cycle. RT-qPCR was conducted according to MIQE-guideline (Bustin et al., 2009).

The Cq values of gene expression analysis were normalized with the arithmetic means of reference genes. The three suitable reference genes Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH), Ubiquitin (UBQ3) and Actin gamma 1 (Actinγ1) (Tabl. 2) were selected using GenEx software. ΔCq data were transposed into $2^{-\Delta Cq}$ without normalisation against control treatments to be comparable with ELISA generated control treatment data. To further calculate the level differences between control and restrictive feeding as x-fold regulation visible in *S.aureus*, *E.coli* as well as in the untreated control cells expression $2^{-\Delta Cq}$ were transposed into $2^{-\Delta\Delta Cq}$ with second normalisation against control feeding group. Therefore level differences as x-fold expression change against the control feeding group could be presented. Outliners were identified and excluded using the GenEx software 5.0.1 (MultiD Analyses AB, Göteborg, Sweden) and its function Grubbs' test.

Statistics

Differences between restriction and control feeding of LF in milk were determined by SAS 9.2 (SAS Institute Inc., Cary, USA). The REML approach was applied to estimate

components of variance in unbalanced data. Fixed effects for time relative to calving, cow and treatment were considered in a MIXED MODEL procedure with repeated measurements to calculated significant differences between control and energy restricted feeding. The effects of time, treatment, and time x treatment interactions were tested. The significance level was *P*-values ≤ 0.05.

Sigma Plot 11.0 (Systat Software, Chicago, USA) was used to calculate significant differences of LF in the stimulated pbMEC between control and restrictive feeding. Gene expression data on $2^{-\Delta Cq}$ level, LF concentration in the cellular protein and in the culture supernatant were analysed by an unpaired T-tests. For failed normality the Mann-Whitney-U-Test was performed. *P*-values ≤ 0.05 were considered as significance level.

Results

Finally energy restricted cows experienced a greater induced energy deficiency (-63 MJ NEL/d) compared to common energetic imbalance in early lactation (- 42 MJ NEL/d) (Gross et al. 2011). Nevertheless, the indicators of metabolic imbalance NEFA and BHB did not reach critical levels. Highest blood concentrations of 0.25mmol/L and 0.6mmol/L for NEFA and BHB were detected during the restriction period, respectively. Intensive metabolic and reproductive screening of the experimental cows was conducted and published elsewhere (Gross et al. 2011).

Lactoferrin in milk

The LF concentration in the milk (Fig.2) increased continuously from the beginning of sampling in the third week pp until week 16pp. In the following week dietary energy

restriction to finally 49% was induced (grey triangles). In the first week control cows showed further increasing LF concentration (mean 147.32µg/mL) compared to restriction cows (mean 69.81µg/mL). The dietary energy restriction induced a significantly decreased milk LF secretion ($P \leq 0.005$). The level difference between control and restriction group persisted throughout the restriction period, but no further significant differences were determined in the second and third week of the induced energy deficiency. Realimentation quickly reestablished increasing LF concentration in the milk of restriction cows and further increased the course of LF within lactation.

Statistical analysis of LF gene expression and LF concentration in pbMEC-total protein and cell culture supernatant

LF gene expression

The qRT-PCR (Fig.3A) determined higher levels of LF in restriction compared to control cells and an additional pronounced effect of the *E.coli* challenge after 24h and even more after 72h incubation time. Lower effects were found in *S.aureus* stimulated cells. Significant differences were found in the untreated cells after 24h in which higher LF expression was found in the restriction group ($P \leq 0.05$). $2^{-\Delta\Delta Cq}$ data presented in the small figure present the level difference in x-fold regulation (difference between control and restriction feeding) and therefore the effect of the restriction feeding on LF gene expression. *E.coli* 24h and 72h induced a 6.5 ± 3.9 SEM and 7.3 ± 3.9 SEM fold up-regulation of LF in energy deprived cells compared to cells from the control feeding group. Also the untreated control cells of the restriction group showed 2.7 ± 1.7 SEM and 2.4 ± 1.2 SEM fold up-regulation of LF after 24h and 72h, respectively. Interestingly LF was 1.5 ± 2.8 SEM fold down-regulated in *S.aureus*

stimulated cells after 24h and only marginal 1.1 ± 0.3 SEM fold up-regulation was found after 72h.

LF concentration in pbMEC- total protein

Cellular LF of pbMEC-total protein (Fig. 3B) was also higher in restriction compared to control fed cells. Significant differences between restriction and control feeding groups were found in *S.aureus* 24h ($P \leq 0.001$), *S.aureus* 72h ($P \leq 0.005$), *E.coli* 24h ($P \leq 0.005$), *E.coli* 72h ($P \leq 0.05$) and untreated control cells after 24h ($P \leq 0.005$) and 72h ($P \leq 0.05$).

LF concentration in pbMEC-supernatant

The accumulation of secreted LF in the culture supernatant over 24h and 72h revealed the clearest effect of the energy deficiency on immune response (Fig 3C). LF concentration in the respective infection groups between control versus energy restriction treatment resulted in highly (*S.aureus* 72h, *E.coli* 24h, *E.coli* 72h: $P \leq 0.005$) and extremely significant (*S.aureus* 24h, C24h, C72h: $P \leq 0.001$) differences (3C). Especially the untreated control cells increased the production of LF considerably after 24h and 72h ($P \leq 0.001$) without being stimulated by a potential pathogen and therefore further confirm the effect of the *in vivo* energy deficiency challenge on the immune response in pbMEC.

The modulatory effect of the feeding regime is revealed in higher LF levels of the restriction compared to the control feeding group. Furthermore, high SEM were calculated for gene expression data and therefore prevent further significant differences. The variation prevented further significant differences. The variation was, however cow- and not assay-dependent, because very low coefficients of variation CV% were calculated for the conducted double approaches in all analyzed matrixes. Clear significant treatment effects were found and could be calculated due to low variation.

Discussion

The LF milk concentration during the experimental period increased up to a maximum during dry period, in accordance with Silanikove et al. (2006). In the present study the detected LF concentrations confirm also the findings of Hiss et al. (2009), but concentrations of LF at the start of the recording in week 3pp were lower.. Hiss et al. (2009) characterized the concentrations in association with blood NEFA concentrations. Enhanced fat mobilization, mirrored by high blood plasma concentrations of non-esterified fatty acids (NEFA) were associated with high milk LF concentrations, whereas low plasma NEFA concentrations were associated with low milk LF concentrations. These findings are contrary to the immune impaired effect of NEB reported in several other studies (Epperson, 2005; Sartorelli et al., 1999). In the present study the measured milk LF concentrations mirrored the changes of additional metabolic parameters of the experimental cows published in Gross et al. (2011), Thus, although the induced energy deficiency was greater than the energy deficit in early lactation, the cows did not reveal signs of critical metabolic instability indicated by elevated blood NEFA and BHB concentrations. Highest NEFA and BHB concentrations of 0.9mmol/L and 0.95mmol/L, respectively, were found in early lactation energy deficit compared to highest concentrations of 0.25mml/L NEFA and 0.65mmol/L BHB during the experimental energy restriction. However, the induced energy deficiency, beginning in week 17pp. markedly reduced the LF concentration for 3 weeks as compared with the control group. During the three weeks of energy restriction LF concentrations are significantly lower compared to the control group. However the metabolic findings in Gross et al. (2011) do not show substantial effects due to the induced energy restriction. This is contrary to Sartorelli et al. (1999) who reported declined LF concentration in states of imbalanced energy metabolism due to impaired neutrophil function. In the present study, increased BHB concentrations, seen during early lactation, were not observed during induced energy deficiency in midlactation

and therefore were not a cause for reduced milk LF concentrations. The experimental cows were able to compensate the induced energy deficiency not primarily by enhanced lipolysis and ketogenesis, but primarily by reduced milk yield. No clinical or subclinical signs of metabolic diseases were detected either (Gross et al., 2011). A reduced milk yield of only 3kg/d was found due to induced energy deficiency (Gross et al., 2011). The reduced energy intake caused a reduced ruminal microbial and mammary and possibly an overall reduced protein synthesis (including mammary LF synthesis). Milk LF concentrations increased immediately after the start of realimentation. Interestingly, significantly higher LF concentrations were found in pbMEC of energy –restricted than control cows because the cell cultures were generated at the last day of the energy deficiency period and were challenged only in their passage under culture conditions in vitro, in which the availability of nutrients was normal, i.e. as controls, thus simulating realimentation in vivo. Although the immune challenge took place in the third passage, the infection evoked a remarkable increase in LF levels in the restriction groups compared to the control fed group and an additional considerable increase in LF production due to exposition to *E.coli*. The low immune response of *S.aureus* stimulated cells can be explained by the character of the induced mastitis. Compared to *E.coli,* which leads to acute and severer forms of mastitis 24h post infection, the gram positive pathogen S.*aureus* invades the mammary gland, induces only a short and relatively weak immune response within the first 3h of invasion (Griesbeck-Zilch et al., 2008; Lahouassa et al., 2007) and thereafter persists nearly undetected and often lifelong. It is further associated with impairment of the immune system, which assists the development of subclinical and chronicle mastitis (Matsunaga et al., 1993).

In the present animal study the incidence of mastitis and of other metabolic related diseases was not greater in the energy-restriction group than in the control group (Gross et al. 2011). Therefore one can presume that the acquired immune system (of which LF was evaluated in

the present study) and the acquired immune system both successfully protected against infections.

Conclusion

LF as member of the innate host defense system is influenced by the dietary energy status. The provoked energy deficiency in mid lactation caused a reduced milk LF concentration. Following realimentation and nutrient re-supply according to requirements, milk LF concentrations immediately increased to levels of controls. The pbMEC derived from energy restricted cows under *in vitro* conditions, even in their third passage, produced more LF than those derived from normally fed cows, suggesting a memory effect, induced by the induced preceeding energy deficiency *in vivo*. Greater LF responses *in vitro* than in unchallenged controls were seen in the presence than in the absence of E.coli but not in the presence of *S.aureus* and effects of *E.coli* were greater in pbMEC derived from energy restricted than control cows. Maybe epigenetic changes occurred during restriction *in vivo* having also effects on the pbMEC *in vitro*. Further investigations need to be done to answer this hypothesis. Nevertheless, the present study indicate 1) evidence of innate immune compensatory mechanisms after an energy deficiency, which is even present in the untreated control cells of the restricted and control fed cows and 2) the suitability of primary cell culture models even in the third passage of cultivation due to an impressive physiological memory ability.

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Figures and Tables

Figure 1

Immunhistostaining of cytokeratine confirms the epithelial identity of the generated primary bovine cell cultures. The successful staining is presented by the positive and negative control.

Figure 2

Milk LF throughout the whole experimental period in µg/mL ± SEM. -○- = control group, -▲- = restriction group; significant level $P \leq 0.05$ *, $P \leq 0.005$**, $P \leq 0.001$ ***

Figure 3

LF in cell culture of control and energy restriction cows after infection with heat inactivated *S.aureus*, *E.coli* and untreated control cells after 24h and 72h incubation time (S24, S72, E24, E72, C24, C72). A: $2^{-\Delta Cq}$ gene expression data, $2^{-\Delta\Delta Cq}$ with normalization against untreated control group, B: LF in MEC-total protein in ng/mL, C: LF in cell culture supernatant in ng/mL; significant level $P \leq 0.05$ *, $P \leq 0.005$**, $P \leq 0.001$ ***

Table 1

Diet compositions of the experimental feeding regime

Table 2

Lactoferrin and reference gene primer pairs for qRT-PCR: sequence, product length in base pairs (bp), annealing temperature in °C and NCBI accession number.

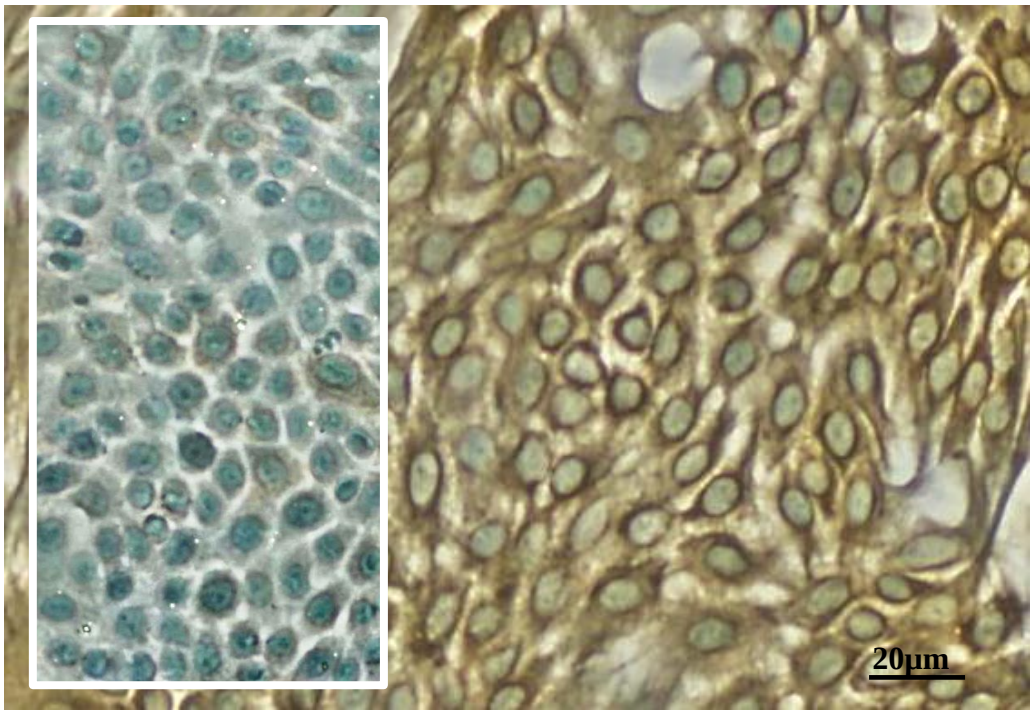

Figure 1

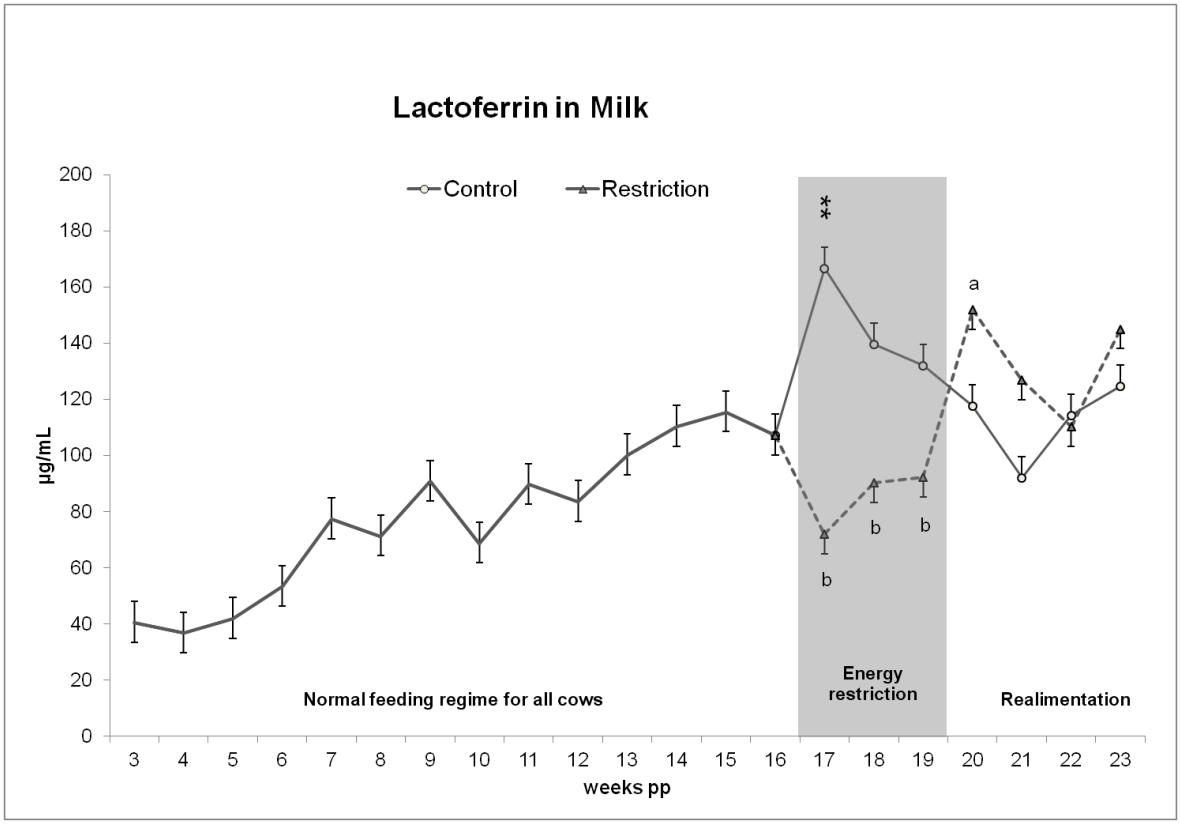

Figure 2

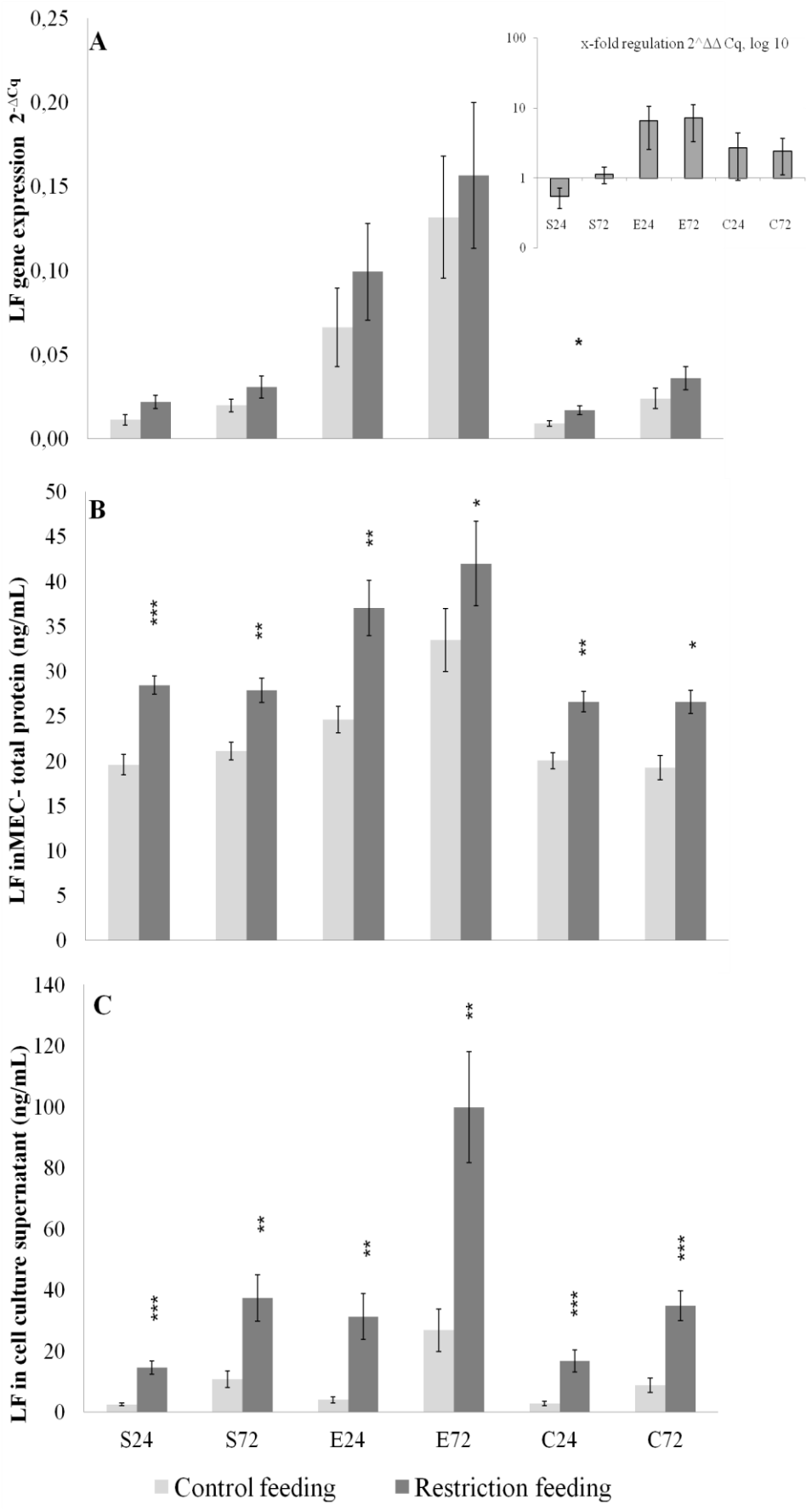
A
LF gene expression 2-ΔCq
0,25
0,20
0,15
0,10
0,05
0,00
x-fold regulation 2^ΔΔCq, log 10
100
10
1
0
S24
S72
E24
E72
C24
C72
*
B
LF inMEC- total protein (ng/mL)
50
45
40
35
30
25
20
15
10
5
0
***
**
**
*
**
*
C
LF in cell culture supernatant (ng/mL)
140
120
100
80
60
40
20
0
***
**
**
**
***
***
S24
S72
E24
E72
C24
C72
Control feeding
Restriction feeding

Figure 3.

Table 1

	PMR1[1]	PMR2[1]	Concentrate[2]
Components (% in DM)			
Grass silage	33.7	21.8	
Corn silage	44.9	29.1	
Hay	6.5	39.4	
Concentrate[3]	14.9	9.7	
Nutrient values			
MJ NE_L/kg DM[4]	6.53	6.24	7.96
Crude fibre (g/kg DM)	214	251	62
Crude ash (g/kg DM)	76	75	76
Crude fat (g/kg DM)	32	28	24
Crude protein (g/kg DM)	146	138	216
ADF (g/kg DM)[5]	254	313	84.1
NDF (g/kg DM)[6]	431	529	184
NFC (g/kg DM)[4,7]	316	230	500
Available crude protein (ACP) (g/kg DM)[4]	143	137	172
Ruminant nitrogen balance (RNB) (g/kg DM)[4]	0.88	0.18	2.37

[1]Partial mixed ration
[2]Additional concentrate was fed in amounts dependent on milk yield and consisted of 14.9% barley, 24.8% maize, 21.8% wheat, 20.1% soybean meal, 15.2% dried sugar beet pulp with molasses, and 3.2% vitamin-mineral-premix including limestone
[3]Concentrate: 7.9% barley, 24.7% wheat, 60.0% soybean meal, 7.3% vitamin-mineral-premix including salt and limestone.
[4]Calculated values
[5]Acid detergent fibre
[6]Neutral detergent fibre
[7]Nonfibre carbohydrates calculated by difference: 100 - (%crude protein + %NDF + %crude fat + %crude ash)

Table 2

Target genes	Sequence		Product	Annealing	Reference
Lactoferrin	forward	cga agt gtg gat ggc aag gaa	215 bp	64 C°	NM_180998.2
	reverse	ttc aag gtg gtc aag tag cgg			
Reference genes					
Actin gamma 1	forward	aactccatcatgaagtgtgacg	233 bp	60°C	NM_001033618
	reverse	gatccacatctgctggaagg			
Ubiquitin 3	forward	agatccaggataaggaaggca t	198 bp	60°C	NM_174133
	reverse	gctccacttccagggtga t			
Glyceraldehyde 3-Phosphate	forward	gtcttcactaccatggagaagg	197 bp	60°C	Berisha et al. 2002
Dehydrogenase	reverse	tcatggatgaccttggccag			

MATABOLIC STATUS AND OESTROUS CYCLE IN DAIRY COWS

Dairy cow reproduction: Does a natural or induced energy deficiency influence oestrous cycle activity?

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22 Abstract

23 A study with 40 multiparous high yielding dairy cows was conducted to investigate the
24 influence of an induced negative energy balance (NEB) on reproductive performance.
25 Energy restriction of 49% was performed for 3 weeks beginning on oestrous cycle day 12 of
26 first oestrous cycle after day 85 post partum (pp). From day 20 to day 150 pp animals were
27 monitored for ovary activity three times weekly using rectal palpation and transrectal
28 ultrasound scanning and were inseminated around day 150pp. Additionally, milk
29 progesterone and milk hydrocortisone were analyzed twice a week. Body condition score
30 and body weight as well as blood glucose, plasma nonesterified fatty acids and plasma β-
31 hydroxybutyrate were recorded weekly. According to oestrous cycle activity before (period 1
32 = natural energy deficiency), during (period 2) and after (period 3) induced energy restriction
33 animals were assigned to the following groups: delayed first ovulation until day 45pp, normal
34 oestrous cycle, prolonged oestrous cycle and shortened oestrous cycle. Sporadic
35 significances but no clear effect of the metabolic state on reproductive performance could be
36 found during period 1 and period 2. Service success and conception rate were also not
37 influenced. Our results demonstrate a remarkable adaptation of reproductive activity to
38 metabolic challenges. Animals were able to compensate natural NEB in period 1 as well as
39 induced NEB (period 2) for preventing metabolic disorders and maintaining reproductive
40 activity. Therefore dietary energy availability had no effect on reproductive performance at
41 more than 85 DIM in the present study. To understand reproductive failures in dairy cows
42 focus should be laid on genetic disposition of high yielding individuals that cope successful
43 with metabolic challenges.

44 Keywords: ovarian cycle - negative energy balance - dairy cows

Introduction

The interaction between nutritional status post partum (pp) and reproductive performance in high yielding cattle is still a serious and sustained problem in dairy cows. Over the past years breeding led to increased milk yields accompanied by declining health (Schlamberger *et al*., 2010) and calving rate (Royal et al., 2000, Schlamberger et al., 2010). High yielding cows often face metabolic and reproductive problems that decrease profitability by increased services per conception, longer calving intervals, higher costs of replacement heifers and veterinarian services (Inchaisri et al., 2010). Interestingly, many but not all dairy cows of high producing breeds are affected by the negative side of high milk yield. There are animals that are able to cope with metabolic challenges post partum without suffering from metabolic and reproductive failures. During early lactation metabolism is affected by high mobilisation of body reserves to compensate the nutrient requirements for the high level of milk production leading to a negative energy balance (NEB). As adequate feed intake cannot be achieved, required energy is provided by mobilization of body mass. Metabolic factors often associated with NEB are decreased blood glucose followed by increased nonesterified fatty acids (NEFA) and β-hydroxybutyrate (BHB), which may lead to metabolic disorder signs (Drackley, 1999). Reproductive failures caused by metabolic instability are anovulation and anoestrus (Scaramuzzi et al., 2006) as well as far reaching consequences for the next conception as retained placenta and endometritis. Different studies have been conducted to improve energy balance (EB) using dietary fat supplements, increased dietary energy content or reduced milking regime (Grummer and Carroll, 1991; Patton *et al*. 2006; Schlamberger *et al*., 2010). However, the improvement of EB due to diet supplements or altered milking regime often lack beneficial effects, especially on reproductive performance. Although a definite relation between dietary energy source and reproductive performance has not been established yet, it is evident that elevated blood insulin and glucose values due to glucogenic diets have a beneficial effect on fertility in contrast to elevated NEFA and BHB blood concentrations (van Knegsel *et al*., 2007). There are significant genetic correlations between body condition score (BCS) dynamics, total body energy content or NEFA concentrations

and metritis that indicate the influence of EB not only on reproductive ability but also on reproductive health (Oikonomou *et al*., 2008). Although the association of BCS with production and reproduction is nonlinear, it is a likely candidate to predict health status in early lactation with an optimum BCS at calving of 3.0 to 3.5 (five point scale). Lower calving BCS indicate reduced production and reproduction whereas BCS >3.5 at calving are associated with lower dry matter intake and milk yield as well as increased risk of metabolic disorders pp (Roche et al., 2009). After calving, re-establishment of cyclicity is dependent on pulsatile LH secretion to allow ovulation of a dominant follicle. During energy deficit in early lactation, pulsatile LH secretion pp can be suppressed and ovarian responsiveness to LH pp can be reduced (Butler, 2000). Vanholder et al. (2005) indicate, that through metabolic and hormonal adaptations caused by NEB, the hypothalamic-pituitary function and further on the follicular growth and development may be affected. They presume that genetic hereditary factors associated with cystic ovarian follicles may promote or increase the functional importance and therefore influence follicular growth or hypothalamic-pituitary function. Another important link between NEB and fertility seems to be the interval to first ovulation. Minimizing this time provides enough time to complete multiple ovarian cycles prior to insemination, which in turn improves conception rate (Butler and Smith, 1989). But even high milk yield seems to have different effects on fertility. In Leitgeb and Van Saun (2009) high milk production during first 40 days of lactation results in impaired conception whereas high peak milk results in longer days open and calving interval. But still, cows with the highest milk yield do not show the lowest fertility. The influence of nutrient state on metabolism seems clear, but on reproduction remains uncertain due to individuals that are not affected by metabolic and reproductive failures during early lactation. In order to investigate the effect of dietary energy on reproduction especially on ovarian cycle activity and insemination success an animal trial with high yielding dairy cows was conducted. To ensure consistent effects of the induced dietary energy deficit in all animals, energy restriction was performed during mid-lactation when normal oestrous cycle was adjusted and a positive metabolic status was re-established.

Materials and Methods

The animal trial had been approved by the responsible committee of the government of Upper Bavaria and was carried out on the research farm Hirschau of the Technische Universität München.

Animals

40 multiparous Red Holstein cows were housed in a free-stall barn and fed a partial mix ratio (PMR1) (Tab. 1). Individual feed intake was measured automatically by electronic load cells and automatic feeders dispensed concentrate. Animals were machine-milked twice daily and milk yield was recorded (Gross et al., 2011). Animals were monitored from day 20 pp until day 150 pp. The experiment was divided into three experimental periods. First 85 days of lactation were classified into period 1 in which all cows were treated equally and received the same partial mixed ration ad lib (PMR1, Tab. 1). In period 2 animals were grouped equally into control group (n=20) and restriction group (n=20) according to the cow specific individually calculated energy balance in period 1. A calculated energy deficit (PMR2) was conducted for three weeks, beginning on approximately day 100 pp, but exactly on day 12 of oestrous cycle after day 85 pp. In the following period 3 of realimentation animals received the same diet as control cows (PMR1). Animals were assigned to the experiment and monitored for metabolic and reproductive screening from day of calving until insemination in period 3 at about day 150 ± 1.2 SEM pp. Detailed information is published in Gross et al. 2011.

Feeding regime

During first five days of lactation a daily PMR1 was offered with additional 1.3kg DM/d concentrate (CONC). PMR1 was calculated for 650kg body mass, an assumed daily feed intake of 16kg DM/d and daily milk production of 21kg. From day 6 until day 35pp additional CONC was increased from 1.8kg DM/d until a maximum of 8.9kg DM/d. Thereafter PMR1 was enhanced by individual CONC adjustment, according to milk yield which was automatically dispensed. During induced energy deficit (period 2) restriction animals received PMR2 (Tab. 1) reduced in energy by hay addition, reduced concentrate content and reduced feed amount (Gross *et al*., 2011). Control cows were fed PMR1 ad libitum, throughout the whole experimental time. In period 3 restriction cows were realimented ad libitum with PMR1 until the end of the experiment (Gross *et al*., 2011).

Energy restriction and experimental period

Induced energy deficit was accomplished according to Gross et al. (2011). Detailed information about feeding regime, metabolic screening and performance can be found there. In short, animals were assigned equally to control and restriction groups by means of their NEB during period 1. According to the difference of dietary energy intake and energy output by maintenance and milk yield during period 1, energy balance was calculated for each cow individually. On the first day 12 of oestrous cycle after average day 85 pp when early postpartum NEB is over and metabolism is re-stabilized dietary energy restriction of 49% of the total requirement was performed for three weeks (period 2). Length of period 2 was chosen to enable one ovulation during energy restriction for determining the effect on cycle length. In the following 8 weeks of realimentation after period 2, animals were fed the same diet ad libitum as control group (PMR1) (Gross *et al*., 2011). In the present work focusing on reproduction, period 3 is defined as begin of realimentation until second ovulation after energy restriction (day 150 ± 1.2 SEM pp). Animals were inseminated at the second oestrus.

Metabolic parameters

For metabolic screening body condition parameters and plasma metabolites were collected and analyzed. Detailed information can be found in Gross et al. (2011). In short, body weight was recorded automatically on electronic scales integrated in the concentrate dispensers every time the cows entered the stations. Body condition score (BCS) were monitored simultaneously once per week. BCS was performed according to Edmonson et al. (1989) in a five point scale. Weekly blood samples were analyzed for glucose, NEFA and BHB. Glucose concentrations were measured by kit no. 61269 from bioMérieux (Genève, Switzerland). Plasma NEFA was analyzed with kit no. FA 115 and BHB with kit no. RB 1007 from Randox Laboratories Ldt. (Schwyz, Switzerland) according to the manufactors instructions. Data are shown in Gross et al. 2011.

Fertility and oestrous cycle groups

Ovulation detection was conducted three times per week by transrectal ultrasound scanning and rectal palpation accompanied by twice a week milk progesterone analysis until insemination around day 150 pp. In terms of reproductive performance in period 1, animals were grouped into delayed first ovulation showing first ovulation after 45 days pp (DOV) and animals with normal first ovulation (NOV), which were again grouped according to oestrous cycle lengths into normal (NOC) of 18-24 days, prolonged (POC) of >24 days and shortened (SOC) oestrous cycle <18 days. Cows with at least one prolonged or shortened oestrous cycle during period 1 were assigned to the POC or SOC group, respectively. DOV cows were not grouped into NOC, POC or SOC in period 1. In period 2 and again in period 3 animals were grouped anew according to length of oestrous cycle into NOC, POC and SOC. After period 3 on average day 150 ± 1.2 SEM pp, first insemination service was conducted (Fig. 1). Data for the parameters services per conception and total conception rate were collected.

Milk progesterone

Progesterone was measured twice per week in fat-free milk samples by an enzyme immuno assay (EIA) as described earlier (Prakash et al., 1988) but using the monoclonal antibody anti-progesterone clone 2H4 (1:3500) from SIGMA-Aldrich (München, Germany). The used label (1:3500) was progesterone-3CMO (Steraloids Inc., Rhode Island, USA.) coupled to horseradish peroxidase (Roche Applied Science Mannheim, Germany). Inter- and intra-assay coefficients of variation were 13.46% and 5.69% respectively. The detection range was from 0.2ng/ml to 3ng/ml in skimmed milk. Milk progesterone results were used to determine ovulation times in addition to ultrasound scanning and rectal palpations. Values >1ng/ml were assigned to preceded ovulation and a functional corpus luteum, values <0.2ng/ml indicated no ovarian activity.

Milk hydrocortisone

Metabolic stress was evaluated by milk hydrocortisone and was measured twice a week in skimmed milk. Measurement of hydrocortisone was done by EIA as developed earlier for plasma and tissue (Sauerwein *et al*., 1991). Hydrocortisone-21-glucuronide (Steraloids) was labeled with horseradish peroxidase (1:12000) (Roche Applied Science, Mannheim, Germany) as described for other steroids earlier (Meyer 1986). The polyclonal antibody (C1 Pool2) in 1:90000 dilution had been raised in rabbits against hydrocortisone-21-hemisuccinate-BSA, its crossreactivities are: hydrocortisone 100%, cortisone 8%, corticosterone 9.5%, prednisolone 18% and dexamethasone <0.1%. Hydrocortisone standards in skimmed milk treated with activated charcoal ranged from 0.1 to 34.5nmol/l. 10µl skimmed milk in duplicates was measured in 96 well microtiter plates using a double antibody technique. For the determination of recoveries, aliquots of skimmed milk were treated with charcoal and then spiked with hydrocortisone (SIGMA-Aldrich, München,

Germany). The mean recovery was 106.48 % ± 11.93 %. Inter- and intra-assay coefficients of variation were 12.38% and 7.42%, respectively.

Statistical Evaluation

The statistical evaluation has been done using Sigma Plot 11.0 (Systat Software, Chicago, USA), the R Project for Statistical Computing (http://www.r-project.org, R Development Core Team, 2011) and the Weka Machine Learning Framework (http:sourceforge.net/projects/weka, Hall *et al*., 2009). First, it was tested for each week whether a significant difference in the range of each metabolic parameter could be observed if three groups are formed in accordance to cycle length and time of the first ovulation (NOC, POC and DOV). Either a one-way Analysis of Variance (ANOVA) for normally distributed data or a Kruskal-Wallis variant working on ranks was applied if Sigma Plot reported non-normal samples (Fig. 3). Multiple Testing Correction was done using sequential Holm-Sidak correction. Differences in the metabolic data for period 2 and 3 were tested by either T-test or via Mann-Whitney Rank Sum test for not normally distributed data (Fig. 5, 7). Each metabolic parameter is expressed as Area Under the Curve (AUC) for period 1. Mean values are used for period 2 and 3. In case of missing values we impute median values across all measured cows. An all-pairwise metabolic parameter correlation matrix was computed for each period (using Pearson correlation). If the correlation of a parameter exceeds a threshold of 0.65 to one or more others two separate models excluding either parameter set were build. A binary classification setting is induced by our grouping procedure: the cycle length fractions are treated as dependent variables (i.e. 2 classes) and the metabolic parameters represent independent measurements. Models have been built for all three periods comparing NOC, POC and DOV, respectively.

A Multiple Logistic Regression (MLR) model was trained using the SigmaPlot Maximum Likelihood Fitting procedure on all cow parameters, the Wald test statistics and associated p-values for each parameter and classification problem (Tab. 3) was reported. In addition, the

229 WEKA logistic function (using a ridge penalty of 1e-8) was used to estimate the predictive
230 power of the logistic models by setting up a leave-one-out (LOO) validation procedure: each
231 cow is left out from the model building process once and is predicted using the model trained
232 on all other cows' parameters. This process yields a constant estimate of the models
233 predictive power. The area under Receiver Operator Characteristic Curve (AUROC) of all
234 single predictions combined is reported. An AUROC value around 0.5 shows that the
235 underlying model is not better than random guessing. Note that the LOO-AUROC is still likely
236 an overestimate due to over-fitting of the model to the observations, yet it is (1) more
237 conservative than training a model on all cows and (2) imposes an upper bound on any k-fold
238 cross-validation procedure i.e. if the predictive power during LOO is low the model will likely
239 perform worse when applied on further observations.

240 A comparison of cycle length for period 2 and 3 between restriction and control was
241 conducted using z-tests (Fig. 4, 6) on the two group proportions, where H_0 assumes equal
242 proportions of restriction and control group. Similarly, z-tests were applied to detect
243 differences in the number of services until conception and the conception rate (Fig. 8).

244

245 Results

246 Period 1

247 In period 1, during first 85 days pp in 6 animals no ovulation could be detected until day 45pp
248 (DOV). 11 animals showed normal oestrous cycles (NOC) and 23 animals showed one or
249 more prolonged oestrous cycle (POC) (Fig. 2A). Time of first ovulation after calving took
250 place later in DOV compared to NOC and POC but did not differ among NOC and POC
251 (Fig.2A). Oestrous cycle lengths in DOV and NOC were constant between 18-24 days
252 whereas the cycle length in POC showed a high variation (Fig.2B). All cows of DOV, NOC
253 and POC showed a negative EB around -40MJ NEL/day in the first week pp. A positive EB
254 was reached at day 40 pp for NOC and day 50 pp for POC and DOV (Fig. 3A). The average

255 milk yield was 35.27 ± 6.72kg/day (fat 5.02 ± 0.74%, protein 3.53 ± 0.52%) for each cow. The
256 energy corrected milk (ECM) yield ((0.38 x fat% + 0.21 x protein% + 1.05) / 3.28 x milk yield
257 kg) (Fischer et al. 2002) was 36.35 ± 6.10kg/day. In period 1 blood metabolites showed
258 expected courses during negative energy balance in early lactation (Fig. 3). Glucose was on
259 low levels of 3.3mmol/L in the second week pp and increased according to energy balance
260 (Fig. 3B). NEFA reached highest levels of 1mmol/L in the second week pp in DOV and
261 showed lower levels but equal courses in NOC and POC, with a significant difference
262 between DOV and POC on day 29 pp (Fig. 3C). BHB concentrations increased up to peak
263 values of 1.2mmol/L with high variation in POC and decreased until base levels were
264 reached on day 50 pp. Similar progressions of DOV and NOC could be found but NOC
265 decreased earlier and showed a significant difference on day 29 pp compared to DOV. Milk
266 hydrocortisone (Fig. 3E) is not influenced by metabolic state during early lactation. The
267 higher value for DOV on day 56 pp resulted of one outlier indicated also by error bars.
268 According to body mass mobilization in period 1 DOV showed lower BCS by trend compared
269 to NOC and POC. This difference was only significant on day 85 pp (Fig. 3F).

270 Period 2

271 During restriction phase (period 2) animals were divided into restriction and control group
272 with 20 animals each. No difference in cycle length between control and restriction group
273 could be found (Fig. 4A). Within the control group 12 cows were assigned to NOC, 7 animals
274 to POC and only 1 cows showed a short ovarian cycle (SOC). In the restriction group 16
275 cows showed normal cycle lengths. Prolonged cycles could be found in 4 animals and no
276 short cycle was detected in the restriction group (Fig. 4B). The average milk yield during
277 period 2 for the control group was 29.91 ± 5.93kg/day (fat 4.35 ± 0.53%, protein 3.37 ±
278 0.30%) (ECM 30.95 ± 5.66 kg/day). Milk yield was slightly reduced in restriction group to
279 27.59 ± 4.92kg/day (fat 4.50 ± 0.66%, protein 3.21 ± 0.28%) (ECM 28.64 ± 4.24 kg/day).
280 Calculated energy balance of 49% reduction during induced energy restriction (period 2) in
281 figure 5A showed more sever negative deficiency than in postpartum NEB with highly

significant differences ($p<0.001$) between restriction and control group for NOC and POC. Short ovarian cycle (SOC) was detected only in control group. Significant differences ($p<0.05$) between restriction and control group for NEFA and BHB within NOC could be found (Fig. 5B, C). Milk hydrocortisone revealed no effect of energy restriction and showed no differences between oestrous cycle groups (Fig. 5D). There were no critical metabolic states detected during energy restriction and no animal had to be treated due to metabolic problems (Tab.2).

Period 3

After energy restriction (period 3) the same tendency in cycle length could be found, but again no clear effects were observed (Fig. 6, 7). During realimentation 13 cows from the restriction group (66.7%) showed normal ovarian cycles compared to 9 control cows (37.5%). 6 prolonged cycles (27.7%) were detected in restriction group compared to 9 cows in control group (35%). In the restriction group only one cow showed a short luteal phase compared to 2 animals in control group. The average milk yield for control group was 27.98 ± 5.53 kg/day (fat 4.31 ± 0.66%, protein 3.43 ± 0.30%, ECM 28.88 ± 5.03kg/day). Surprisingly the average milk yield for the restriction group was slightly higher: 28.67 ± 5.44kg/day (fat 4.33 ± 0.74%, protein 3.42 ± 0.28%, ECM 29.54 ± 4.98 kg/day). Restriction cows still showed mean lower energy balances in NOC and POC, where as SOC cows were in a positive balance compared to control SOC (Fig. 7A). Blood NEFA concentrations were on low levels in control and restriction group and showed no differences between oestrous cycle groups (Fig. 7B). Restriction animals with normal oestrous cycle activity had significant higher BHB concentrations (Fig. 7C) than control cows in NOC ($p<0.05$). BHB and hydrocortisone (Fig. 7D) were higher in SOC, but no significance was found. In Figure 8A data of insemination success and services per conception of the restriction and control group are presented, but there was no significant effect due to previous energy restriction. Conception rate of first service was 64% in the restriction group and 54% in the control group. Second service rate was 29% and 23% for restriction and control animals, respectively. Whereas third service till

309 conception was 7% for restriction and 15% for control group. After 4th service conception rate
310 was 8% for control animals. No restriction cow received more than 3 services until
311 conception. The overall conception rate (Fig. 8B) in restriction and control group was 70%
312 and 68%, respectively, showing again no influence of the provoked energy deficiency.

313 Multiple logistic regression

314 The effect of metabolic parameters on oestrous cycle length and time of first ovulation was
315 tested by multiple logistic regression for period 1, 2 and 3 separately. All-pairwise metabolic
316 parameter correlation matrix for each period (using Pearson correlation) showed a
317 correlation for NEFA and BHB / EB above 0.65 or rather under -0.65. Therefore two separate
318 models excluding either parameter set were built. WEKA was used to set up a leave one out
319 (LOO) cross-validation which was then taken to evaluate the predictive power of each model.

320 Note that the LOO approach reflects the basic assumption of most experiments quite well: a
321 model build on a subset of cows should hold if it is applied to further individuals. LOO yields
322 an upper border on this performance since the training skips only one instance per model.
323 Yet, this validation procedure is more conservative than any model build on the complete
324 data set which would easily overfit the underlying model. The area under the ROC curve
325 values (AUROC) is reported for the combined folds of the LOO procedure. An estimated
326 result above 0.6 would indicate that the model and thus the parameters have at least weak
327 predictive power considering group separation. None of the AUROC values exceed this
328 threshold. As shown in table 4 none of the measured parameters differs between our groups
329 and may therefore not provide decision support on this data set. In some cases the number
330 of cows per group (class dist, Tab. 4) is highly uneven and results in artificially small AUROC
331 values. Here, the complete data are additionally inspected without any model assumption at
332 all. All raw data used during the MLR training are provided in Fig. 9. No indications of
333 divergent distributions among the respective classes were observed as well. The LOO
334 results are consistent with the Wald test statistics given by logistic regression models trained
335 with SigmaPlot on the complete data set. In summary, no effect of the metabolic parameters

on oestrous cycle length or time of first ovulation in all three periods could be found (Tab. 3, 4, Fig. 9).

Discussion

In the current study we investigated the influence of a natural negative energy balance in early lactation and an induced energy deficit, conducted around day 85pp for 3 weeks, on ovary activity and cycle length. Furthermore, time of realimentation after energy restriction was related to ovary function and the insemination success after the induced energy deficit was determined. To point the metabolic challenge during common and induced NEB as well as the realimentation phase, blood glucose, blood NEFA and blood BHB were determined throughout out the experiment and the reproductive findings were referred to them. The early NEB and the induced dietary energy restriction of finally 49% revealed neither metabolic nor reproductive parameters. Marginal significant effects between metabolic challenge and reproductive parameters were found, but only on low significant levels and without explicit allocation. Our metabolic data showed the known characteristics of early lactation with elevated concentrations of blood NEFA and BHB (Fig. 3). According to the classification of (Huszenicza et al., 1988) which was also used in Kessel et al. (2008), only 16 animals had higher BHB values than 1mmol/L during first 85 days pp. In contrast to Kessel et al. (2008) NEFA and BHB values decreased already on day 20pp and reached base values on day 50 indicating the end of negative energy balance. Comparing these findings to cycle activity in period 1 POC showed highest but mostly not significant BHB values compared to NOC and DOV whereas DOV had higher NEFA values compared to NOC and POC by trend. These findings are confirmed by Patton et al. (2006) who found no association of NEFA and BHB in early lactation with resumption of cyclicity but are contrary to other studies (Kafi and Mirzaei, 2010). Furthermore, increased ketone concentrations in early lactation were shown to elevate the risk of delayed ovarian activity (Reist et al., 2000) and is considered to be an indicator of the severity of negative energy balance (Vanholder et al., 2006). This confirms

363 our findings of only 6 animals with DOV, as the phase of critical BHB concentrations
364 indicated by more than 1mmol/L in early lactation is shorter compared to other studies and
365 fewer animals in our trial have delayed first ovulations. On the other hand these animals do
366 not show exceeded BHB concentrations, only one cow has elevated BHB concentrations
367 higher than 1mmol/L. However, BHB concentrations in POC during first 3 weeks of lactation
368 revealed higher levels compared to NOC (Fig. 3C). Therefore we suggest that the metabolic
369 imbalance might influence prolonged luteal phases but has no effect on time of first ovulation
370 pp in our experiment. Never the less, our animals were able to endure common NEB
371 postpartum and the provoked NEB and did not contract metabolic derived dysfunctions or
372 showed higher disease susceptibility within the experimental periods (Tab. 2).

373 During dietary energy restriction (period 2) cycle activity was also not influenced. This is
374 confirmed by BHB, which only exceeds the threshold of 1mmol/L in 4 single measurements
375 and NEFA being elevated over 0.6mmol/L in only 2 samples. Although restriction and control
376 group have low but significant different NEFA and BHB levels and energy balance was even
377 more negative during restriction phase than in early lactation (-62.7± 1.8 MJ NEL/d, Fig.3) no
378 negative effect of the induced energy deficiency on reproduction was revealed, whereas in
379 other studies higher mean concentrations of these metabolites were found (2009, Perkins et
380 al., 2002). This may explain the low reproductive responsiveness of induced NEB in the
381 present work, as elevated BHB concentrations, more than NEFA, seem to have a strong
382 negative correlation with reproductive performance (Oikonomou et al., 2008). However, the
383 restriction intensity to almost only half of the requirement (49%) for up to 3 weeks which even
384 exceeded the energy deficit in early lactation has not been performed in any other study than
385 in the present trial conducted by Gross et al. (2011). Different restriction levels can be found
386 in literature using NEFA and BHB as indicators for the restriction severity. Perkins et al.
387 (2002) investigated disease susceptibility and used a restriction level to 80% of maintenance
388 energy requirements for two weeks. Another group performed 60% of calculated net energy
389 for lactational requirements for seven days in order to challenge immune function (Moyes et
390 al., 2009). For simulating ketosis 50% of control intake for up to 14 days was fed (Loor et al.,

2007) or even only 16% energy intake of the control group for 60h in order to investigate energy homeostasis and metabolic adaptations was conducted (Kuhla et al., 2007). But no work examined reproductive performance according to metabolic challenge. Furthermore the animals in our study commenced ovulation before the feeding restriction was imposed. According to Burke et al. (2010) this may have protected the energy restricted cows from more considerable negative effect on oestrous cycle as well as the finding that the ability of performing several oestrous cycles improves not only cyclicity during dietary restriction (Burke et al., 2010), but also services per conception and conception rate (Butler and Smith, 1989, Butler, 2000). Although there was no significant influence, there is a trend in restrictively fed animals showing almost more stable ovarian cycles and less prolonged and short cycles during and after restriction time than control animals. One could presume that energy deficit does not have an effect reproductive performance, but almost improves it. These findings only by trend however might be due to lower NEFA and BHB concentrations as a compensation of the energy restriction phase during realimentation (period 3) due to reversion to normal diet and feed intake in the restriction group compared to control cows. This phenomenon in trend can also be detected in elevated milk yield (Gross *et al*., 2011) after restriction compared to the control group. However, our overall findings suggest no significant influence of the restriction phase on reproductive failures. Compared to the findings of Burke et al. (2010) showing lower first (50%) and second (47%) service rates than in the present study, but a final pregnancy rate of 93% among all treatments, our final pregnancy rate of only 69% among both control and restriction group can be explained by monitoring only 4 services. Animals with more than 4 inseminations were not recorded anymore. Furthermore, cows were inseminated after the experimental phase. Therefore time of first service on around day 150 is even later than conducted in practice. But in terms of the performed treatment all pregnant cows calved without problems and healthy calves were born. Never the less, the performed energy restriction seems to be not long enough to provoke severe effects on reproductive action. Furthermore through a successful conditioning during transition period prior to the experiment, animals might have been able to

endure early lactation without exposure of the well known metabolic and reproductive disorders and to be protected metabolically of the effects induced during energy restriction. On the other hand milk yield decreased (Gross *et al*., 2011) and body mass was mobilized considerable during early postpartum NEB (Fig. 3F). However, the present results demonstrate a high metabolic adaptation of the selected dairy cows that are able to compensate for common and provoked NEB without developing severe metabolic and reproductive disorders. Beside the metabolic challenge animal health was not affected in any ways. The documentation of the experimental cows' health status is summarised in Table 2. No serious problems according to the induced energy deficit appeared. The results of our comprehensive study point, that an energy deficiency alone might not be the reason for metabolic imbalance and reproductive failures in dairy cattle. Moreover there seems to be no relationship between energy deficiency in early and midlactation and the well known metabolic diseases emerging in the state of energetic imbalance, which were also considered to influence reproductive ability in high yielding cows. More research is needed to elucidate the more complex reasons of negative energy balance with the biological mechanisms which prevent the appearance of fertility problems in some resistant high yielding milk cows compared to those animals that suffer from metabolic instability in the early post partial phase.

Conclusion

The negative correlation between high milk yield and reproductive performance is still a serious problem in milk production and seems more complex than often presumed. The high energy requirements for lactation pp and the subsequent energetic imbalance were meant to be responsible for metabolic and reproductive dysfunctions. But there are animals which developed mechanisms to overcome common NEB without being affected by reproductive dysfunction and metabolic diseases. In our comprehensive animal study, the provocation of an energy deficiency of up to 49% of total energetic requirements in midlactation after the

446 phase of early postpartum NEB resulted in almost no effect on metabolic stability and
447 reproductive ability. Our data show that there is no connection between a 49% energy
448 deficiency for 3 weeks and metabolic imbalance on the one hand as well as fertility problems
449 in terms of oestrous cycle and insemination success on the other hand. Therefore an energy
450 deficiency alone seems not sufficient to induce the focused reproductive failures in our dairy
451 cows.

452

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459

Figures and Tables

Table 1: Composition and nutrient values of experimental diets and concentrate.

Table 2: Number of health disorders during experimental periods

Table 3: Associated p-values for each metabolic parameter and classification problem applying Wald test statistics for multiple logistic regression.

Table 4: Overview of Area under Receiver Operator Characteristics for leave-one-out Multiple Regression Models. Since NEFA and BHB/EB show absolute correlations above 0.65 or rather under -0.65 in period 1 we train three independent models here. The row AUROC includes both, while “Not BHB, EB” and “NEFA” exlude BHB and EB or NEFA, respectively.

Figure 1: Experimental design of the animal trail. Period 1 (day (d) of calving (0 post partum (pp)) till the 12^{th} day of the oestrous cycle after 85^{th} pp) covers the natural energy deficit. Oestrous cycle length and day of ovulation was recorded from day 20 pp on. The cows were group according to their first ovulation in normal first ovulation (NOV) before day 45 pp or delayed first ovulation (DOV) after day 45 pp. The NOV group was furthermore divided according to their oestrous cycle length in normal oestrous cycle (NOC) or prolonged oestrous cycle (POC). Cows with at least one prolonged oestrous cycle during period 1 were assigned to the POC group. The numbers of cows in each group are shown. Cows were equally divided in control (black numbers of cows) and restriction group (red numbers of cows) in period 2. A restrictive feeding of 49% of total requirement was performed for 3 weeks. Cows were assigned anew according to their oestrous cycle length during period 2 to NOC, POC or SOC (shortened oestrous cycle). A realimentation was conducted in period 3 followed by an insemination on the second oestrus after period 2 (day 150 ± 1.2 SEM pp). The cows were grouped again into NOC, POC or SOC according to their first oestrous cycle length in period 3.

Figure 2: A) Time of first ovulation in days in milk (DIM) and length of oestrous cycle in days in milk (DIM) during period 1 according to NOC (normal oestrous cycle), POC (prolonged oestrous cycle) and DOV (delayed first ovulation). B) Variation of oestrous cycle length (days) in period 1 for NOC, POC and DOV. The box plots show the range of 10% to 90% percentiles of the data points. All outliners of this range are indicated as dots. The horizontal line within the box indicates the median. Significant differences are marked as *** with $p<0.001$.

Figure 3: Influence of natural negative energy balance (A) during period 1 on metabolic parameters in blood (B, C, D) and milk (E) and body mass mobilization indicated by BCS (F). Values are given as mean ± SEM. Significant levels are * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 4: Variation of oestrous cycle length in days (d) during energy restriction (period 2) according to control and restriction group (A) and distribution of normal (NOC), prolonged (POC) and shortened oestrous cycle length (SOC) within control and restriction group (B). Data are given as mean values ± SEM in A and as numbers of cows (n) in B.

Figure 5: Mean values ± SEM of energy deficiency (A), blood NEFA (B), blood BHB (C) and milk hydrocortisone (D) during restriction phase (period 2) on oestrous cycle length according to control and restriction group. Significant levels are * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

Figure 6: Variation of oestrous cycle length in days (d) (A) and distribution of normal (NOC), prolonged (POC) and shortened oestrous cycle (SOC) (B) during realimentation (period 3) according to control and restriction group. Data in A are given as mean values ± SEM and as number of cows (n) in B.

Figure 7: Effect of energy deficiency (A), blood NEFA (B), blood BHB (C) and milk hydrocortisone (D) during realimentation (period 3) on oestrous cycle length according to control and restriction group. Data are given in mean values ± SEM. Significant levels are * $p<0.05$.

Figure 8: Insemination rate (A) and total conception rate (B) according to control and restriction group. Data are given as percentage rates (%) according to number of services (n) (A) and according to pregnant and not-pregnant state until 4th insemination.

Figure 9: Scattered Dataset Visualisation. The data for period 1 to 3 is shown scatterd by parameter values and colored by group membership. Normal noise was added to the group dimension (0 or 1) to ease visual inspection. The groups are discussed in figure 1.

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585

586 Table 1

	PMR1[1]	PMR2[1]	Concentrate[2]
Components (% in DM)			
Grass silage	33.7	21.8	
Corn silage	44.9	29.1	
Hay	6.5	39.4	
Concentrate[3]	14.9	9.7	
Nutrient values			
MJ NE_L/kg DM[4]	6.53	6.24	7.96
Crude fibre (g/kg DM)	214	251	62
Crude ash (g/kg DM)	76	75	76
Crude fat (g/kg DM)	32	28	24
Crude protein (g/kg DM)	146	138	216
ADF (g/kg DM)[5]	254	313	84.1
NDF (g/kg DM)[6]	431	529	184
NFC (g/kg DM)[4,7]	316	230	500
Available crude protein (ACP) (g/kg DM)[4]	143	137	172
Ruminant nitrogen balance (RNB) (g/kg DM)[4]	0.88	0.18	2.37

[1]Partial mixed ration
[2]Additional concentrate according to milk yield,consisting of 14.9% barley, 24.8% maize, 21.8% wheat, 20.1% soybean meal, 15.2% dried sugar beet pulp with molasses, and 3.2% vitamin-mineral-premix including limestone
[3]Concentrate: 7.9% barley, 24.7% wheat, 60.0% soybean meal, 7.3% vitamin-mineral-premix including salt and limestone.
[4]Calculated values
[5]Acid detergent fibre
[6]Neutral detergent fibre
[7]Nonfibre carbohydrates calculated by difference: 100 - (%crude protein + %NDF + %crude fat + %crude ash)

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Table 2

Health disorder	Period 1[1]	Period 2[2]		Period 3[3]	
		R	C	R	C
Mastitis and other udder related problems	8	-	2	4	1
Reproductive tract related problems	2	-	-	-	-
Claw problems	9	2	-	-	2
Milk fever	3				
Total	22	2	2	4	3

[1]Period 1: calving until day 85 pp

[2]Period 2: induced energy deficit

[3]Period 3: realimentation period

[4]R: restriction cows

[5]C: control cows

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599 Table 3

Oestrous cycle	Period 1		Period 2	Period 3	
	NOC[1] vs DOV[2]	NOC vs POC[3]	NOC vs SOC[4]	NOC vs SOC	NOC vs POC
	P[6] 0.146	*P*[6] 0.253	*P*[6] 0.177	*P*[6] 0.189	*P*[6] 0.0.096
Metabolic parameters	*P*[7]	*P*	*P*	*P*	*P*
Constant	0.45	0.19	0.79	0.85	0.24
EB[5]	0.33	0.32	0.14	0.63	0.28
BCS	0.22	0.42	0.15	0.94	0.52
NEFA	0.14	0.39	0.71	0.6	0.64
BHB	0.52	0.56	0.3	0.16	0.65
Glucose	0.13	0.06	0.19	0.74	0.37
Hydrocortisone	0.45	0.21	0.16	0.3	0.17

[1]normal oestrous cycle

[2]delayed first ovulation

[3]prolonged oestrous cycle

[4]shortened oestrous cycle

[5]energy balance

[6]P-value Pearson Chi Square

[7]P-value Wald Statistic

600

601 Table 4

	Period 1		Period 2	Period 3	
	NOV[1] vs. DOV[2]	NOC[3] vs. POC[4]	NOC vs. POC	NOC vs. POC	NOC vs. SOC[5]
Not BHB,EB	0.533	0.405	-	-	-
Not NEFA	0.429	0.614	-	-	-
AUROC	0.4	0.564	0.532	0.504	(0.875)[6]
Class Dist	36/6	24-Nov	28/11	18/13	28/1

[1]normal first ovulation
[2]delayed first ovulation
[3]normal oestrous cycle
[4]prolonged oestrous cycle
[5]shortened oestrous cycle
[6]artefact of highly uneven class distribution

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Figure 1

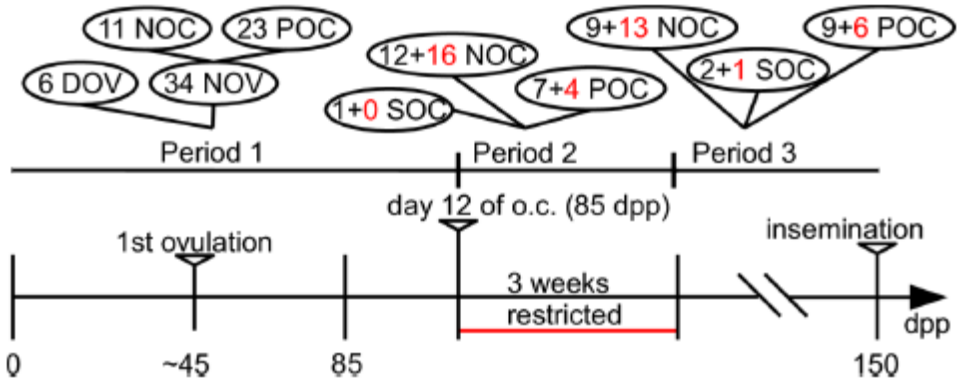

11 NOC
23 POC
6 DOV
34 NOV
12+16 NOC
1+0 SOC
7+4 POC
9+13 NOC
9+6 POC
2+1 SOC
Period 1
Period 2
Period 3
day 12 of o.c. (85 dpp)
1st ovulation
insemination
3 weeks
restricted
dpp
0
~45
85
150

Figure 2

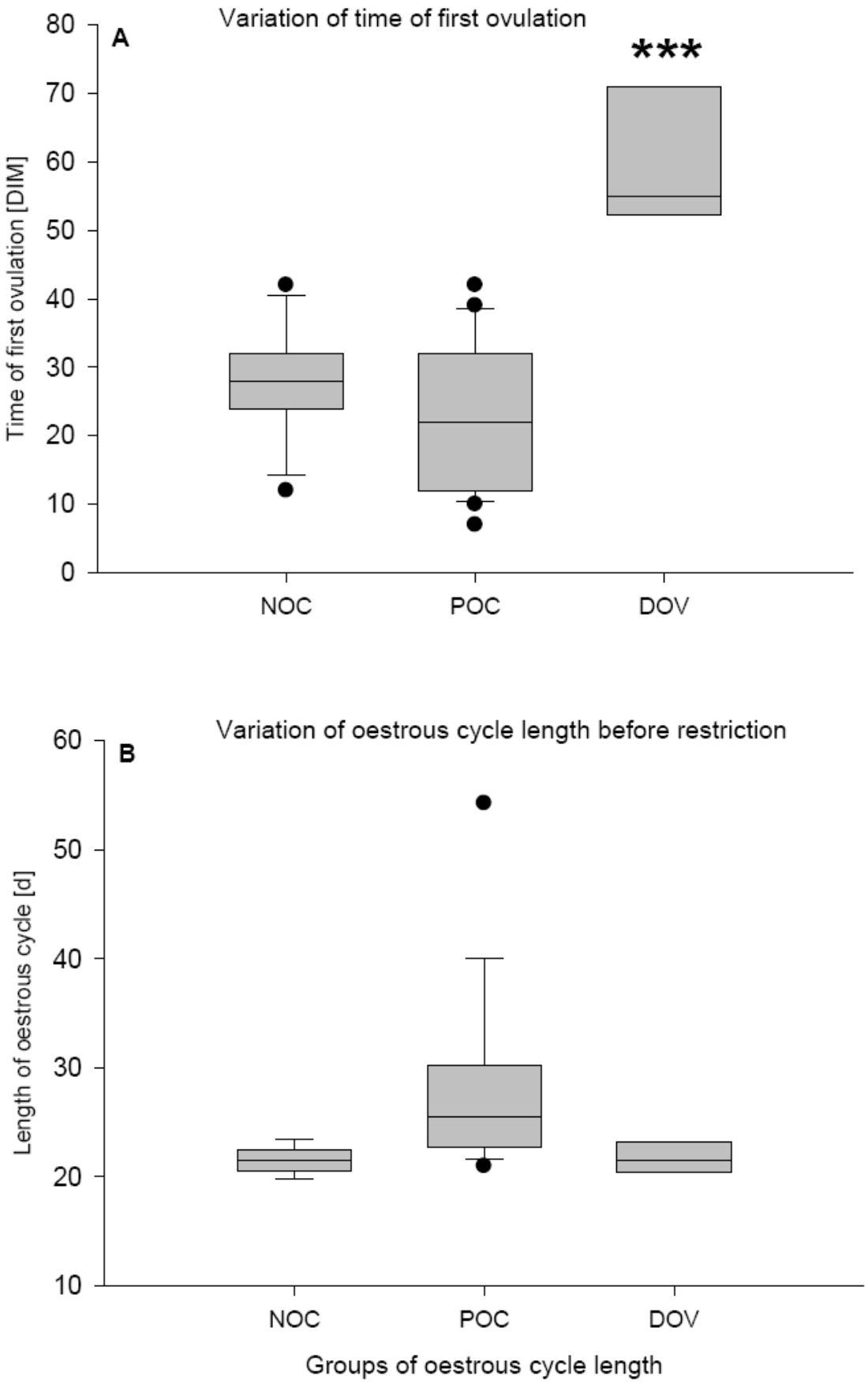

A
Variation of time of first ovulation
***
Time of first ovulation [DIM]
80
70
60
50
40
30
20
10
0
NOC
POC
DOV
B
Variation of oestrous cycle length before restriction
Length of oestrous cycle [d]
60
50
40
30
20
10
NOC
POC
DOV
Groups of oestrous cycle length

Figure 3

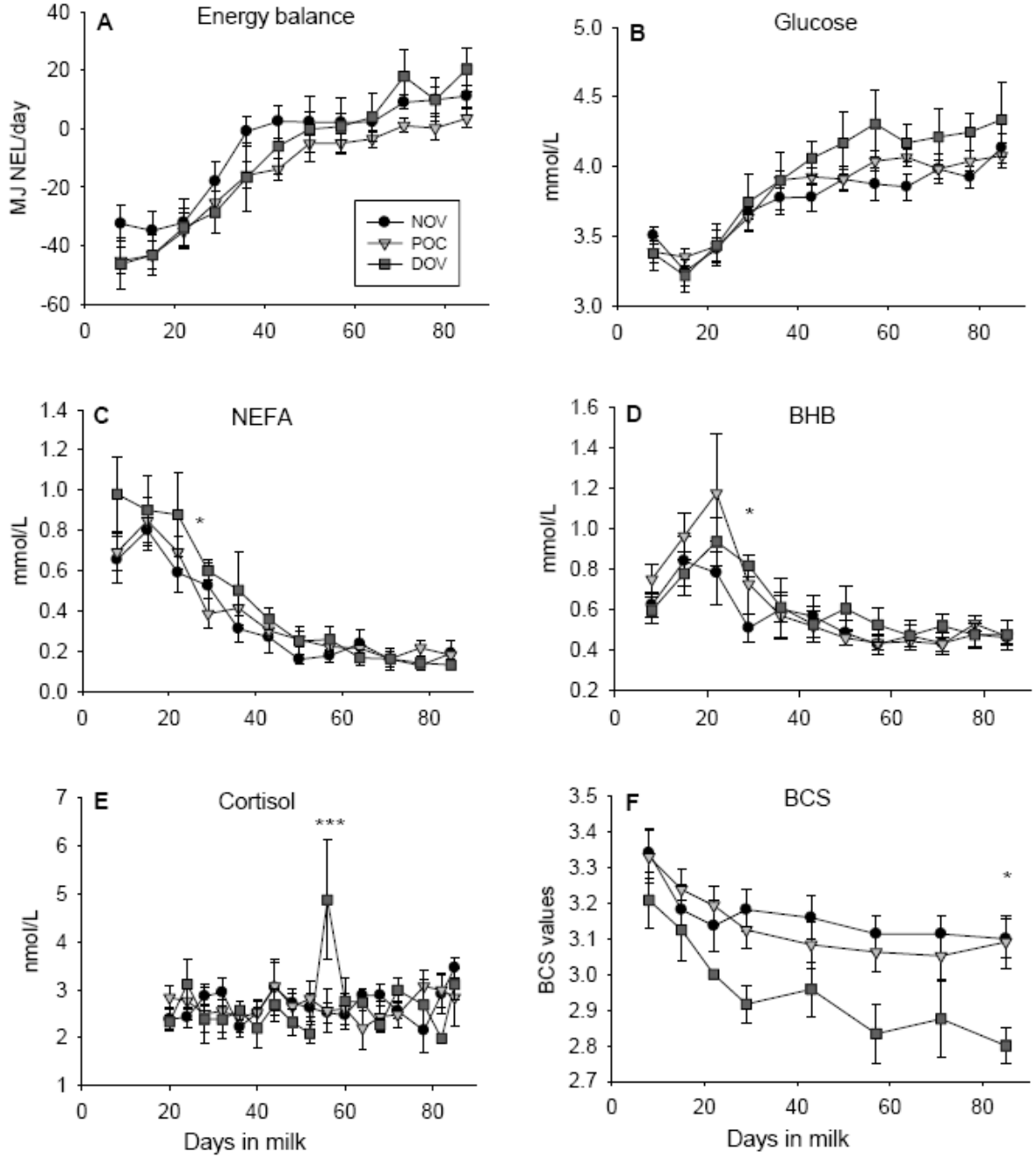

A
Energy balance
MJ NEL/day
NOV
POC
DOV
B
Glucose
mmol/L
C
NEFA
mmol/L
D
BHB
mmol/L
E
Cortisol
nmol/L
***
F
BCS
BCS values
Days in milk
Days in milk

Figure 4

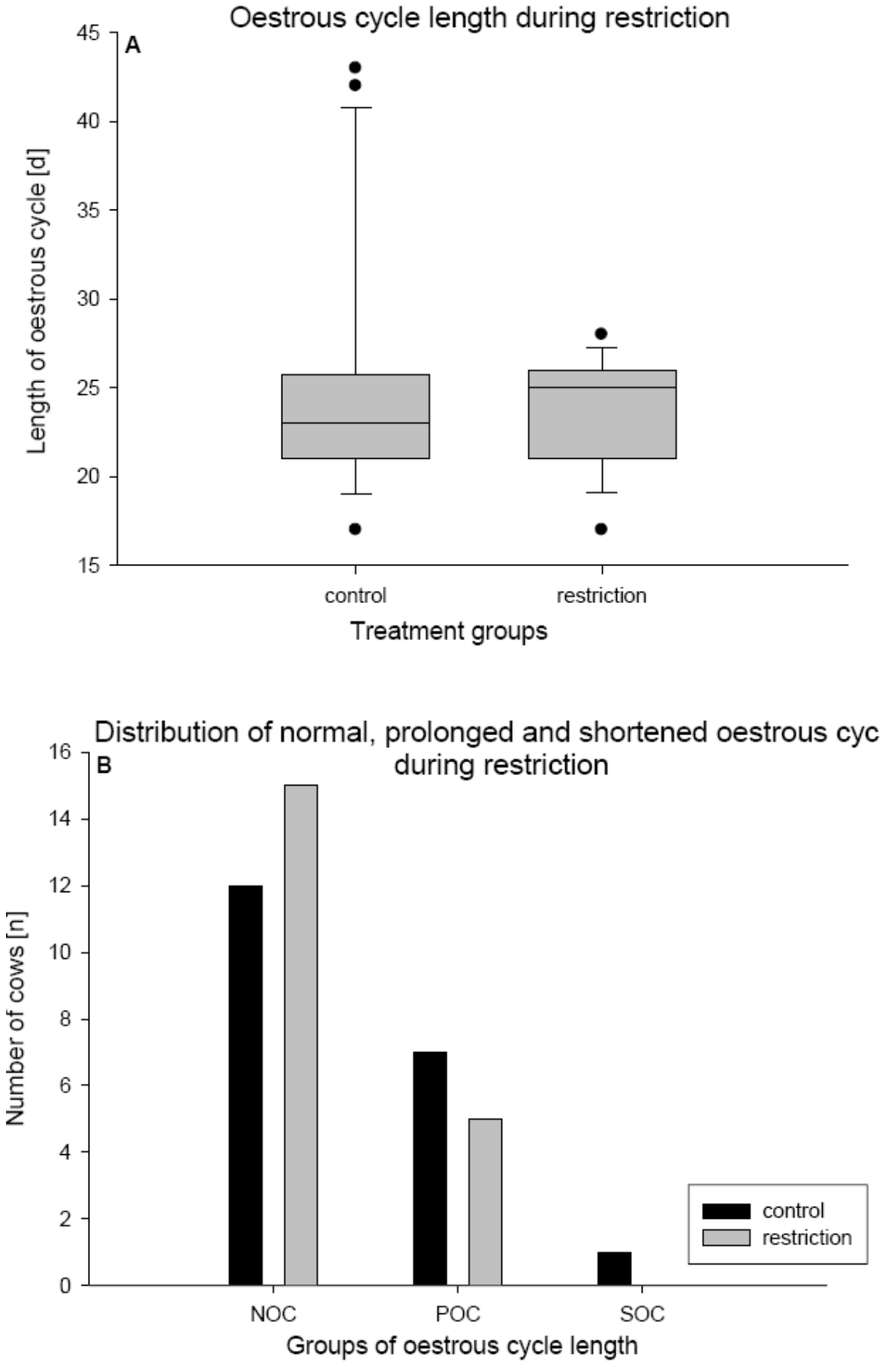

Oestrous cycle length during restriction
A
Length of oestrous cycle [d]
45
40
35
30
25
20
15
control
restriction
Treatment groups
Distribution of normal, prolonged and shortened oestrous cyc
during restriction
B
Number of cows [n]
16
14
12
10
8
6
4
2
0
control
restriction
NOC
POC
SOC
Groups of oestrous cycle length

Figure 5

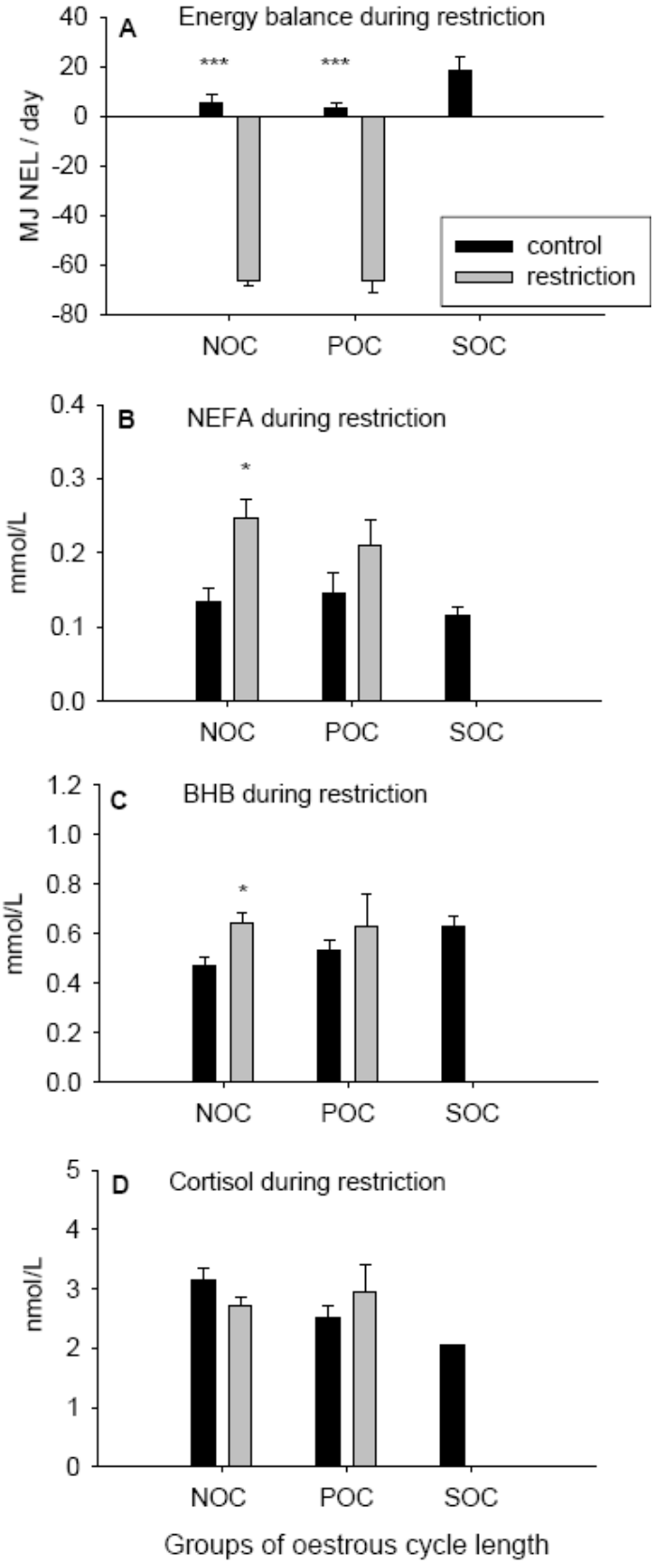

A
Energy balance during restriction
MJ NEL / day
40
20
0
-20
-40
-60
-80
***
***
control
restriction
NOC
POC
SOC
B
NEFA during restriction
mmol/L
0.4
0.3
0.2
0.1
0.0
*
NOC
POC
SOC
C
BHB during restriction
mmol/L
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NOC
POC
SOC
D
Cortisol during restriction
nmol/L
5
4
3
2
1
0
NOC
POC
SOC
Groups of oestrous cycle length

Figure 6

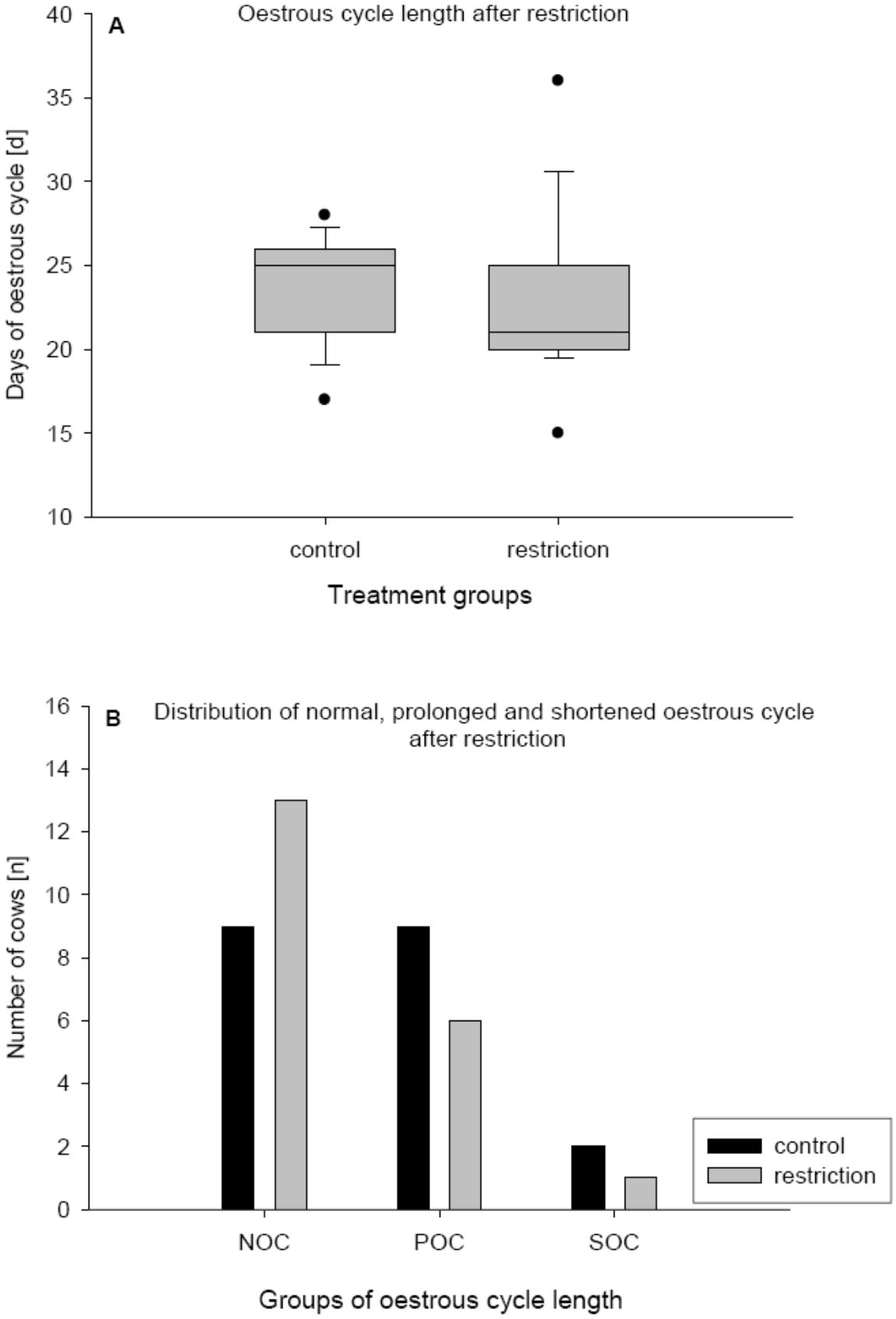

A
Oestrous cycle length after restriction
Days of oestrous cycle [d]
40
35
30
25
20
15
10
control
restriction
Treatment groups
B
Distribution of normal, prolonged and shortened oestrous cycle after restriction
Number of cows [n]
16
14
12
10
8
6
4
2
0
control
restriction
NOC
POC
SOC
Groups of oestrous cycle length

Figure 7

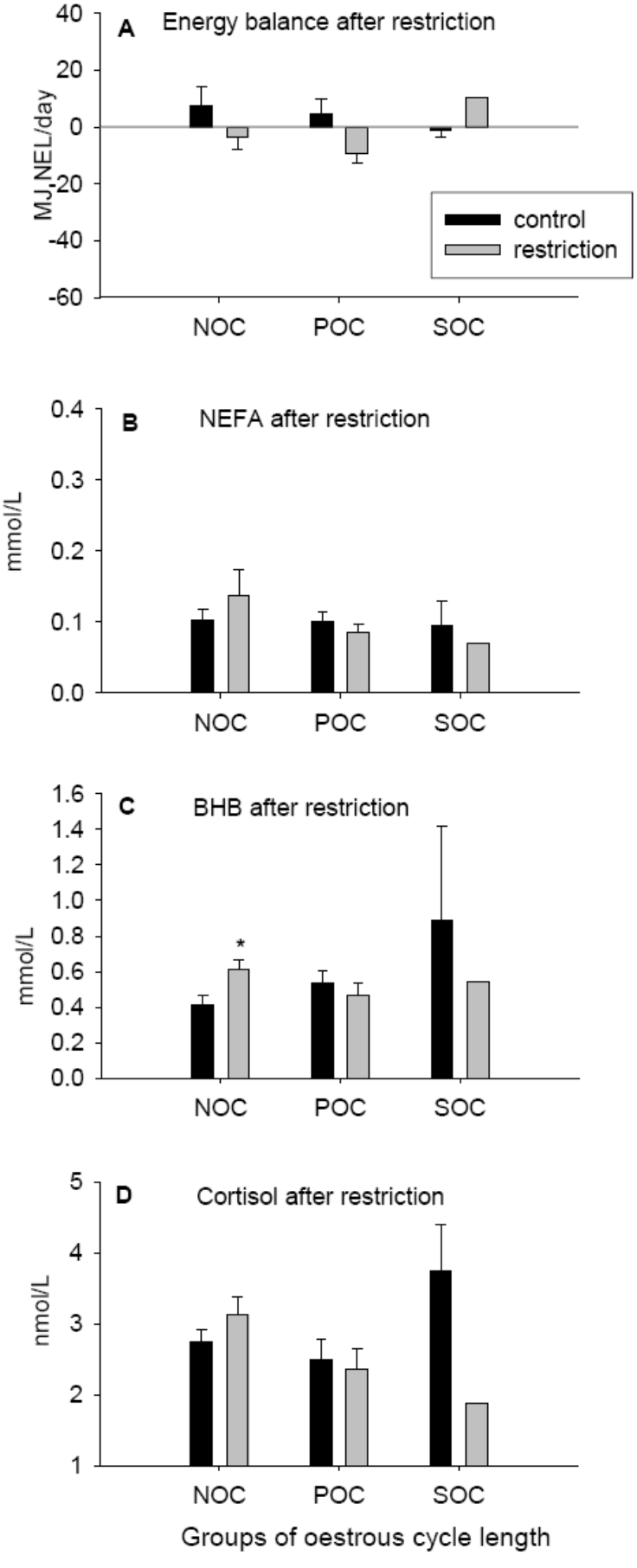

A
Energy balance after restriction
MJ,NEL/day
40
20
0
-20
-40
-60
control
restriction
NOC
POC
SOC
B
NEFA after restriction
mmol/L
0.4
0.3
0.2
0.1
0.0
NOC
POC
SOC
C
BHB after restriction
mmol/L
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
NOC
POC
SOC
D
Cortisol after restriction
nmol/L
5
4
3
2
1
NOC
POC
SOC
Groups of oestrous cycle length

Figure 8

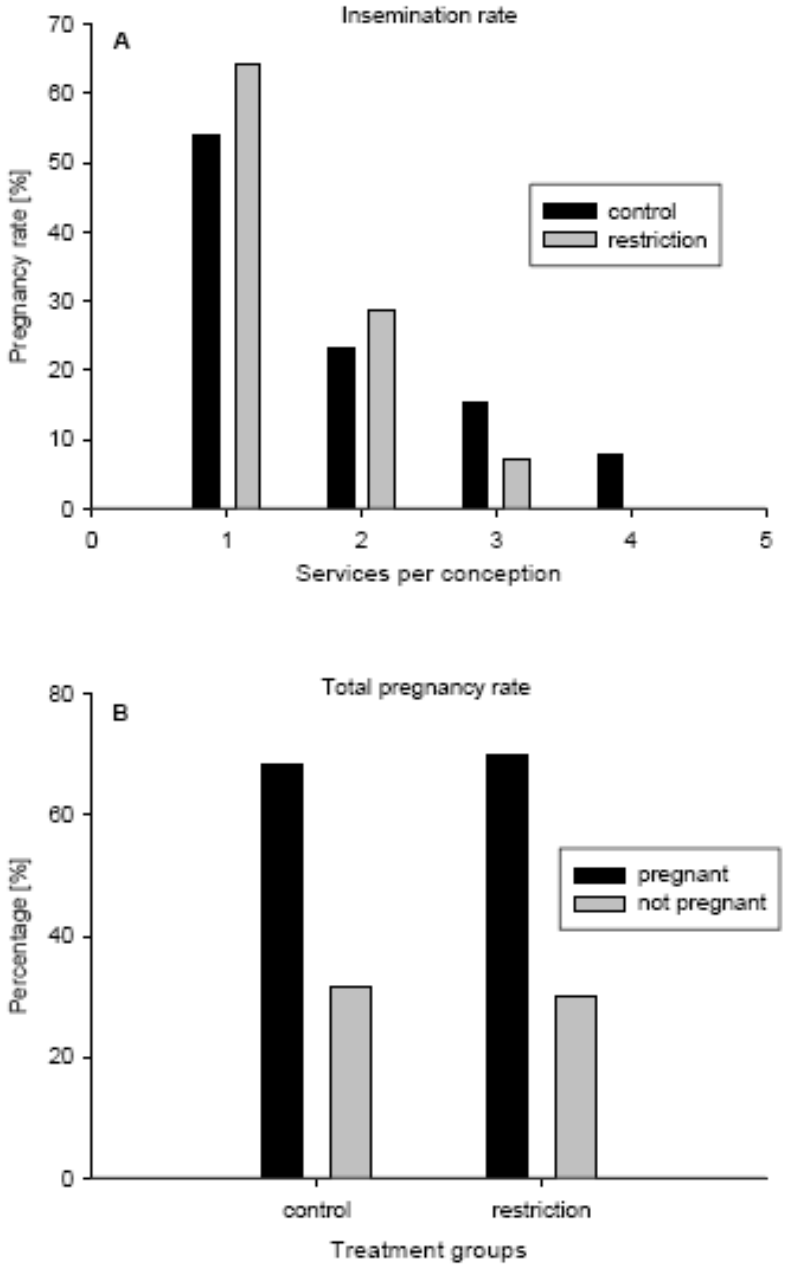
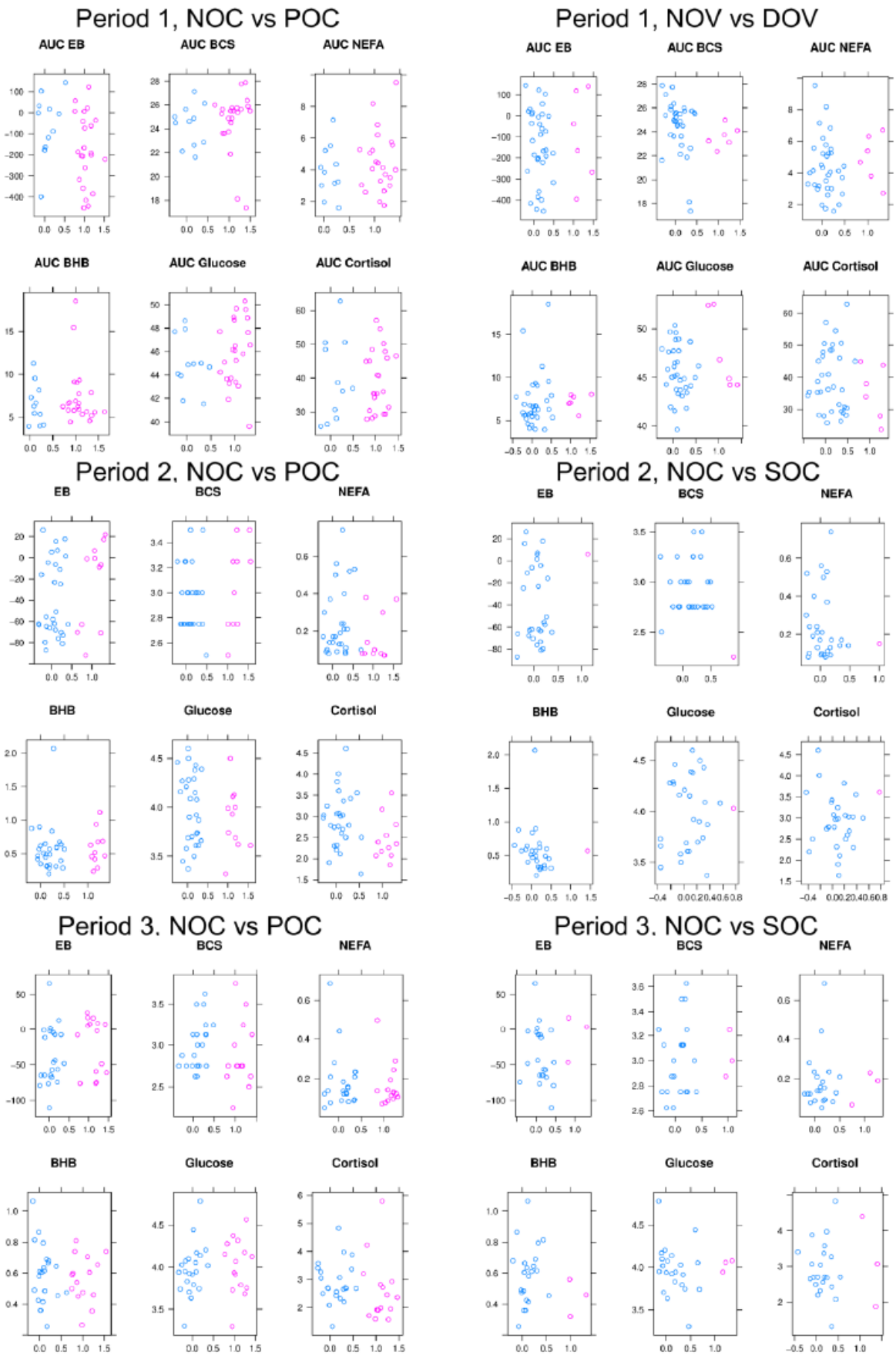

Insemination rate
A
Pregnancy rate [%]
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Services per conception
control
restriction
Total pregnancy rate
B
Percentage [%]
80
60
40
20
0
control
restriction
Treatment groups
pregnant
not pregnant

Figure 9


Period 1, NOC vs POC
AUC EB
AUC BCS
AUC NEFA
AUC BHB
AUC Glucose
AUC Cortisol
Period 1, NOV vs DOV
AUC EB
AUC BCS
AUC NEFA
AUC BHB
AUC Glucose
AUC Cortisol
Period 2, NOC vs POC
EB
BCS
NEFA
BHB
Glucose
Cortisol
Period 2, NOC vs SOC
EB
BCS
NEFA
BHB
Glucose
Cortisol
Period 3. NOC vs POC
EB
BCS
NEFA
BHB
Glucose
Cortisol
Period 3. NOC vs SOC
EB
BCS
NEFA
BHB
Glucose
Cortisol