

*Full Length Research Paper***Metabolic status and oestrous cycle in dairy cows****K. Danowski¹, J. J. Gross³, K. Gellrich¹, T. Petri⁵, H. A. Van Dorland³, R. M. Bruckmaier³,
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A study with 40 multiparous high yielding dairy cows was conducted to investigate the influence of an induced negative energy balance (NEB) on reproductive performance. Energy restriction of 49% was performed for 3 weeks beginning on oestrous cycle day 12 of first oestrous cycle after day 85 post partum (pp). From day 20 to day 150 pp animals were monitored for ovary activity three times weekly using rectal palpation and transrectal ultrasound scanning and were inseminated around day 150 pp. Additionally, milk progesterone and milk hydrocortisone were analyzed twice a week. Body condition score and body weight as well as blood glucose, plasma nonesterified fatty acids and plasma β -hydroxybutyrate were recorded weekly. According to oestrous cycle activity before (Period 1 = natural energy deficiency), during (Period 2) and after (Period 3) induced energy restriction animals were assigned to the following groups: Delayed first ovulation until day 45 pp, normal oestrous cycle, prolonged oestrous cycle and shortened oestrous cycle. Sporadic significances, but no clear effect of the metabolic state on reproductive performance could be found during Periods 1 and 2. Service success and conception rate were also not influenced. Our results demonstrate a remarkable adaptation of reproductive activity to metabolic challenges. Animals were able to compensate natural NEB in Period 1 as well as induced NEB (Period 2) for preventing metabolic disorders and maintaining reproductive activity. Therefore dietary energy availability had no effect on reproductive performance at more than 85 days in milk in the present study. To understand reproductive failures in dairy cows focus should be laid on genetic disposition of high yielding individuals that cope successful with metabolic challenges.

Key words: 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

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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 effects 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 (Drackley, 1999). Reproductive failures caused by metabolic instability are indicated by the inhibition of ovulation and oestrus (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 a BCS >3.5 at calving is 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 can be reduced (Butler, 2000). Vanholder et al. (2006) indicate that, the hypothalamic-pituitary function and further on the follicular growth and development may be affected by metabolic and hormonal adaptations caused by NEB. 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 (2008) 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. An animal trial with high yielding dairy cows was conducted to investigate the effect of dietary energy on reproduction, especially on ovarian cycle activity and insemination success. 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) (Table 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 libitum (PMR1, Table 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 \pm 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 1.3 kg DM/d concentrate (CONC). PMR1 was calculated for 650 kg body mass, an assumed daily feed intake of 16 kg DM/day and daily milk production of 21 kg. From day 6 until day 35 pp additional CONC was increased from 1.8 kg DM/day until a

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

Components (% in DM)	PMR1 ¹	PMR2 ¹	Concentrate ²
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

¹Partial mixed ration; ²Additional 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; ³Concentrate: 7.9% barley, 24.7% wheat, 60.0% soybean meal, 7.3% vitamin-mineral-premix including salt and limestone; ⁴Calculated values; ⁵Acid detergent fibre; ⁶Neutral detergent fibre; ⁷Nonfibre carbohydrates calculated by difference: 100 - (%crude protein + %NDF + %crude fat + %crude ash)

maximum of 8.9 kg DM/day. Thereafter PMR1 was enhanced by individual CONC adjustment according to milk yield. During induced energy deficit (Period 2) restriction animals received PMR2 (Table 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. A dietary energy restriction of 49% of the total requirement was performed for three weeks (Period 2) starting on the first day 12 of oestrous cycle after average day 85 pp, when early pp NEB was over and the metabolism was re-stabilized. The 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 the beginning of realimentation until the second ovulation after the energy restriction (day 150 ± 1.2 SEM pp). Animals were inseminated at the second oestrus.

Metabolic parameters

Body condition parameters and plasma metabolites were collected

and analyzed for the metabolic screening. 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. The Body condition score (BCS) was 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 Ltd. (Schwyz, Switzerland) according to the manufacturers 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 to 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 (Figure 1). After Period 3 on average day 150 ± 1.2 SEM pp, first insemination service was conducted. Data for the parameters services per conception and total conception rate were collected.

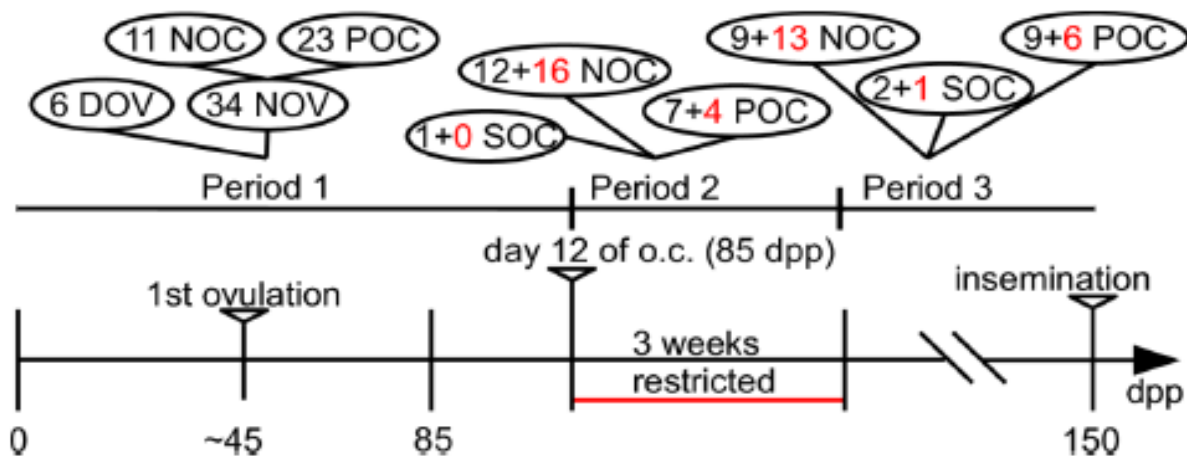


Figure 1. Experimental design of the animal trail. Period 1 (day (d) of calving (0 post partum (pp)) till the 12th day of the oestrous cycle after 85th pp) covers the natural energy deficit. Oestrous cycle length and day of ovulation was recorded from day 20 pp on. The cows were group according to their 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.

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., 1987) 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 0.2 to 3 ng/ml in skimmed milk. Milk progesterone results were used to determine ovulation times in addition to ultrasound scanning and rectal palpations. Values >1 ng/ml were assigned to preceded ovulation and a functional corpus luteum, values <0.2 ng/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 Inc., Rhode Island, USA) was labeled with horseradish peroxidase (1:12000) (Roche Applied Science, Mannheim, Germany) as described for other steroids earlier (Meyer and Guven, 1986). The polyclonal antibody (C1 Pool 2) in 1:90000 dilution had been produced in rabbits against hydrocortisone-21-hemisuccinate-BSA, its cross reactivities were: 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 34.5 nmol/l. 10 µl 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 was done using SigmaPlot 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 for not normally distributed data (Figure 3). Multiple testing correction was done using sequential Holm-Sidak correction. Differences in the metabolic data for Periods 2 and 3 were tested by either T-test or via Mann-Whitney Rank Sum test for not normally distributed data (Figures 4 and 5). Each metabolic parameter is expressed as area under the curve (AUC) for Period 1. Mean values are used for Periods 2 and 3. Mean values across all measured cows were imputed in case of missing values. An all-pairwise metabolic parameter correlation matrix was computed for each period (using Pearson correlation). If the correlation of a parameter exceeded a threshold of 0.65 two

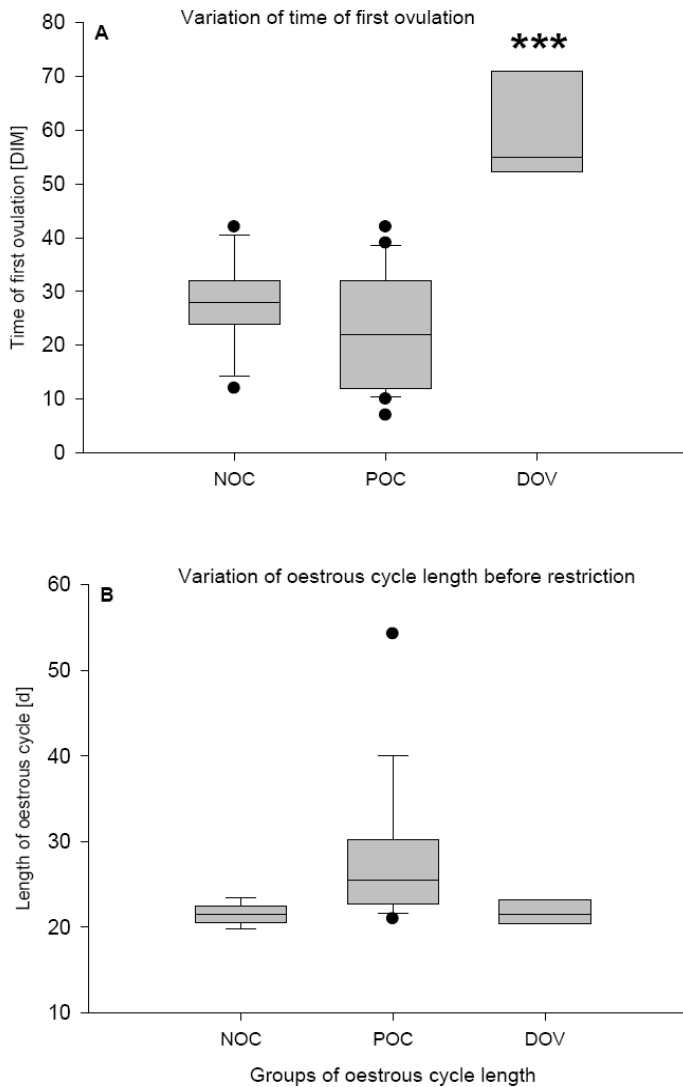


Figure 2. (A) Time of first ovulation in days in milk (DIM) and length of oestrous cycle (DIM) during Period 1 according to NOC (normal oestrous cycle), POC (prolonged oestrous cycle) and DOV (delayed first ovulation). (B) Variation of oestrous cycle length (days) in Period 1 for NOC, POC and DOV. The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

separate models excluding either parameter set were built. A binary classification setting was induced by our grouping procedure: the cycle length fractions were treated as dependent variables (that is, 2 classes) and the metabolic parameters represent independent measurements. Models were 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 is reported in Table 3. 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 procedure: Each cow is left out from the model building process once and is predicted

using the model trained on all other cows' parameters. This process yields a constant estimate of the models predictive power. The area under receiver operator characteristic curve (AUROC) of all single predictions combined is reported. An AUROC value around 0.5 shows that the underlying model is not better than random guessing. Note that the LOO-AUROC is still likely an overestimate due to over-fitting of the model to the observations, yet it is (1) more conservative than training a model on all cows and (2) imposes an upper bound on any k-fold cross-validation procedure, that is, if the predictive power during LOO is low the model will likely perform worse when applied on further observations. A comparison of cycle length for Periods 2 and 3 between restriction and control was conducted using z-tests on the two group proportions, where H_0 assumes equal proportions of restriction and control group. Similarly, z-tests were applied to detect differences in the number of services until conception and the conception rate (Figure 6).

RESULTS

Period 1

In Period 1, during the first 85 days pp no ovulation could be detected in 6 cows until day 45 pp (DOV). 11 animals showed normal oestrous cycles (NOC) and 23 animals showed one or more prolonged oestrous cycle (POC) (Figure 1). Time of first ovulation after calving took place later in DOV compared to NOC and POC ($p < 0.001$), but did not differ among NOC and POC ($p = 0.164$) (Figure 2A). Oestrous cycle lengths in DOV and NOC were constant between 18 and 24 days whereas the cycle length in POC showed a high variation (Figure 2B). All cows of DOV, NOC and POC showed a negative EB around -40 MJ NEL/day in the first week pp. A positive EB was reached at day 40 pp for NOC and day 50 pp for POC and DOV (Figure 3A). The average milk yield was 35.27 ± 6.72 kg/day (fat $5.02 \pm 0.74\%$, protein $3.53 \pm 0.52\%$) for each cow. The energy corrected milk (ECM) yield ($(0.38 \times \text{fat } \% + 0.21 \times \text{protein } \% + 1.05) / 3.28 \times \text{milk yield kg}$) (Fischer et al., 2002) was 36.35 ± 6.10 kg/day. In Period 1, blood metabolites showed expected courses during NEB in early lactation (Figure 3). Glucose was on low levels of 3.3 mmol/L in the second week pp and increased according to energy balance (Figure 3B). NEFA reached highest levels of 1 mmol/L in the second week pp in DOV and showed lower levels, but equal courses in NOC and POC, with a significant difference between DOV and POC on day 29 pp ($p < 0.05$) (Figure 3C). BHB concentrations increased up to peak values of 1.2 mmol/L with high variation in POC and decreased until base levels were reached on day 50 pp. Similar progressions of DOV and NOC could be found, but NOC decreased earlier and showed a significant difference on day 29 pp compared to DOV ($p < 0.05$). Milk hydrocortisone (Figure 3E) was not influenced by the metabolic state during early lactation. The higher value for DOV on day 56 pp resulted of one outlier indicated also by error bars. According to body mass mobilization in Period 1 DOV showed lower BCS by trend compared to NOC and POC. This difference was only significant on

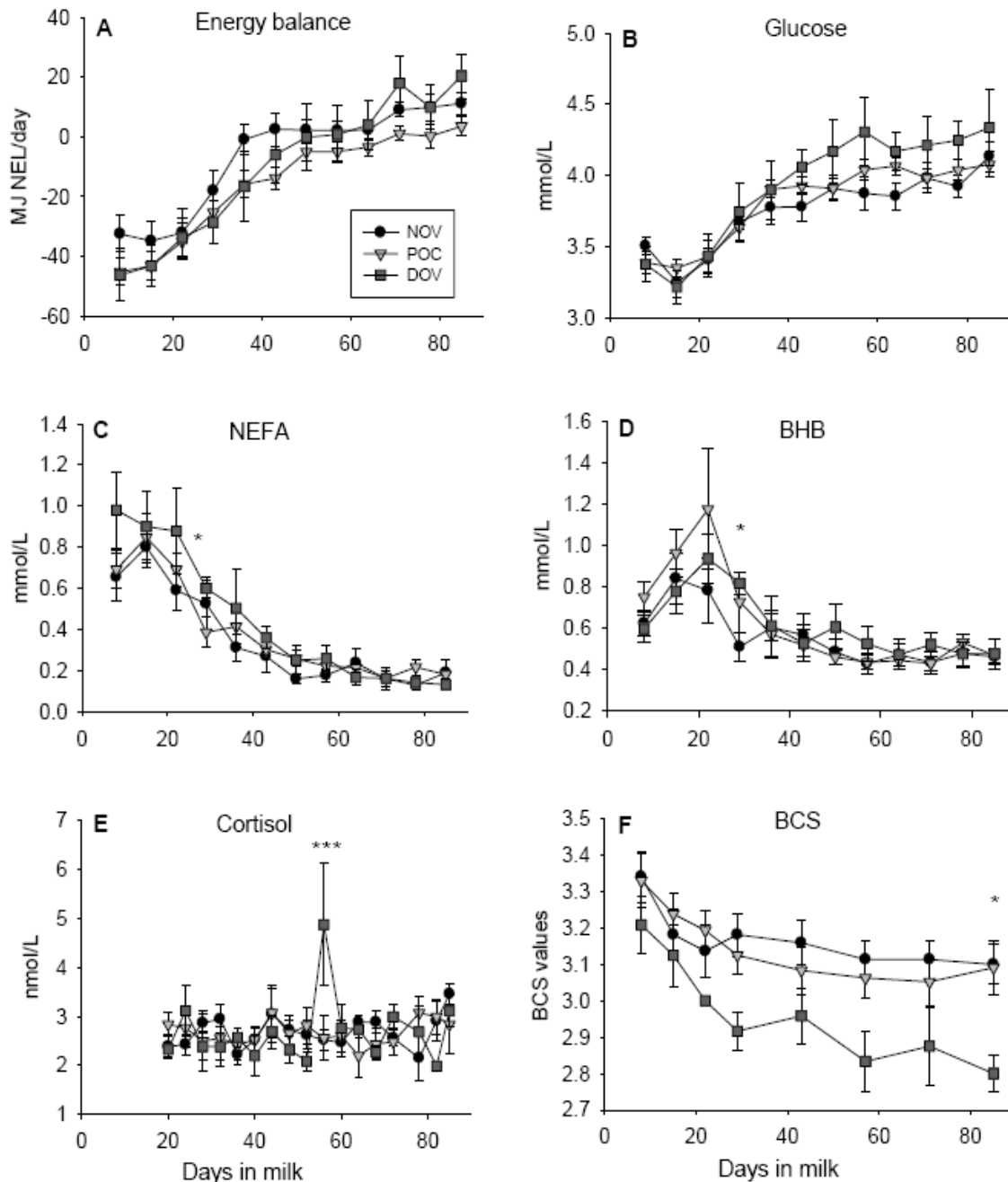


Figure 3. Influence of natural negative energy balance (A) during Period 1 on metabolic parameters in blood (B, C, D) and milk (E) and body mass mobilization indicated by BCS (F). Values are given as mean \pm SEM. Significant levels are * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

day 85 pp (Figure 3F).

Period 2

During restriction phase (Period 2) animals were divided into restriction and control group with 20 animals each. No difference in cycle length between control (24.8 ± 1.5 day) and restriction group (23.4 ± 1.3 day) could be

found. Within the control group 12 cows were assigned to NOC, 7 animals to POC and only 1 cow showed a short ovarian cycle (SOC). In the restriction group 16 cows showed normal cycle lengths. Prolonged cycles could be found in 4 animals and no short cycle was detected in the restriction group (Figure 1). The average milk yield during Period 2 for the control group was 29.91 ± 5.93 kg/day (fat $4.35 \pm 0.53\%$, protein $3.37 \pm 0.30\%$) (ECM 30.95 ± 5.66 kg/day). Milk yield was slightly reduced in restriction

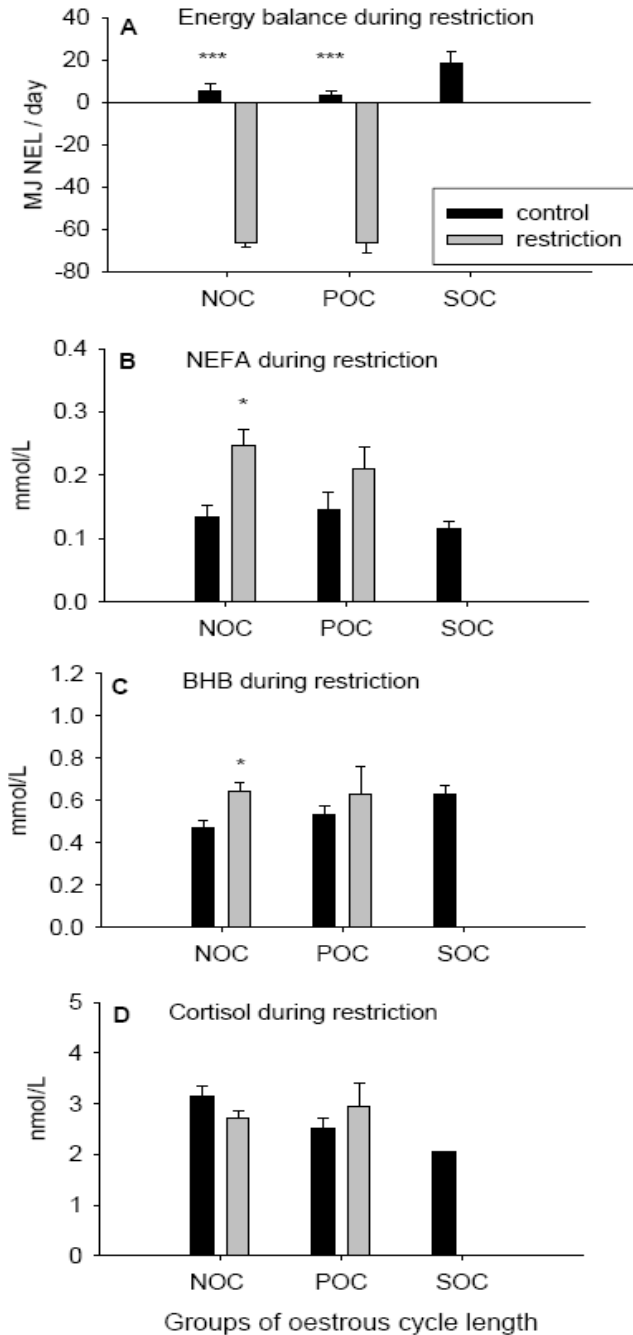


Figure 4. Mean values ± SEM of energy deficiency (A), blood NEFA (B), blood BHB (C) and milk hydrocortisone (D) during restriction phase (Period 2) on oestrous cycle length according to control and restriction group. Significant levels are *p < 0.05, **p < 0.01 and ***p < 0.001.

group to 27.59 ± 4.92 kg/day (fat 4.50 ± 0.66%, protein 3.21 ± 0.28%) (ECM 28.64 ± 4.24 kg/day). Calculated energy balance of 49% reduction during induced energy restriction (Period 2) showed more severe negative deficiency than in pp NEB with highly significant differences (p < 0.001) between restriction and control group for NOC and POC (Figure 4A). Short ovarian cycle

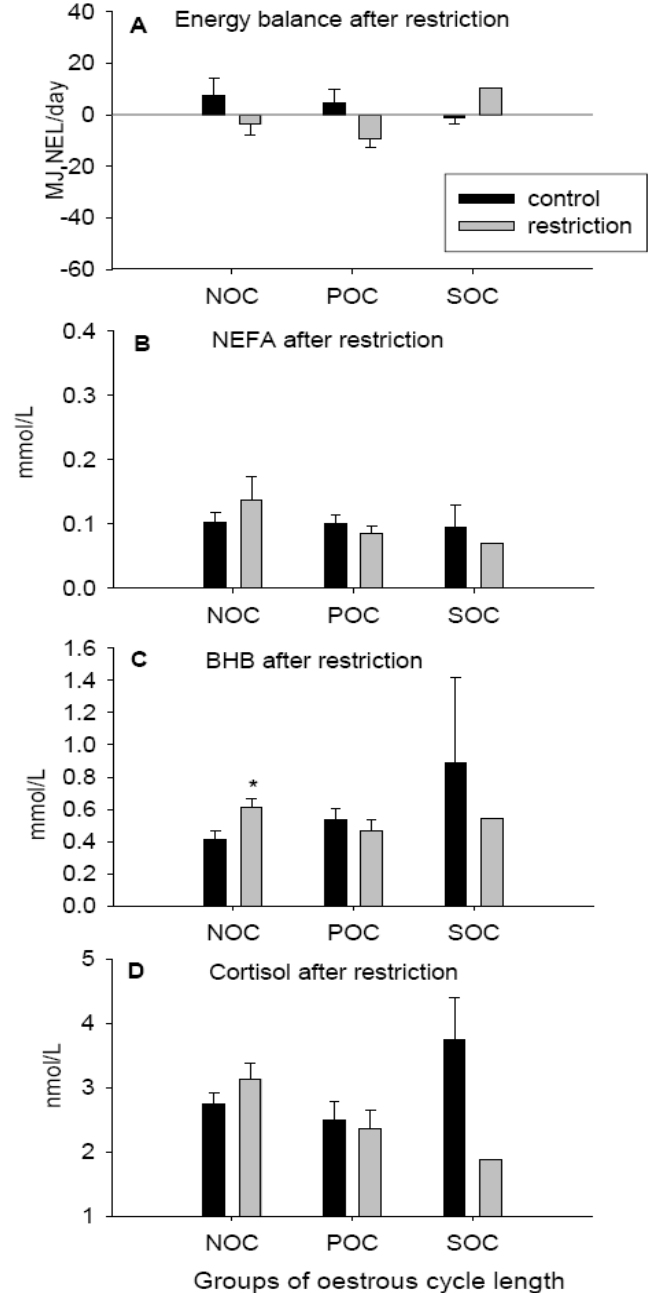


Figure 5. Effect of energy deficiency (A), blood NEFA (B), blood BHB (C) and milk hydrocortisone (D) during realimentation (Period 3) on oestrous cycle length according to control and restriction group. Data are given in mean values ± SEM. Significant levels are *p < 0.05.

(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 (Figures 4B and C). Milk hydrocortisone revealed no effect of energy restriction and showed no differences between oestrous cycle groups (Figure 4D). There were no critical metabolic states detected during energy restriction and no animal had to be treated due to

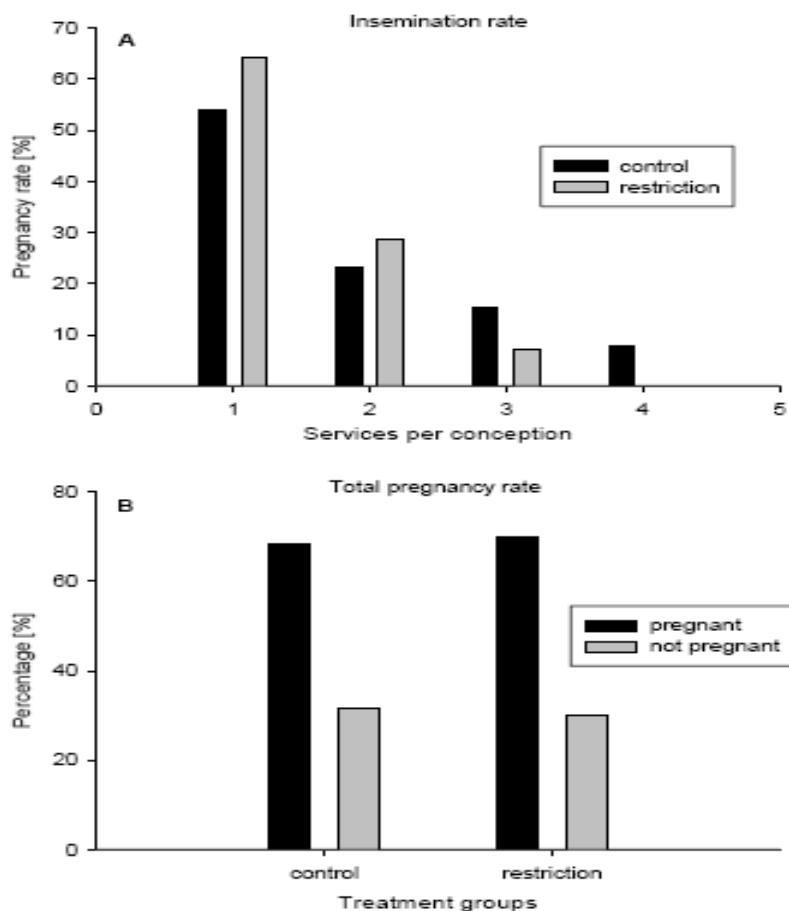


Figure 6. Insemination rate (A) and total conception rate (B) according to control and restriction group. Data are given as percentage rates (%) according to number of services (n) (A) and according to pregnant and not-pregnant state until 4th insemination (B).

Table 2. Number of health disorders during the experimental periods.

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

¹Period 1: calving until day 85 pp; ²Period 2: induced energy deficit; ³Period 3: realimentation period; ⁴R: restriction cows; ⁵C: control cows.

metabolic problems (Table 2).

Period 3

After energy restriction (Period 3) the same tendency in

cycle length could be found, but again no clear effects were observed (control group 23.7 ± 0.8 day, restriction group 22.7 ± 1.1 day). 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

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

Oestrous cycle	Period 1		Period 2	Period 3	
	NOV ¹ vs DOV ²	NOC ³ vs POC ⁴	NOC vs POC ⁴	NOC vs SOC ⁵	NOC vs POC
	<i>P</i> ⁶ 0.146	<i>P</i> ⁶ 0.253	<i>P</i> ⁶ 0.177	<i>P</i> ⁶ 0.189	<i>P</i> ⁶ 0.0.096
Metabolic parameter	<i>P</i> ⁷	<i>P</i>	<i>P</i>	<i>P</i>	<i>P</i>
Constant	0.45	0.19	0.79	0.85	0.24
EB ⁸	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	0.60	0.64
BHB	0.52	0.56	0.30	0.16	0.65
Glucose	0.13	0.06	0.19	0.74	0.37
Cortisol	0.45	0.21	0.16	0.30	0.17

¹normal first ovulation; ²delayed first ovulation; ³normal oestrous cycle; ⁴prolonged oestrous cycle; ⁵shortened oestrous cycle; ⁶*P*-value Pearson Chi Square; ⁷*P*-value Wald Statistic; ⁸energy balance.

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 (Figure 1). The average milk yield for control group was 27.98 ± 5.53 kg/day (fat $4.31 \pm 0.66\%$, protein $3.43 \pm 0.30\%$, ECM 28.88 ± 5.03 kg/day). Surprisingly the average milk yield for the restriction group was slightly higher: 28.67 ± 5.44 kg/day (fat $4.33 \pm 0.74\%$, protein $3.42 \pm 0.28\%$, 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 (Figure 5A). Blood NEFA concentrations were on low levels in control and restriction group and showed no differences between oestrous cycle groups (Figure 5B). Restriction animals with normal oestrous cycle activity had significant higher BHB concentrations (Figure 5C) than control cows in NOC ($p < 0.05$). BHB and hydrocortisone (Figure 5C and D) were higher in SOC cows, but no significance was found. In Figure 6A 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 conception was 7% for restriction and 15% for control group. After 4th service conception rate was 8% for control animals. No restriction cow received more than 3 services until conception. The overall conception rate (Figure 6B) in restriction and control group was 70 and 68%, respectively, showing again no influence of the provoked energy deficiency.

Multiple logistic regression

The effect of metabolic parameters on oestrous cycle

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

The LOO approach reflects the basic assumption of most experiments quite well: A model build on a subset of cows should hold, if it is applied to further individuals. LOO yields an upper border on this performance since the training skips only one instance per model. Yet, this validation procedure is more conservative than any model build on the complete data set which would easily overfit the underlying model. The area under the roc curve values (AUROC) is reported for the combined folds of the LOO procedure. An estimated result above 0.6 would indicate that the model and thus the parameters have at least weak predictive power considering group separation. None of the AUROC values exceed this threshold. As shown in Table 4 none of the measured parameters differs between our groups and may therefore not provide decision support on this data set. In some cases the number of cows per group (class dist, Table 4) is highly uneven and results in artificially small AUROC values. Here, the complete data are additionally inspected without any model assumption at all. All raw data used during the MLR training are provided in Figure 7. No indications of divergent distributions among the respective classes were observed as well. The LOO results are consistent with the Wald test statistics given by logistic regression models trained 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 (Tables 3

Table 4. Overview of area under the receiver operator characteristics (AUROC) for leave-one-out multiple regression models.

	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	0.400	0.564	0.532	0.504	(0.875) ⁶
Class distribution	36/6	24/11	28/11	18/13	28/1

Since NEFA and BHB/EB show absolute correlations above 0.65 or rather under -0.65 in Period 1 three independent models were trained. The row AUROC includes both, while "Not BHB, EB" and "NEFA" exclude BHB and EB or NEFA, respectively. ¹normal first ovulation; ²delayed first ovulation; ³normal oestrous cycle; ⁴prolonged oestrous cycle; ⁵shortened oestrous cycle; ⁶artefact of highly uneven class distribution.

and 4; Figure 7).

DISCUSSION

In the current study we investigated the influence of a natural NEB in early lactation and an induced energy deficit, conducted around day 85 pp 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 out the metabolic challenge during common and induced NEB as well as the realimentation phase, blood glucose, blood NEFA and blood BHB were determined throughout the experiment and the reproductive findings were referred to them. The early NEB and the induced dietary energy restriction of finally 49% revealed strong differences in neither metabolic nor reproductive parameters. Marginal significant effects between the 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. 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 1 mmol/L during first 85 days pp. In contrast to Kessel et al. (2008) NEFA and BHB values decreased already on day 20 pp and reached base values on day 50 pp indicating the end of NEB. Comparing these findings to cycle activity in Period 1, POC showed highest, but mostly not significant BHB values compared to NOC and DOV. 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 NEB (Vanholder et al., 2006). This confirms our findings of only 6 animals with DOV, as the phase of

critical BHB concentrations indicated by more than 1 mmol/L in early lactation is shorter compared to other studies and fewer animals in our trial have delayed first ovulations. On the other hand these animals do not show exceeded BHB concentrations, only one cow had elevated BHB concentrations higher than 1 mmol/L. However, BHB concentrations in POC during first 3 weeks of lactation revealed higher levels compared to NOC. Therefore we suggest that the metabolic imbalance might influence prolonged luteal phases, but has no effect on time of first ovulation pp in our experiment. Nevertheless, our animals were able to endure common NEB pp and the provoked NEB and did not contract metabolic derived dysfunctions or showed higher disease susceptibility within the experimental periods.

During dietary energy restriction (Period 2) cycle activity was also not influenced. This is confirmed by BHB, which only exceeds the threshold of 1 mmol/L in 4 single measurements and NEFA being elevated over 0.6 mmol/L in only 2 samples. No negative effect of the induced energy deficiency on reproduction was revealed although restriction and control group had low, but significant different NEFA and BHB levels and energy balance was even more negative during restriction phase than in early lactation (-62.7± 1.8 MJ NEL/day). In other studies higher mean concentrations of these metabolites were found (Perkins et al., 2002). This may explain the low reproductive responsiveness of induced NEB in the present work, as elevated BHB concentrations, more than NEFA, seem to have a strong negative correlation with reproductive performance (Oikonomou et al., 2008). However, the restriction intensity to almost only half of the requirement (49%) for up to 3 weeks, which even exceeded the energy deficit in early lactation, has not been performed in any other study than in the present trial conducted by Gross et al. (2011). Different restriction levels can be found in literature using NEFA and BHB as indicators for the restriction severity. Perkins et al. (2002) investigated disease susceptibility and used a restriction level to 80% of maintenance energy requirements for two weeks. Another group performed 60% of calculated net energy for lactational requirements for seven days in order to challenge immune function (Moyes et al., 2009).

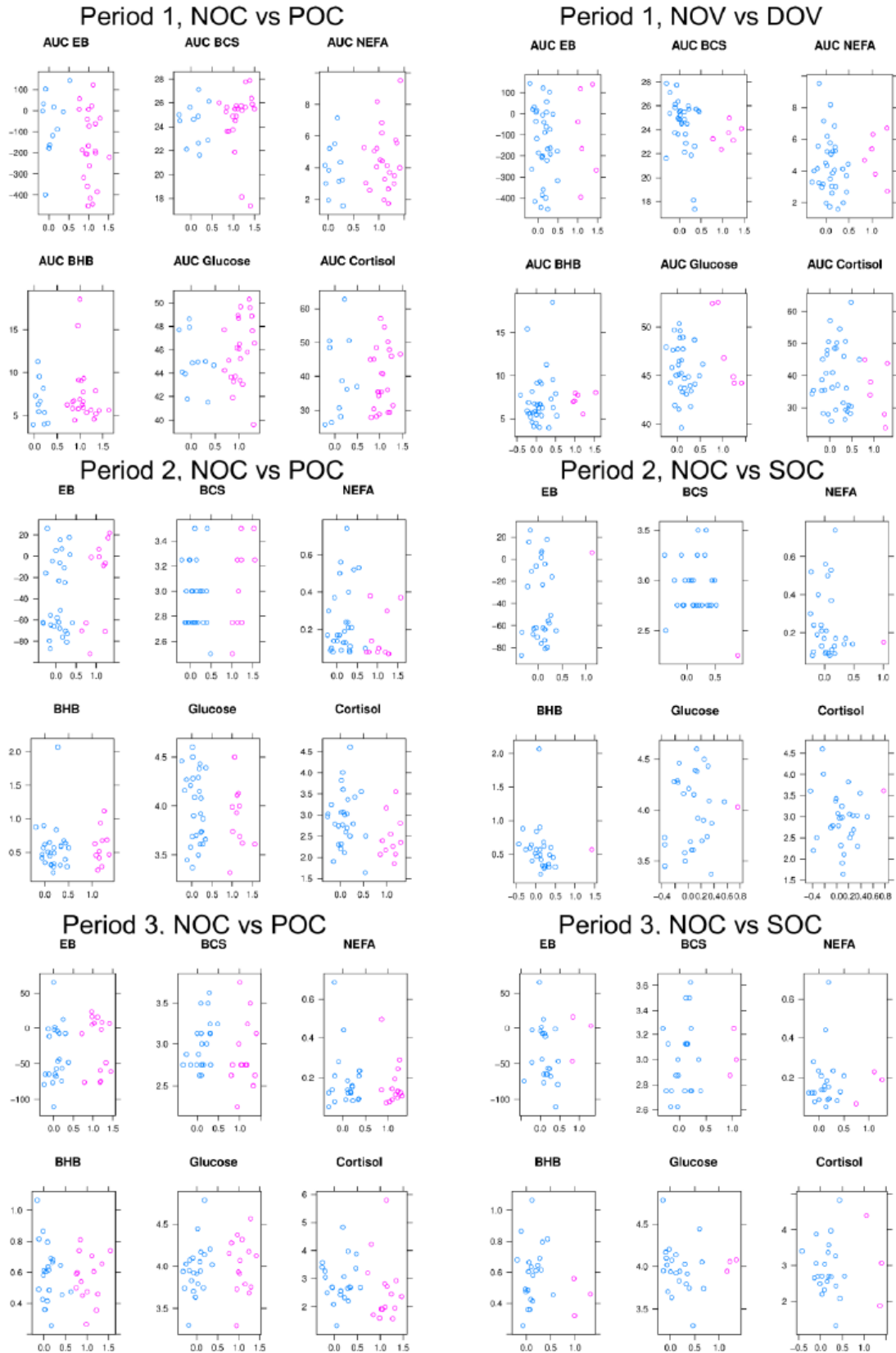


Figure 7. Scattered dataset visualisation. The data for Periods 1 to 3 is shown scattered by parameter values and colored by group membership (NOC,NOV: blue, POC, SOC, DOV: pink). Normal noise was added to the group dimension (0 or 1) to ease visual inspection. The groups are discussed in Figure 1.

For simulating ketosis 50% of control intake for up to 14 days was fed (Lor et al., 2007) or even only 16% energy intake of the control group for 60 h 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 on reproductive performance, but almost improves it. These findings, by trend only, 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 can be also 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% in 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 the 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 pp NEB. 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. No serious problems according to the induced energy deficit appeared. The results of our comprehensive study point out, 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 NEB 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 pp 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 phase of early pp NEB resulted in almost no effect on metabolic stability and reproductive ability. Our data show that there is no connection between a 49% energy deficiency for 3 weeks and metabolic imbalance on the one hand as well as fertility problems in terms of oestrous cycle and insemination success on the other hand. Therefore an energy deficiency alone seems not sufficient to induce the focused reproductive failures in dairy cows.

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REFERENCES

- Burke CR, Williams YJ, Hofmann L, Kay JK, Phyn CV, Meier S (2010). Effects of an acute feed restriction at the onset of the seasonal

- period on reproductive performance and milk production in pasture-breeding grazed dairy cows. *J. Dairy. Sci.* 93:1116-1125.
- Butler WR (2000). Nutritional interactions with reproductive performance in dairy cattle. *Anim. Reprod. Sci.* 60-61:449-457.
- Butler WR, Smith RD (1989). Interrelationships between energy balance and postpartum reproductive function in dairy cattle. *J. Dairy. Sci.* 72:767-783.
- Drackley JK (1999). ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier. *J. Dairy. Sci.* 82:2259-2273.
- Edmonson AJ, Lean IJ, Weaver D, Farver T, Webster G (1989). A Body Condition Scoring Chart for Holstein Dairy Cows. *J. Dairy. Sci.* 72:68-78.
- Fischer B, Mäurer H, Engelhard T, Haacker W (2002). Zur Futteraufnahme und Energieversorgung von Kühen in der frühen Trockenstehphase feed intake and energy supplementation of far-off dry cows. *Veterinarija Ir Zootechnika. T.* 19:41-48.
- Gross J, van Dorland HA, Bruckmaier RM, Schwarz FJ (2011). Performance and metabolic profile of dairy cows during a lactational and deliberately induced negative energy balance with subsequent realimentation. *J. Dairy. Sci.* 94:1820-1830.
- Grummer RR, Carroll DJ (1991). Effects of dietary fat on metabolic disorders and reproductive performance of dairy cattle. *J. Anim. Sci.* 69:3838-3852.
- Hall M, Frank E, Holmes G, Pfahringer B, Reutemann P, Witten IH (2009). The WEKA data mining software: An update. *CM SIGKDD Explorations Newsletter* 11:10-18.
- Huszenicza G, Haraszti J, Molnar L, Solti L, Fekete S, Ekes K, Yaro AC (1988). Some metabolic characteristics of dairy cows with different post partum ovarian function. *Zentralbl.Veterinarmed.A* 35:506-515.
- Inchaisri C, Jorritsma R, Vos PL, van der Weijden GC, Hogeveen H (2010). Economic consequences of reproductive performance in dairy cattle. *Theriogenology* 74:835-846.
- Kafi M, Mirzaei A (2010). Effects of first postpartum progesterone rise, metabolites, milk yield, and body condition score on the subsequent ovarian activity and fertility in lactating Holstein dairy cows. *Trop. Anim. Health Prod.* 42:761-767.
- Kessel S, Stroehl M, Meyer HH, Hiss S, Sauerwein H, Schwarz FJ, Bruckmaier RM (2008). Individual variability in physiological adaptation to metabolic stress during early lactation in dairy cows kept under equal conditions. *J. Anim. Sci.* 86:2903-2912.
- Kuhla B, Kuhla S, Rudolph PE, Albrecht D, Metges CC (2007). Proteomics analysis of hypothalamic response to energy restriction in dairy cows. *Proteomics* 7:3602-3617.
- Leitgeb E, van Saun RJ (2008). Interrelationships between production and reproduction in German dairy cows. In '25th World Buiatrics Congress'. (Eds Szenci O and Bajcsy ACs) pp. 148-155.
- Loor JJ, Everts RE, Bionaz M, Dann HM, Morin DE, Oliveira R, Rodriguez-Zas SL, Drackley JK, Lewin HA (2007). Nutrition-induced ketosis alters metabolic and signaling gene networks in liver of periparturient dairy cows. *Physiol. Genomics* 32:105-116.
- Meyer HHD, Guven B (1986). Improvement of microtitration plate enzyme immunoassays for steroid determination by a "second antibody technique". *J. Steroid Biochem.* 25(Suppl).
- Moyes KM, Drackley JK, Salak-Johnson JL, Morin DE, Hope JC, Loor JJ (2009). Dietary-induced negative energy balance has minimal effects on innate immunity during a *Streptococcus uberis* mastitis challenge in dairy cows during midlactation. *J. Dairy. Sci.* 92:4301-4316.
- Oikonomou G, Valergakis GE, Arsenos G, Roubies N, Banos G (2008). Genetic profile of body energy and blood metabolic traits across lactation in primiparous Holstein cows. *J. Dairy. Sci.* 91:2814-2822.
- Patton J, Kenny DA, Mee JF, O'Mara FP, Wathes DC, Cook M, Murphy JJ (2006). Effect of milking frequency and diet on milk production, energy balance, and reproduction in dairy cows. *J. Dairy. Sci.* 89:1478-1487.
- Perkins KH, VandeHaar MJ, Burton JL, Liesman JS, Erskine RJ, Elsasser TH (2002). Clinical responses to intramammary endotoxin infusion in dairy cows subjected to feed restriction. *J. Dairy. Sci.* 85:1724-1731.
- Prakash BS, Meyer HH, Schallenberger E, van de Wiel DF (1987). Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *J. Steroid. Biochem.* 28:623-627.
- Reist M, Koller A, Busato A, Kupfer U, Blum JW (2000). First ovulation and ketone body status in the early postpartum period of dairy cows. *Theriogenology* 54:685-701.
- Roche JR, Friggens NC, Kay JK, Fisher MW, Stafford KJ, Berry DP (2009). Invited review: Body condition score and its association with dairy cow productivity, health, and welfare. *J. Dairy. Sci.* 92:5769-5801.
- Royal M, Mann GE, Flint AP (2000). Strategies for reversing the trend towards subfertility in dairy cattle. *Vet. J.* 160:53-60.
- Sauerwein H, Dursch I, Meyer HH (1991). Quantitation of glucocorticoid receptors in bovine skeletal muscle: topographical distribution, sex effect and breed comparisons. *J. Steroid Biochem. Mol. Biol.* 39:941-945.
- Schlamberger G, Wiedemann S, Viturro E, Meyer HH, Kaske M (2010). Effects of continuous milking during the dry period or once daily milking in the first 4 weeks of lactation on metabolism and productivity of dairy cows. *J. Dairy. Sci.* 93:2471-2485.
- van Knegsel AT, van den BH, Dijkstra J, Kemp B (2007). Effects of dietary energy source on energy balance, metabolites and reproduction variables in dairy cows in early lactation. *Theriogenology* 68(1):S274-S280.
- Vanholder T, Opsomer G, de KA (2006). Aetiology and pathogenesis of cystic ovarian follicles in dairy cattle: A review. *Reprod. Nutr. Dev.* 46:105-119.