



## Animal models to study the impact of nutrition on the immune system of the transition cow



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### ABSTRACT

The immune system is particularly challenged in transition cows as marked physiological changes occur in this period which are driven by late gestation, partus and onset of lactation. As a consequence, the metabolic and nutritional state of the cow also changes significantly with possible implications for the plasticity and flexibility of the immune system. In order to understand how the balance between metabolism, nutritional status and the immune system is maintained under challenging conditions, such as an infection, various animal models can be used which specifically manipulate the nutritional status through various feeding and management strategies. Such models aim at exploring the immunological response to a challenge under largely varying nutritional and metabolic states.

As energy balance (EB) is strongly associated both with the metabolic state and with the immunoreactivity of the cows the manipulation of the EB by either influencing energy intake or energy excretion with milk, or by both, offers model opportunities for studying EB effects on the immune system. For example, assigning cows with a higher body condition score (BCS) at least 6 weeks prior to calving to an energy-dense diet exceeding the energy requirement in combination with a decelerated increase in the concentrate feed proportion *post partum* was shown to be effective in inducing a ketotic metabolic state under *ad libitum* feeding conditions. Compared to an adequately managed control group this model allows studying immune responses in the transit period and in dependence on dietary interventions.

### 1. Introduction

Dairy cows experience pronounced physiological changes during the transition from late gestation to early lactation. These changes include endocrine, metabolic and nutritional adaptations in an effort to adjust for the dynamics of the energy and nutrient requirement for the late exponential increase of conception products, for lactogenesis, calving, the onset of lactation and galactopoiesis (e.g. Bell, 1995). Based on these physiologically distinct events and processes it is clear that the immune system is involved and interacts at different levels of metabolism (Fig. 1) in order to maintain a nutritional status capable to cope with environmental challenges, such as infections. Thus, regulation of homeostasis as a dynamic steady state aims at supporting of different physiological states. These processes of orchestrated and coordinated control of metabolism are referred as to homeorhesis (Bauman and Currie, 1980). Thus, homeorhesis can be regarded as the process version of homeostasis (Gilbert, 2000) and can also be considered as the qualitative and quantitative insensitivity of a living system to the exogenous signals acting on it (Mamontov, 2007).

The homeorhetic adaptations associated with the transit period require distinct dietary adjustments to meet the specific requirement for energy and nutrients. Deviations from the fulfilment of these demands might influence the metabolism and possibly the immuno-reactivity of cows. Therefore, the knowledge of dietary factors influencing the different metabolic states of the transition cow is a precondition for investigating the consequences for immune responses.

### 2. General considerations for investigation of the relationships between the nutritional and metabolic state and the immune system by using animal models

To understand the complexity and the assumed plasticity of the homeorhetic regulation of different physiological states of the cow it seems to be helpful to simplify the main interrelationships contributing to the health of the animal (Fig. 1). The aim of the immune system is to protect the animal from environmental factors potentially disturbing its current physiological steady state. In order to stay responsive, the energy and nutrient requirement of the immune system needs to be met.

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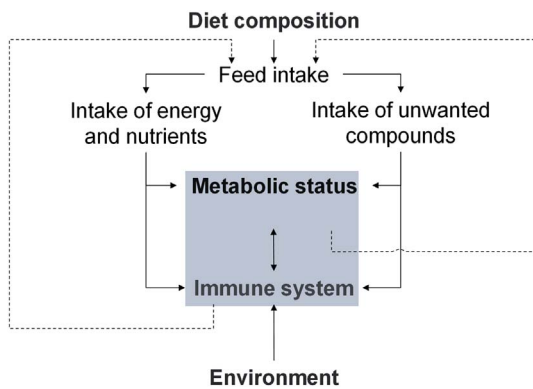


Fig. 1. Possible implications of nutrition and other environmental factors on the functionality of the immune system; for further details see text.

On the other hand it is clear that the nutritional status and therefore the functionality of the immune system might also be influenced by the diet composition and feed intake level. Diet composition itself might largely influence the level of feed intake and is therefore related not only to qualitative but also to quantitative aspects of maintaining the steady state between metabolism and immune system and the environment in general. However, estimation of energy and nutrient costs for immune responses is difficult due to the complexity of the immune system which is not confined to one individual organ, tissue or cell type but extending on many of them. Nevertheless, the lysine requirement for the maintenance of the immune system of chickens was estimated to vary between 0.5 and 2% relative to the total lysine requirement and to increase up to 10% upon immune stimulation (Klasing, 2007). More generally, the maintenance and use costs for acute phase responses are higher than for specific, lymphocyte-mediated immunity, which in turn, requires rather high developmental costs (Klasing, 2004). The traditional factorial approach of deriving energy and nutrient requirement assumes the costs of basal immune functions to be a part of it (Rauw, 2012). However, in situations of limited resource availability energy could be directed away from immune functions to other physiological processes essential for survival under maintenance conditions. Although severe malnutrition is generally recognized to compromise the resistance to human infections (Rauw, 2012; Rytter et al., 2014) not all immune functions are adversely affected (Rytter et al., 2014). Moreover, extending the concept of homeorhesis to other physiological and pathological states, including nutrition, infections are accepted to shift metabolic priorities, *i.e.* nutrient and energy use for immunological processes to cope with the infection. Here, the inflammation-associated pro-inflammatory cytokines orchestrate a homeorhetic response diverting nutrients and energy away from tissue growth and lactation to support immune response.

Moreover, the diet not only contains nutrients but also unwanted compounds such as mycotoxins and anti-nutritive substances which might adversely affect the metabolism but also the immune system (*e.g.* Dänicke et al., 2016; Winkler et al., 2014). An over-stimulated immune system might induce pro-inflammatory cytokines which in turn decrease feed and consequently nutrient intake (Bradford et al., 2015). Based on these facts it becomes clear that nutrition and feeding play a decisive role among the environmental factors challenging the steady state between metabolism, nutritional status and the immune system. Therefore, it seems to be justified to separate the nutrition from the rest of the environment (Fig. 1).

In order to understand how the steady state between metabolism, nutritional status and the immune system is maintained under challenging conditions, such as an infection, various animal models can be used which specifically manipulate the nutritional status through various feeding strategies. Such models aim at exploring the immunological response to a challenge under largely varying nutritional

and metabolic states.

These models can further be specified to particularly refer to the mentioned physiological states and might be characterized as follows:  
Non-lactating, non-gravid cows:

- Models for studying the role of lipogenesis/anabolism not biased by lactation and gravidity

Transition cows:

- Non-lactating, gravid cows: Models for studying the role of lipogenesis/anabolism and late gravidity not biased by lactation
- Lactating cows: Models for studying the combined role of lactation, lipolysis/catabolism, lipogenesis/anabolism and early gravidity

While the first model type clearly enables the investigation of the isolated effects of energy and nutrient supply on energy metabolism and immune system, the separation of the transition period into two phases can only be didactically since both physiological states are closely associated within the production and reproduction cycle. Depending on the time-span covered from late gestation of the preceding reproduction cycle to early gravidity of the following cycle both model types can be combined to transition cow models.

Energetically, the non-lactating, non-gravid cow needs energy only for maintenance purposes. In this situation the energy balance would be at steady state and numerically amount to zero. However, when energy intake exceeds this maintenance requirement, for example due to overfeeding, the energy balance becomes positive and might even become excessively positive. Based on its adult state, such a cow retains the excessive energy mainly as fat in various depots, such as subcutaneous, omental and retroperitoneal adipose tissues.

From an energetic viewpoint the situation of the transition dairy cow is characterized by a decrease in energy intake close to parturition and a slow increase after calving (Fig. 2). While during (late) gestation only the energy requirement for maintenance and fetus growth are needed to be met, the onset of lactation means an explosion of energy expenditure for milk energy. As this requirement cannot be met due to a stagnating and slowly increasing feed and energy intake the resulting energy balance becomes negative. This negative energy balance (NEB) is accompanied by an increase in blood concentrations of non-esterified fatty acids (NEFA) and ketone bodies such as beta-hydroxy-butyrate (BHB) as a reflection of lipolysis and insufficient NEFA utilization.

The models can be characterized and explored at different hierarchic levels such as whole animal (cow), tissue, cell and molecule while various key parameters indicative for model response are often used to describe the effects of nutritional and metabolic manipulations.

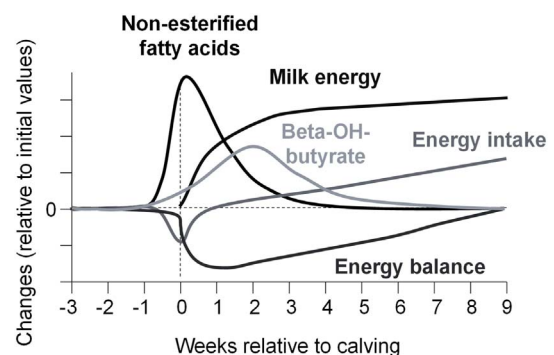


Fig. 2. Important physiological events in the transition period with relevance for metabolism and immune system.

### 3. Energy balance as an integrative and quantitative indicator for the nutritional and metabolic state

The energy balance (EB) is the difference between energy intake (EI) and quantifiable energy expenditures by the cow. For non-lactating and non-gravid cows the energy maintenance requirement ( $E_M$ ) is the only energetic expenditure:

$$EB = EI - E_M$$

For non-lactating late-gravid cows an extra energy expenditure for energy retention in fetus and fetus-related products ( $E_F$ ) has to be considered in balancing of energy:

$$EB = EI - E_M - E_F$$

For lactating cows the milk energy excretion ( $E_{\text{milk}}$ ) is the main energy expenditure besides the energy maintenance requirement:

$$EB = EI - E_M - E_{\text{milk}}$$

In any of the three cases, the energy balance is the proportion of energy retained in the body. When this balance is applied to the net energy system the energy balance represents (mainly) the sum fat and protein energy retention. When this balance is negative (NEB) the cow loses varying proportions of body fat and protein and consequently body weight. A positive EB means the opposite. As the EB includes both fat and protein energy at varying proportions the physiological meaning of the so-calculated difference can be ambiguous. Fat accretion as well as the proportions between fat and protein energy in the EB vary depending on physiological state, including parity (e.g. Raschka et al., 2016; Schroder and Staufenbiel, 2006; von Soosten, 2012; von Soosten et al., 2012) and might additionally be influenced by dietary variables.

Besides the energetic composition of the EB and its possible consequences for the nutritional and metabolic status the absolute value of EB depends on the accuracy of variables determining this balance. While energy excretion with milk can be easily and accurately determined the preciseness of EI depends on the energy system used, including its robustness to varying diet compositions.

$E_M$  is another source for variation in calculating the EB due to methodological aspects in estimating this requirement and for physiological reasons. From a methodological viewpoint it has to keep in mind that  $E_M$  is commonly estimated according to the so-called factorial approach which means a regressive assignment of ingested metabolizable energy to metabolic body weight, to milk energy and to live weight change. The regression coefficient assigned to the metabolic body weight equals the metabolizable  $E_M$ . An inherent feature of this approach is the correlation between the estimated regression coefficients. A positive linear relationship between  $E_M$  and partial utilization of ME for milk energy could clearly be demonstrated as a result of a meta-analysis (Gruber et al., 2007). Therefore, a high  $E_M$  is also associated with a high partial utilization of ME for milk energy with the consequence that calculation of energy requirement within a particular energy evaluation system is consistent. However, in calculating the EB the  $E_M$  is used isolated from the energy requirement for milk energy. As milk energy is not dependent on a particular energy evaluation system systematic and inherent variations of the calculated energy balance are the result. Currently, the  $E_M$  varies between 0.289 and 0.349 MJ NEL/kg metabolic body weight across several countries. Moreover, newer calculations based on a broad database from Germany revealed that the  $E_M$  is generally higher than hitherto assumed (Gruber et al., 2007). By using this database various evaluation strategies further revealed that  $E_M$  is approximately 21% lower for cows in a NEB than for cows in a positive EB and that it decreases by about 31% from 1st to 8th month of lactation before it increases again. Furthermore, lactation number and breed are further sources of variation. These statistically based conclusions clearly have a physiological meaning. For example, not only a primiparous cow differs in its chemical composition from a pluriparous

cow but also cows in similar physiological states might differ in chemical composition as discussed above. Based on the marked differences between energy expenditure for fat and protein turnover it becomes clear that using a similar constant for estimating energy maintenance requirement will result inevitably in variation of energy balance. As individual cows might have different chemical body compositions and metabolic states the individual energy maintenance requirement can probably not be regarded as being constant. Coupling the  $E_M$  to the metabolic body weight can probably not completely compensate for the discussed variations.

Taken together, the advantage of a simple equation for calculating the EB is ultimately also a source of variation due to the discussed assumptions. These variations need to be considered when interpreting and discussing the associations between EB and the immune system.

Irrespective of this variation EB has been associated with metabolic diseases, such as ketosis, insulin resistance (Bell, 1995; Holtenius and Holtenius, 1996, 2007), hepatolipidosis (Bobe et al., 2004; Hammon et al., 2009; Herdt, 2000; Holtenius, 1993; Schulz et al., 2014; Wensing et al., 1997), hypocalcaemia (Mulligan et al., 2006), digestive disorders, such as ruminal acidosis (Esposito et al., 2014), abomasal displacement (Shaver, 1997), compromised fertility (Fenwick et al., 2008), an impairment of the immune system (Morris et al., 2009; van Kneysel et al., 2012), and an increased incidence of infectious diseases such as metritis, mastitis, laminitis (Collard et al., 2000; Goff, 2006; LeBlanc, 2010).

### 4. Beta-hydroxy butyrate (BHB) and non-esterified fatty acids (NEFA) as non-quantitative indicators for lipolysis and energy balance

Although the EB as an indicator for energy status can easily be calculated other parameters such as blood levels of BHB and NEFA indicative for energy metabolism and particularly for NEB are used. The apparent association between EB, blood concentrations of glucose, BHB and NEFA were used to define a physiological imbalance index (PI) which is based on the regression of the mentioned 3 blood variables indicative for energy metabolism on EB (Ingvarsen and Moyes, 2015; Moyes et al., 2013). Prepartal PI was shown to be a more reliable predictor for postpartal infectious diseases such as metritis than EB, BHB and glucose alone. These relationships make clear that postpartal immune-suppression can be explained more reliably by more than one parameter indicative for the energetic and metabolic status of the cow. Nevertheless, many attempts have been made to relate these parameters to each other and to immune traits aiming at identifying diagnostic tools which can be implemented on a routine basis. On the other hand, the identification of parameters which most reliably describe the relationship between energy status of the cow and her immune-competence is useful for developing animal models addressing the relationships between metabolic and immunological status.

Cows assigned p.p. either to a group with high or low NEFA levels showed clear associations between the extent of NEB and gene expression of enzymes involved in NEFA utilization and ketogenesis (Van Dorland et al., 2012).

The easy way of calculating the EB and its advantage as a quantitative term describing the energy metabolism is defeated by the necessity to quantitatively record feed intake and milk excretion. While recording of milk yield is a part of common dairy farm management the quantitative-individual recording of feed and thus EI is confined to experimental farms or facilities. Therefore, cut-off values for the non-quantitative BHB and NEFA blood levels are used for distinguishing of individual cows with a high probability of undergoing an excessive NEB and a higher risk for production diseases (McArt et al., 2013). Moreover, as blood BHB levels can be determined on a routine diagnostic basis this energy balance indicator is well suited for epidemiological studies investigating the associations between blood BHB concentrations and the prevalence of important production diseases such as

metritis, mastitis and lameness. For example, a survey of a total of 5884 cows from 528 dairy farms from Croatia, Germany, Hungary, Italy, Poland, Portugal, Serbia, Slovenia, Spain, and Turkey revealed 1.5, 9.5 and 5.0 times greater odds for developing metritis, clinical ketosis, and displaced abomasum, respectively, in subclinical ketotic cows when a cut-off BHB concentration of 1.2 mMol/L was set (Suthar et al., 2013). These results principally agree with an earlier literature survey suggesting a 3 to 8 times higher risk for a displaced abomasum, a 3 times higher risk for metritis together with a higher probability for subclinical endometritis and an increased duration and severity of mastitis when blood BHB levels were higher than 1.2 mMol/L (LeBlanc, 2010).

Compromised udder defense capacity of cows in an NEB were related to hyperketonemia. In this situation, a poor metabolic state of leukocytes, including lower enzyme activities for energy metabolism, was hypothesized as a possible reason for a compromised functionality as evidenced by a reduced chemotactic activity (Suriyasathaporn et al., 2000).

On the other hand, the limitations of BHB and NEFA as indicators for energy balance becomes clear from observations where cows that were grouped according to their BHB and NEFA levels did not differ in their p.p. NEB (Kessel et al., 2008). Moreover, BHB and NEFA levels are obviously closer associated to the physiological p.p. NEB than to an even more pronounced NEB artificially induced by a qualitative feed restriction (dietary energy dilution) at about day 100 of lactation due to different endocrine and metabolism prerequisites (Gross et al., 2011a; Gross et al., 2011b; Gross et al., 2013).

Taken together, the epidemiological based associations between BHB and the prevalence of postpartal infectious diseases provide some evidence that energetic and nutritional status of the cow is related to her immune-competence in this critical physiological period. Furthermore, based on these relationships, BHB levels appear to be suited as a critical endpoint for development of animal models investigating the consequences of a compromised energy status on the immune system.

## 5. BHB and NEFA implications for the immune system

Both NEB - as a balance parameter of many processes involved in energy metabolism - and particular indicators of lipolysis such as NEFA and BHB have been implicated in immuno-suppressive and -modulating effects (Adewuyi et al., 2005). These conclusions were mainly drawn from *in vitro* or *ex vivo* dose-response studies evaluating the viability, functionality or proliferative response of immune cells isolated from blood or spleen of cows.

A BHB level of about 1.5 mMol/L has been identified as critical on the function of bovine myeloid and erythroid precursor cells (Hoeben et al., 1999) and the respiratory burst activity of bovine neutrophil granulocytes (Hoeben et al., 1997). On the other hand, others found lower concentrations to be effective in the suppression of the proliferation of peripheral blood mononuclear cells (PBMC) (Targowski and Klucinski, 1983) and the phagocytic activity of polymorphonuclear granulocytes. An experimentally provoked ketosis resulted in a significantly reduced interferon (IFN) production of concanavalin-A (ConA)-stimulated PBMC (Filar et al., 1992; Kandefer-Szerszen et al., 1992).

In addition, a negative correlation was found between the phytohemagglutinin (PHA)-stimulated IFN synthesis, the blastogenic response of PBMC and circulating total ketone bodies. This correlation covered the range of 0.6, 1.25 and 4.25 mMol ketone bodies/L corresponding to healthy cows, and cows suffering from a subclinical and clinical ketosis, respectively (Kandefer-Szerszen et al., 1992). Supporting this negative relationship, a comparable ConA-stimulated PBMC proliferation has been reported for lactating and dry cows having mean systemic BHB concentrations of 1.13 and 0.6 mMol/L, respectively, while a significant reduction was noticed for ketotic cows showing an average BHB concentration of 2.03 mMol/L (Sato et al.,

1995). In contrast, significantly higher BHB concentrations of up to 6.25 mMol/L have not suppressed ConA-stimulated PBMC proliferation (Franklin et al., 1991). Moreover, the proliferative response of ConA-stimulated PBMC isolated from transition cows with a higher body condition score which were characterized by a high prevalence of subclinical ketosis (BHB > 1.2 mMol/L) was not compromised compared to cows with a lower body condition score (Schulz et al., 2015). Subjecting these PBMC to increasing BHB-concentrations *in vitro* resulted in a dose-dependent decrease of the stimulation index which became significant at BHB concentrations of > 5 mMol/L. The corresponding IC<sub>50</sub> were estimated at 6.9 and 6.6 mMol/L for cows with a lower and higher BCS, respectively.

*In vitro* experiments with bovine PBMC exposed to increasing doses of a mixture of a fatty acid mixture typically mobilized from the body fat reserves (C16: 0, C16: 1, C18: 0, C18: 1, C18: 2) and thus mimicking the p.p. NEFA profile have revealed a dose-dependent reduction of the pokeweed mitogen (PWM), PHA and ConA-stimulated lymphocyte proliferation (Lacetera et al., 2004). The drop became significant at concentrations of 0.5 to 2.0 mMol/L, depending on the mitogen used. Immunoglobulin M and IFN secretion by these PBMC were significantly depressed at even lower concentrations of 0.125 mMol/L. From these results, it was concluded that the NEFA plasma level exerts a direct influence on the function of the PBMC and thus on the immunological situation of the postpartum dairy cow in particular. In contrast, a nearly similar fatty acid mixture, but without linoleic acid, was shown to affect proliferation at much lower concentrations whereby an IC<sub>50</sub> of 0.81 mMol/L was estimated. It needs to be stressed that both experiments (Lacetera et al., 2004; Renner et al., 2013) were carried out as dose-response experiments covering the NEFA concentration range to be expected in the production cycle of a dairy cow. Based on literature compilation the NEFA concentrations are usually lower than 0.2 mMol/L for cows being in a positive energy balance and increase to 0.5 and 1.0 mMol/L during the last week, and to 0.8 and 1.2 mMol/L during the last 2–3 days prior to parturition where the maximum level is reached. Thereafter, the levels decrease again down to values lower than 0.3 mMol/L within a period of about 6 weeks. Levels of > 0.7 mMol/L after calving are considered to indicate severe NEB (Adewuyi et al., 2005). Irrespective of possible slight deviations from this general pattern with regard to absolute values and kinetics it becomes obvious that NEFA levels which were shown to be inhibitory for proliferation and function of bovine PBMC were in the lower physiological range. Moreover, as fatty acid pattern of NEFA also contributes to variation of the proliferative response of PBMC it seems reasonable to assume that different effects might be observed even at similar NEFA levels observable in in the transition period and the whole reproductive cycle. This makes clear that PBMC might become quite refractory to mitogen stimulation at the onset of NEB when NEFA levels are high. However, *ex vivo* proliferation assays using PBMC collected during the entire transit period revealed inconclusive results. While a parturition related significant drop was found by Renner et al. (2012), others failed to demonstrate a decrease in the period of the p.p. NEFA peak (e.g. Drong et al., 2017; Schulz et al., 2015; Tienken et al., 2015a).

*In vitro* investigations of the interactions between NEFA and BHB on proliferation of PBMC isolated from cows at day 61 p.p. revealed a significant drop when NEFA was added while BHB failed to reduce proliferation (Ster et al., 2012). The proliferation decrease observed when cells were incubated with NEFA and BHB together was of the same order of magnitude as compared to NEFA addition alone. Further dose-response studies confirmed the vulnerability of bovine PBMC for the anti-proliferative effects low NEFA doses *in vitro*. Even concentrations of 0.013 mMol/L significantly decreased proliferation of PBMC while IFN secretion started to decrease at higher concentrations (Ster et al., 2012).

However, in comparing physiological NEFA levels with those proven to be inhibitory *in vitro* it needs to be considered that *in vitro* conditions differed between experiments (e.g. chemicals used for



preparation of fatty acid mixtures, glucose contents in media etc.) and that *in vitro* micro-environmental milieu might mimic *in vivo* conditions not perfectly with respect to e.g. nutrients, metabolites, hormones and cytokines. Among these metabolites particularly ketone bodies such as BHB are of interest as discussed above. Regressing either BHB or NEFA levels of cows in NEB on *ex vivo* ConA-stimulation index (MTT assay) revealed only weak coefficients of determination ( $r^2$ ) of only 0.13 and 0.20, respectively (Dänicke et al., 2012). However, a combined multiple quadratic regression of BHB and NEFA on stimulation index increased the  $r^2$  to 0.56. Thus, 56% of the variation in PBMC stimulation ability could be explained by the combined effects of BHB and NEFA. Regression coefficients suggested an increase in stimulation index with decreasing NEFA levels and increasing BHB concentrations (< 1.5 mMol/L). Attempts to explain a higher proportion of variance by including *in vivo* glucose levels in the regression model failed which suggests glucose levels less important in influencing PBMC stimulation than BHB and NEFA.

On the other hand, *in vitro* lymphocyte proliferation tests suggested interactions between glucose and ketone body levels and demonstrated a ketone body-mediated suppression of PWM-stimulated proliferation at low glucose levels (Franklin et al., 1991). These results hint at substrate-driven variations in fuel use by activated immune cells. Resting T-cells which are activated, for example by mitogens, switch from oxidative phosphorylation to aerobic glycolysis to provide glycolytic intermediates as a source of carbon for synthesis of DNA and proteins which are critical for cell proliferation (Donnelly and Finlay, 2015; Finlay, 2012). Other substrates, such as ketone bodies also serve as fuel substrates and interfere with glucose metabolism. For example, murine lymphocytes were shown to be capable of utilizing ketone bodies such as acetoacetate and BHB whereby the rates of utilization were not altered by starvation of the cell donors (Ardawi and Newsholme, 1984). Moreover, unlike glucose utilization, ConA-stimulation did not alter the rate of acetoacetate utilization. On the other hand ketone bodies decreased glucose utilization and pyruvate oxidation (Ardawi and Newsholme, 1984).

T cells being an important sub-population of the PBMC fraction were also phenotypic characterized and related to several physiological conditions, including lipolysis and blood NEFA levels. The ratio between CD4+ and CD8+ T-cells is considered to be important in maintaining T cell homeostasis and immune regulation in cows (Mehrzhad and Zhao, 2008). The authors considered a ratio of ~2.5 as physio-immunologically normal. A higher lymphoproliferative response of T cells was found in primiparous compared to pluriparous cows which corresponded to CD4+/CD8+ ratios of ~2.5 and ~4.0, respectively. Moreover, a gradual increase in dietary energy supply resulted in a significant decrease of the CD4+/CD8+ ratio from 2.5 to 2.1 in non-gravid and non-lactating cows (Fig. 3). Time and treatment (body condition score, energy supply) around calving influenced the

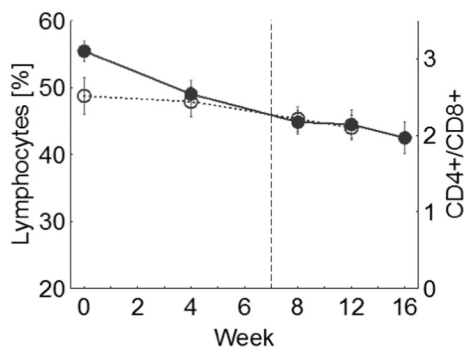


Fig. 3. Lymphocyte proportion (●,  $p < 0.05$ ) in blood and CD4+ to CD8+ ratio (○,  $p < 0.05$ ) in dependence on gradual increase of dietary concentrate feed proportion from 0 to 60% (week 0–8) and a continued feeding of the energy-dense diet to non-lactating, non-gravid cows (Dänicke et al., 2016).

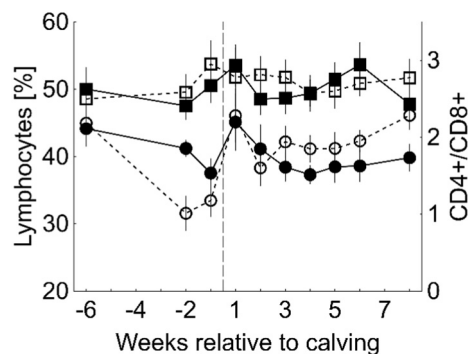


Fig. 4. Lymphocyte proportion (●,  $p_{\text{week} \times \text{treatment}} < 0.05$ ) in blood and CD4+ to CD8+ ratios (■,  $p_{\text{week} \times \text{treatment}} < 0.05$ ) levels in blood of cows with a higher BCS that were fed an energy-dense diet a.p. (60% concentrate feed proportion) and subjected to a decelerated concentrate feed proportion increase p.p. (○, □) in comparison to adequately fed cows with a lower BCS (●, ■) (Drong et al., 2017).

CD4+/CD8+ ratio in an interactive manner (Fig. 4). Furthermore, CD4+/CD8+ ratios were significantly increased p.p. in cows fed a diet with a higher non-fiber carbohydrate content a.p. This change was paralleled by a significant increase in the IF- $\gamma$ /IL-4 mRNA-ratio in PBMC (Ohtsuka et al., 2006). The CD4+/CD8+ ratio correlates positively with the NEFA levels with a steeper increase in early lactating cows compared to non-gravid and non-lactating cows (Fig. 5). It might be assumed that CD4+ and CD8+ cells respond differently to increasing NEFA levels while the steeper slope of the CD4+/CD8+ ratio in early lactating cows might indicate effects of differences in fatty acid composition of NEFA with consequences as discussed above.

The specific role of hyperketonemia for mammary immune response was studied in cows being in a steady state or positive EB by infusion of BHB up to a level of 1.7 mMol/L. Thus, this protocol enabled studying the putative BHB immuno-modulating effects independent of elevated NEFA levels or other typical p.p. endocrine and metabolite fluctuations. It could be shown that induced hyperketonemia increased mRNA abundance of SAA and Hpt (as a trend) in the mammary gland. A subsequent intramammary LPS challenge resulted in a less pronounced increase in somatic cell counts and a stronger increase in IL-8 and IL-10 mRNA expression in hyperketotic cows. Therefore, hyperketonemia exerts immune-modulating effects by influencing the expression of cytokines giving rise to increased mastitis susceptibility (Zarrin et al., 2014).

## 6. Development of models

### 6.1. Factors for manipulating the nutritional and metabolic state

#### 6.1.1. Energy supply

The degree of p.p. NEB can generally be increased either by a decrease in energy intake or by an increase in milk energy excretion in the most critical period immediately after calving. An increase in milk excretion can be achieved by milking the cows thrice daily instead of twice (Fenwick et al., 2008; Morris et al., 2009). This increase in milking frequency increases the severity of NEB and is therefore suited to generate experimental groups differing in the extent of the NEB. The opposite also works meaning that once daily milking in the most sensitive period after calving reduces the milk energy output whereby also experimental groups differing in the magnitude of NEB can be created (see Pierre Lacasse et al., this issue).

A decrease in energy intake as another measure to increase the severity of NEB can be achieved either quantitatively or qualitatively. A quantitative reduction in energy intake means a restriction of the amount of feed and thus energy while a qualitative decrease is managed through an energetic dilution of the daily ration.

Fasting triggers lipolysis and reduces the activities of glucose 6-

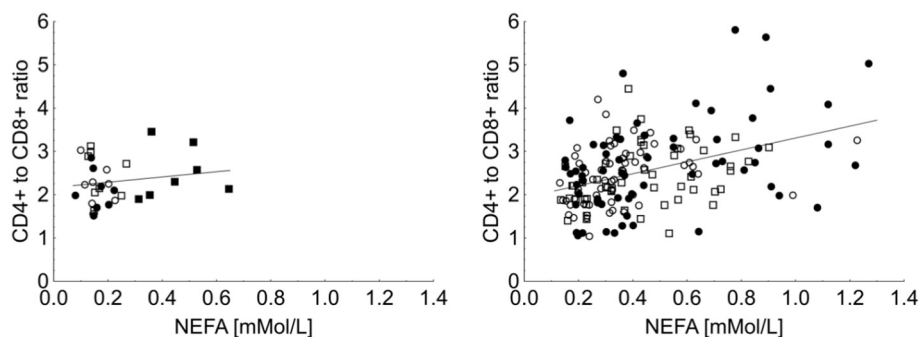


Fig. 5. Relationship between non-esterified fatty acid (NEFA) concentration in blood and ratio between CD4+ and CD8+ lymphocytes.

phosphate dehydrogenase, 6-phosphogluconate dehydrogenase and NADP-isocitrate dehydrogenase in adipose tissues suggesting a decreased lipogenesis (Vernon, 1980). Similarly to adipose tissue, pentose phosphate pathway is downregulated in the mammary gland in response to fasting while feed restriction stimulates this anabolic pathway (Guinard-Flament et al., 2006). Therefore, feed restriction rather promotes lactose synthesis and consequently milk excretion in general for osmotic reasons. Therefore, feed restriction rather than fasting would support the intended NEB.

Fasting does time-dependently alter the eating behavior (Patterson et al., 1998) of cows and therefore the involved metabolic and endocrine mechanisms which might produce an additional source of variation when energy metabolism of fasted and *ad libitum*-fed cows is compared.

Thus, while a restriction of the amount of feed might induce additional metabolic stress to the animal potentially confounding the intended solely nutritional manipulation of metabolism a qualitative energy restriction still allows *ad libitum* feeding and thus a normal feeding behavior.

Several strategies can be employed to manage this energetic dilution such as a decrease in the concentrate feed proportion, a decrease of feedstuffs with higher energy concentrations within the concentrate feed proportion or a decelerated increase of the concentrate feed proportion after calving.

Feeding of high-concentrate diets up-regulates various enzymes of energy metabolism and lipid turnover in bovine adipose tissues, such as ATP-citrate lyase, glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, NADP-malate dehydrogenase, NADP-isocitrate dehydrogenase, glycerol 3-phosphate dehydrogenase and lipoprotein lipase (Vernon, 1980). Particularly the increase in the capacity of NADPH synthesis appears to be influenced by feeding high-concentrate diets.

Phosphorylation of hormone sensitive lipase (HSL) at Ser 660 and Ser 563 was significantly increased at day 21 p.p. in retroperitoneal adipose tissue (RPAT) in cows fed a ration with a high concentrate proportion of 60% starting from calving but not in subcutaneous adipose tissue (SCAT). In contrast, no effects were noticed in either fat depot in cows fed a lower concentrate feed proportion of 30%. These group differences were associated with a significantly less severe NEB, higher propionate and lower BHB blood levels in cows fed the high-concentrate diet (Friedrichs et al., 2014; Friedrichs et al., 2016; Locher et al., 2011). Lipolysis in both fat depots was associated with an increased phosphorylation of adenosine monophosphate-activated protein kinase (AMPK) $\alpha$ 1 and of the ratio of pAMPK $\alpha$ 1 to AMPK $\alpha$ 1 irrespective of concentrate feed feeding regimen (Locher et al., 2012). In addition, at day 21 p.p. free fatty acid receptor (FFAR) 2-mRNA abundance was 2.5-fold higher in RPAT of the energetically underfed group suggesting a decrease in lipolysis while expression in SCAT was only marginally influenced by different concentrate feed proportions (Friedrichs et al., 2014; Friedrichs et al., 2016).

Besides lipid metabolism the oxidative status of the cow might also

be influenced by dietary energy supply. Cows fed a diet with a lower energy concentration a.p. displayed a reduced glutathione peroxidase (GPX) activity in serum when compared to their energetic oversupplied counterparts which was discussed to result from a generally lower oxidative burden due a relative lower metabolic rate (Bühler et al., 2018). Gene expression levels of superoxide dismutase (SOD) 2, (ADP-ribose) polymerase 1 (PARP1) and xanthine dehydrogenase (XDH) were upregulated in response to feeding an energy-dense diet a.p. suggesting energy effects on mitochondrial respiratory chain, ROS formation and regulation of oxidative stress induced cell death (Bühler et al., 2018). Oxidative stress issues of the transition cow are further elaborated elsewhere (Sordillo, 2016; Sordillo et al., this issue).

Feeding of a ration with high concentrate feed proportion of 60% during late gestation resulted in an increased proportion of early apoptotic PMN and PBMC in blood with the effect being more pronounced in PBMC (Bühler et al., 2016). Moreover, a higher energy supply was also shown to increase the CD14+ monocytes in cows (Rontved et al., 2005).

All the discussed metabolic and immunological responses to varying dietary energy densities cannot be evaluated detached from ruminal metabolism and metabolites which form the prerequisite for all scenarios beyond the gastro-intestinal barrier. Besides the pivotal role of propionate for gluconeogenesis and thus for energy metabolism (Aschenbach et al., 2010), it has been suggested that lipopolysaccharides (LPS) as constituents of Gram-negative bacteria are increasingly transferred across the rumen mucosa and acting as endotoxins when this barrier is compromised by an acute or subacute ruminal acidosis (Khafipour et al., 2007; Khafipour et al., 2009; Sato, 2015; Zebeli and Metzler-Zebeli, 2012). This stage might develop under the conditions of a high dietary supply with propionate precursors. Therefore, the rumen is not only the site where a large portion of energy is generated in form of propionate and other volatile fatty acids but ruminal microbiota, its metabolites and constituents are itself potent stimuli of the innate immune system as they are capable of triggering an acute phase reaction (APR) when entering the liver or systemic circulation. LPS belong to the so-called pathogen-associated molecular pattern (PAMP) which is recognizable by highly conserved surface molecules (pattern-recognition receptors, PRR) (Akira et al., 2006) such as CD14, TLR4 and MD-2. Together with other co-receptor molecules this recognition induces a signal transduction cascade at which end inflammatory cytokines are released to initiate the APR (e.g. Aderem and Ulevitch, 2000; Akira et al., 2006; Freudenberg et al., 2008). In the course of the APR acute phase proteins (APP) are formed, mainly by the liver but also by extra-hepatic tissues. Haptoglobin (Hpt) appears to be the most relevant APP in cattle as it increases more dramatically than those of SAA and CRP (Murata et al., 2004; Petersen et al., 2004). In addition, Hpt was identified as adipokine in cows, with the expression appearing to be independent of the size of the adipose tissues and to vary according to parturition and parity (Saremi et al., 2012). The main function of Hpt is to bind hemoglobin and consequently iron whereby a bacteriostatic effect is achieved. Moreover, Hpt influences neutrophil function and

modulates other immune reactions as well (Murata et al., 2004; Petersen et al., 2004).

Based on the discussed functional chain between dietary driven ruminal LPS release and possible initiation of a systemic APR, the relationships between dietary concentrate proportion as determining factor for the development of a ruminal acidosis, ruminal LPS generation and systemic concentrations of APP were examined (for review see Zebeli and Metzler-Zebeli, 2012). For example, both an increase in dietary barley concentration and a longitudinal increase in dietary concentrate proportion increased significantly the ruminal LPS concentration in lactating and non-lactating cows, respectively (Dänicke et al., 2016; Zebeli et al., 2010). However, APP were only increased in the experiment with lactating cows while Hpt remained unaltered even after a dramatic increase in the dietary concentrate proportion from 0 to 60% in non-lactating cows.

Taken together, manipulation of metabolic and nutritional state by feeding diets differing in energy concentration with the aim to influence energy intake and consequently the extent of EB or NEB is mediated via rumen fermentation and might have direct consequences for the innate immune system.

### 6.1.2. Body condition

It is common knowledge that cows calving overconditioned are at a higher risk of an accelerated lipolysis and a more severe NEB.

Therefore, creating experimental groups differing in their body condition at calving is another tool for inducing differences in the p.p. metabolism and NEB.

Differences in body condition at calving can be achieved by an energetic oversupply during the last weeks of pregnancy. The success of such a dietary manipulation strongly depends on the length of this overfeeding period and on the specific body condition of the cows at the beginning of the dietary intervention.

Body condition score (BCS) is a useful and easily to determine indicator for production, reproduction and health of cows; particularly the loss of body condition after calving is related to NEB (Roche et al., 2009). In this review it has been suggested that for many production and health parameters the association to BCS is non-linear (Roche et al., 2009) which might hamper its usefulness as a predictor for all nutritional and metabolic states. Nevertheless, the optimum BCS according to a 5-point-scale ranges between 3.0 and 3.25 whereby a lower BCS is related to a compromised production and reproduction while a BCS higher than 3.5 is associated to a reduced dry matter intake shortly after calving and to an increased susceptibility to metabolic diseases (Roche et al., 2009). While Holtenius and Holtenius (2007) demonstrated a linear negative relationship between BCS and the insulin sensitivity index RQUICKI at calving Schulz et al. (2014) failed to demonstrate a difference between cows with a lower or higher BCS. In interpreting these results it needs to be considered that the former experiment covered the whole range of BCS varying between 1 and 5 while the BCS differences in the latter experiment were rather small (2.94 vs. 2.59). As adipocytes are the main source of leptin it is not surprising that positive correlations exist between a.p. BCS and serum leptin levels (Hachenberg et al., 2007).

Cows being at a higher body condition at calving did not lose more body fat in the transit period when compared to their thin conditioned counterparts in spite of markedly higher NEFA levels. Moreover, lipogenic and lipolytic capacities of isolated adipocytes did not differ between these groups. On the contrary, fat cows lost more muscle fiber area and were characterized by a more pronounced fatty infiltration of the liver (Reid et al., 1986). A lower white blood cell count was detected in the fat cows during week 1 after parturition. Although overconditioned transition cows with a high BCS showed a significantly higher NEFA peak than cows with a medium or low BCS the glucose levels were not different between these groups at any time (Lacetera et al., 2005). This observation led the authors to conclude that evaluation of blood glucose levels is not very helpful when it comes to the

prediction of immunological consequences of overconditioning of transition cows.

Similar to early lactating cows, an increase in energy balance and BCS of non-lactating non-gravid cows was associated with a significant decrease in RQUICKI mostly driven by a sustained increase in blood glucose levels (Dänicke et al., 2014; Locher et al., 2015) rather than by fluctuations in NEFA concentrations seen in early lactating cows.

With regard to nutritional manipulation it is worth mentioning that BCS during the first 4 weeks after calving can hardly be influenced by feeding due to the natural insulin resistance and the uncoupling of the somatotrophic axis. Energy intake stimulates lipogenic enzymes when this period has passed (Roche et al., 2009) giving rise to an increase in BCS and energy balance.

A lower IFN- $\gamma$  secretion of PBMC was observed for transition cows with a high BCS compared to those with a medium or low BCS while the DNA synthesis of PBMC was not significantly different between these 3 BCS groups (Lacetera et al., 2005). Moreover, a significant negative correlation between blood NEFA levels and IFN- $\gamma$  secretion by PBMC was reported by these authors. IFN- $\gamma$  has been implicated in the activation of the indolamine-2,3-dioxygenase (IDO) the enzyme responsible for degradation of tryptophan to kynurenine (Schröcksnadel et al., 2006). The ratio between kynurenine and tryptophan has therefore been suggested as an indicator for IDO activity and immune-activation (Okamoto et al., 2007; Schröcksnadel et al., 2003; Taylor and Feng, 1991). The IDO-mediated decrease in tryptophan levels down-regulates T-cell metabolism by slowing protein synthesis ultimately resulting in a compromised immuno-reactivity of these cells (Schröcksnadel et al., 2006). Late gestating cows with a higher BCS were shown to develop a lower kynurenine to tryptophan ratio p.p. compared to cows with a lower BCS (Fig. 6). Whether down-regulated tryptophan degradation in cows with a higher BCS could be related to the lower IFN- $\gamma$  secretion of PBMC as observed in overconditioned cows (Lacetera et al., 2005) needs to be clarified further. Moreover, as a result of a targeted blood metabolomic approach particularly the kynurenine/tryptophan ratio was identified to be increased in cows that left the productive life early when compared to healthy cows (Huber et al., 2016). This increase was interpreted as the inability of those cows to avoid metabolic imbalance. As the ratio was increased over longer periods of time without episodes of acute diseases a low-grade chronic inflammation was assumed. Based on these findings, monitoring this ratio was suggested for screening of balanced and non-balanced metabolic phenotypes (Huber et al., 2016).

BCS was also associated to monocyte counts. Compared to cows with a higher BCS those with a lower BCS showed increased counts of intermediate monocytes (CD14 + CD16+, intM) and non-classical monocytes (CD14-CD16+, ncM) at 7 days p.p. This effect was even more pronounced when only diseased cows with a lower BCS were evaluated separately (Eger et al., 2015) hinting at a more pro-

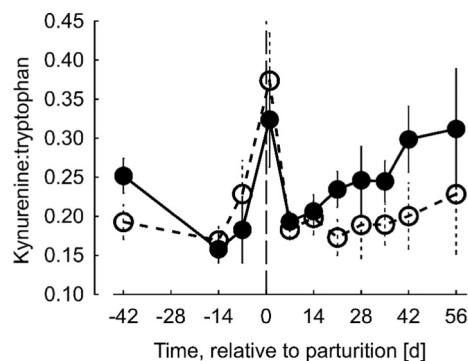


Fig. 6. Kynurenine to tryptophan ratio in blood of cows with a higher BCS that were fed an energy-dense diet a.p. (60% concentrate feed proportion) and subjected to a decelerated concentrate feed proportion increase p.p. (○) in comparison to adequately fed cows with a lower BCS (●) ( $p_{\text{week} \times \text{treatment}} < 0.05$ ) (Hüther et al., 2016).



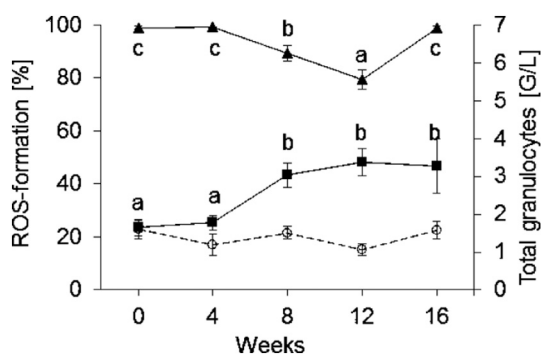


Fig. 7. Granulocyte numbers (■), unstimulated (basal, ○) and stimulated (▲) ROS formation of granulocytes in blood in dependence on gradual increase of dietary concentrate feed proportion from 0 to 60% (week 0–8) and a continued feeding of the energy-dense diet to non-lactating, non-gravid cows (ac - different superscripts indicate significant differences,  $p < 0.05$ ) (Dänicke et al., 2016).

inflammatory situation. It has been shown *in vitro* that monocyte glucose uptake is reduced after parturition (Eger et al., 2015) and is downregulated *in vivo* when mammary glucose uptake increases at the onset of lactation (Eger et al., 2016) independent of BCS. The negative non-linear relationship between the expression of glucose transporter GLUT3 relative to GLUT1 and the lactose synthesis suggests a decrease in the glucose transport affinity of monocytes irrespective of a lower or higher BCS. These results support the prioritization of lactose and thus milk production with possible consequences for the energy metabolism of monocytes.

A higher BCS is apparently associated with an increased oxidative stress (Bernabucci et al., 2005) which is mirrored by a significant negative correlation between BCS and serum ferric reducing ability (FRA) (Bühler et al., 2018). A continuous increase of BCS which was induced by a longitudinal gradual increase in energy supply caused a steady increase of total granulocyte numbers in non-gravid and non-lactating cows. While basal ROS-formation activity remained uninfluenced the stimulated oxidative burst first decreased and later increased in the course of dietary manipulation of energy supply (Fig. 7). Prepartum energetically oversupplied cows with a higher BCS displayed higher total granulocyte counts a.p. and a lower basal oxidative burst activity both a.p. and p.p. when compared to cows fed adequately and having a lower BCS (Fig. 8).

Taken together, the body condition of cows is strongly related to the nutritional status with possible consequences for metabolism and immune-competence. Thus, an integration of BCS pre-selection in developing models for examining the relationships between EB as a

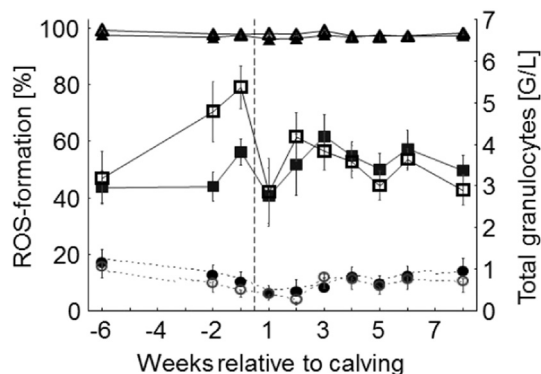


Fig. 8. Granulocyte numbers (■□,  $P_{\text{week} \times \text{treatment}} < 0.05$ ), unstimulated (basal, ●○,  $P_{\text{week} \times \text{treatment}} < 0.05$ ) and stimulated (▲△,  $P_{\text{week} \times \text{treatment}} > 0.05$ ) ROS formation of granulocytes in blood of cows with a higher BCS that were fed an energy-dense diet a.p. (60% concentrate feed proportion) and subjected to a decelerated concentrate feed proportion increase p.p. (□○△) in comparison to adequately fed cows with a lower BCS (●●▲) (Drong et al., 2017).

quantitative net-indicator for the nutritional status and the immune system might be useful.

## 6.2. Models making use of manipulating factors

The relevance of NEB and of associated parameters such as BHB and NEFA for the immune system of the transition cow has been discussed in the previous chapters.

Models to investigate the impact of the nutritional status on the immune system require significant differences in the readouts between the positive and the negative control group. These differences can be achieved by employing one of the discussed factors influencing the EB or by a combination of more factors (Table 1). In particular, these factors include the duration of p.p. feed restriction, a decelerated increase in p.p. energy supply, duration of high a.p. energy supply, overfeeding of cows with a higher BCS and different milking frequencies. These factors vary in relevance for manipulating NEB and combination of various factors might increase the intensity of manipulation.

A very efficient effect on EB results from a reduction of milk energy excretion. Once milking per day instead of the common practice of milking the cows twice a day during the first weeks of lactation resulted in a significant reduction of blood NEFA and BHB levels, and an increased glucose concentration (Loiselle et al., 2009). Although immunological parameters such as chemotaxis, phagocytosis and oxidative burst of PMN, proliferation of PBMC and antibody production to ovalbumin showed clear effects of parturition, there were no effects of the obvious differences in the degree of NEB. The only parameter which was significantly influenced by treatments was the  $\text{IFN-}\gamma$  secretion by PBMC which was higher in cows milked once a day. The differences in EB between the treatment groups can even be more increased in that cows are milked once per day or even 3 times per day. In this case the splenocyte transcriptome was significantly influenced by EB manipulation (Fenwick et al., 2008; Morris et al., 2009). It was found that a total of 545 genes were differently regulated in severe NEB cows compared to their medium NEB counterparts. Pathway analysis showed an association between severe NEB and NRF2-mediated oxidative stress, mitochondrial dysfunction and endoplasmic reticulum (ER) stress. Besides these effects with direct or indirect consequences for the immune response important immune-related genes were regulated, such as natural killer signaling. Moreover IL-15, BCL-2, and  $\text{IFN-}\gamma$  were downregulated (Morris et al., 2009). Peripheral blood PMN isolated from cows with an induced NEB and subsequently challenged intramammary with *Streptococcus uberis* were characterized by an altered expression of genes involved in immune response when compared to cows in a positive EB (Moyes et al., 2010).

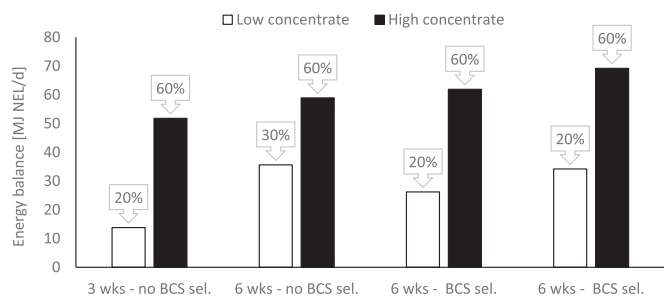
No correlations were found in another experiment between plasma NEFA and BHB levels and the hepatic expression of NRF2-target genes. These results were discussed as a missing link between NEB and NRF2-regulated genes in p.p. inflammation (Gessner et al., 2013). ER stress in the liver has been proposed to induce hepatic and subsequently also systemic inflammation (Ringseis et al., 2015) underlining the role of the liver as a secondary immunological organ. On the other hand, the liver itself is the target for induction of ER stress through the effects of endocrine mediators such as various cytokines, metabolites such as NEFA and BHB, and LPS (Ringseis et al., 2015). ER stress results in an induction of the unfolded protein response (UPR) and genes involved in this UPR were shown to be upregulated in the 1st week p.p. and suggested to be associated with inflammation and hyperketonemia (Gessner et al., 2014). Prepartum differences in BCS and feeding of a high concentrate diet to higher conditioned cows did obviously not affect the UPR in early lactation as indicated by an unchanged expression of involved genes (Drong et al., 2017). Similarly, mRNA abundance of nuclear factor- $\kappa$ B target genes, such as C-reactive protein (CRP), haptoglobin (HP), serum amyloid A 3 (SAA3) and tumor necrosis factor (TNF) and of NRF2-target genes remained uninfluenced by



**Table 1**  
Evaluation of various models for manipulation of the metabolic state of cows and consequences for some immunological traits (Petzold et al., 2014; Graugnard et al., 2013).

	Relevance of manipulation					Intensity of manipulation				
	+	++	++	++	+++	+	++	++	++	+++
Measure to increase severity of negative energy balance										
Duration of p.p. feed restriction	+	0 h	0 h	0 h	0 h	0 h	0 h	0 h	0 h	0 h
Decelerated increase in p.p. energy supply	++	No	No	Yes	No	Yes	No	Yes	No	No
Duration of high a.p. energy supply	++	3 wks	4 wks	6 wks	8-12 wks	6 wks	6 wks	6 wks	6 wks	0 wks
Overfeeding of cows with a higher BCS	+++	No	No	No	No	No	No	Yes	No	No
Twice milking vs. once per day	+++	No	No	No	No	No	No	No	No	Yes
Strength of effects compared to control groups:										
Energy balance	a.p.	<0.05 (†)	<0.05 (†)	<0.05 (†)	<0.05 (†)	<0.05 (†)	<0.05 (†)	<0.05 (†)	<0.05 (†)	<0.05 (†)
BHB	p.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
NEFA	a.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05 (†)	<0.05 (†)	<0.05 (†)
Glucose	p.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Liver-fat	a.p.	n.s.	<0.05 (†)	<0.05 (†)	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05 (†)
Total bilirubin	p.p.	n.s.	<0.05 (†)	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Haptoglobin	a.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	<0.05 (†)	<0.05 (†)	n.s.
White blood picture	p.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
ROS formation by PMN	a.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Stimulation ability of PBMC	p.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
CD4+ to CD8+ ratio	a.p.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	p.p.	Petzold et al., 2014; Petzold et al., 2013	Graugnard et al., 2013	Tienken et al., 2015a; Tienken et al., 2015b	Geelen and Wensing, 2006	Tienken et al., 2015a; Tienken et al., 2015b	Drong et al., 2016; Drong et al., 2017; Schulz et al., 2015; Schulz et al., 2014	Loiselle et al., 2009		

Notes: Increase in number of crosses means an increase in the intensity of manipulation or of the relevance of a particular manipulation measure; wks. weeks; a.p., ante partum; p.p., post partum; n.s., not significant ( $p > 0.05$ ); BHB, beta-OH-butyrate; NEFA, non-esterified fatty acid; †, increase; ‡, decrease; PBMC, peripheral blood mononuclear cells; PMN, polymorphonuclear leucocytes; empty cells indicate that parameters were not reported.

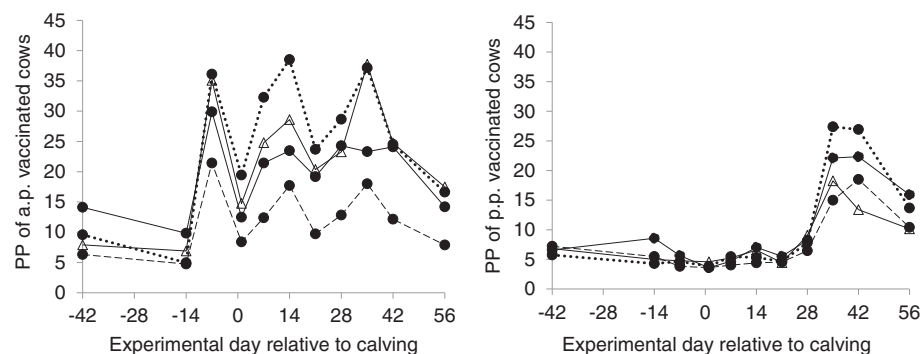


**Fig. 9.** Energy balance of Holstein dairy cows in the prepartum period in dependence on duration of feeding diets with a low or high concentrate proportion and on pre-selection according to the body condition score (BCS) (BCS was either not considered; no BCS sel., or cows with a higher BCS were assigned to the diets with a high concentrate proportion, BCS sel.) Data from Drong et al. (2016), Petzold et al. (2013), Schulz et al. (2014), Tienken et al. (2015b).

assigning cows with a higher BCS to the energy-dense diet (Drong et al., 2017).

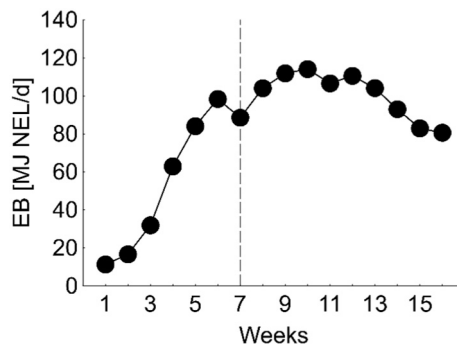
Other models try to influence the EB from the intake site, i.e. by differences in the energy supply. This strategy aims at increasing the energy supply prior to calving and/or a decrease of energy intake after calving in order to predispose the cow for an increased lipolysis p.p. (Table 1). The differences in the mean EB between treatment in the prepartum period increase with increasing duration of the period of an energetic oversupply and with larger differences in the concentrate feed proportions (Fig. 9). The particular role of the length of the a.p. dietary manipulation general agrees with the conclusion by Geelen and Wensing (2006) who evaluated 10 experiments with > 150 cows (Table 1). Their “Utrecht fatty liver model of dairy cows” primarily focused of inducing hepatolipidosis in cows which is a common feature of severe NEB.

With regard to immunological consequences of this model it was found that cows who developed fatty livers were compromised to develop a comparable tetanus toxoid specific immunoglobulin G response following vaccination (Wentink et al., 1997), a compromised functionality of PMN (Zerbe et al., 2000) and a decreased lymphocytic infiltration of skin allotransplants (Wentink et al., 1999). Other models which were also successful in manipulating NEB, BHB and NEFA levels in blood failed to influence various immunological traits (Table 1). Specific antibody formation to vaccination with various antigens was either shown to be affected by NEB manipulation (Drong et al., 2017; Wentink et al., 1997) or not (Loiselle et al., 2009). The experiment by Drong et al. (2017) showed significant interactions between treatment

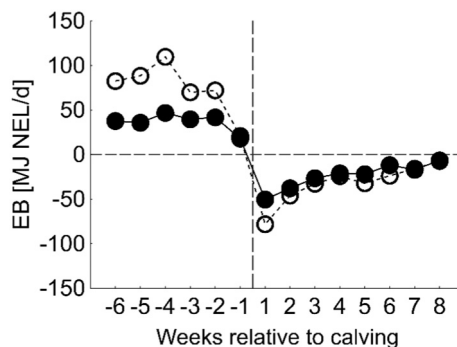


**p-values:**

	a.p.	p.p.
Treatment	0.227	0.065
Vaccination status	< 0.001	< 0.001
Treatment x vaccination status	0.014	< 0.001



**Fig. 11.** Energy balance (EB) in dependence on gradual increase of dietary concentrate feed proportion from 0 to 60% (week 0–8) and a continued feeding of the energy-dense diet to non-lactating, non-gravid cows (p < 0.05) (Dänicke et al., 2014).



**Fig. 12.** Energy balance (EB) of cows with a higher BCS that were fed an energy-dense diet a.p. (60% concentrate feed proportion) and subjected to a decelerated concentrate feed proportion increase p.p. (○) in comparison to adequately fed cows with a lower BCS (●) (p<sub>week x treatment</sub> < 0.05) (Drong et al., 2016).

(Table 1) and status of vaccination (before or immediately after parturition) to bovine viral diarrhoea virus (BVDV) demonstrating that not only NEB manipulation influenced antibody titer formation but also the timing of vaccination. Starting the vaccination immediately after parturition in the period of NEB clearly produced lower antibody titers compared to the prepartum vaccination irrespective of further treatments (Fig. 10). These results would support the view that lactation is a resource-prioritized process that leaves less nutrients and energy for immune responses. Evidence for this assumption comes from immune-challenge experiments during the reproduction period of various species as reviewed earlier (Rauw, 2012). It could be shown that p.p. parasitic load is often increased in this period suggesting a

**Fig. 10.** Antibody titers to bovine viral diarrhoea virus (BVDV), expressed as mean percentage positive value (PP) of ante partum (day -42 and -14, left) and post partum (day +1 and +28, right) vaccinated cows. Cows received a low concentrate (Δ) diet with a concentrate:roughage ratio of 20:80 (n = 7 and 5) or a high concentrate (●) diet with a concentrate:roughage ratio of 60:40 ante partum. Postpartal concentrate:roughage ratio was changed stepwise from 30:70 to 50:50 within 2 weeks in the low concentrate group (Δ) and 3 weeks in the high concentrate groups (●). Treatments included no supplementation (–) (n = 5 and 8), the administration of a monensin controlled-release capsule (---) (n = 6 and 7) at d -21 until d 56 relative to calving (Drong et al., 2017).

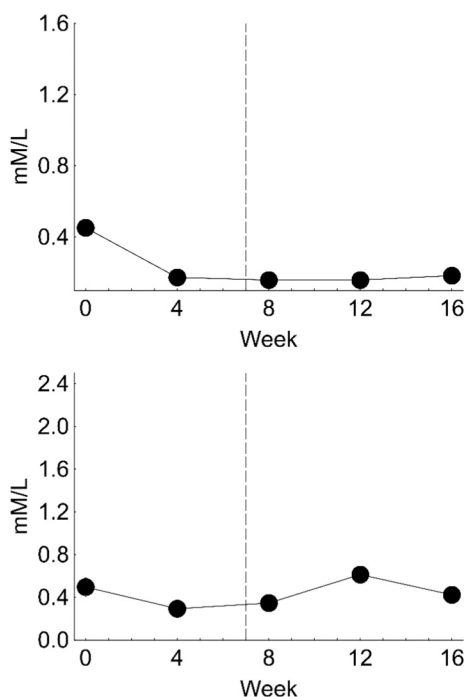


Fig. 13. Non-esterified fatty acids (NEFA, upper panel,  $p < 0.05$ ) and beta-hydroxybutyrate (BHB, lower panel,  $p < 0.05$ ) levels in blood in dependence on gradual increase of dietary concentrate feed proportion from 0 to 60% (week 0–8) and a continued feeding of the energy-dense diet to non-lactating, non-gravid cows ( $p < 0.05$ ) (Dänicke et al., 2014).

compromised immuno-reactivity against parasites. Moreover, both adaptive humoral and cellular responses to specific antigens are compromised under these conditions.

Associating the EB to metabolic and immunological traits both for transition cows and non-gravid, non-lactating cows enables to examine particularly the role of a positive EB in the presence or absence of lactation or gravidity. For example, the EB during weeks 6 and 3 a.p. in energetically oversupplied late-gestating cows is comparable to the EB of non-gravid, non-lactating cows fed a ration with a similar concentrate feed proportion of 60% (Fig. 11, Fig. 12). Similar to EB, the blood levels of NEFA and BHB (Fig. 13, Fig. 14), the lymphocyte proportions and the CD4+ to CD8+ ratio were comparable in this period (Fig. 3, Fig. 4). In contrast, PBMC stimulation ability appeared to be higher in non-gravid, non-lactating cows whereby the lower stimulation ability in late-gestating cows might be a reflection of the gravidity associated anti-inflammatory state of the cow (Fig. 5).

## 7. Conclusions

The models described in this paper can be viewed as principle possibilities to manipulate the metabolic and nutritional state of cows for investigating immunological consequences. The immune parameters used as indicators are rather limited to a small piece of the immune system; the defense system which is not confined to a single organ or tissue but is spread all over the body and tissues. Moreover, even marked nutrition mediated metabolic manipulations might result in variable immunological outputs. Furthermore, it needs to be stressed that the associations between EB and immune-competence are rather descriptive, variable and often inconclusive. Although EB is a quantitative net-indicator for the energy status of the cow it is also accompanied by many physiological processes which might be related to immune-responses in a more causative way than the more descriptively generated EB.

Based on this intricacy, it is increasingly acknowledged that the response of a complex biological system, such as a cow, to

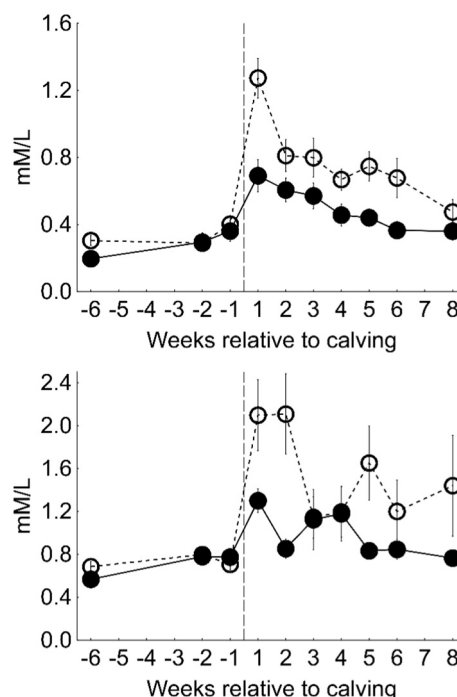


Fig. 14. Non-esterified fatty acids (NEFA, upper panel,  $p_{\text{week} \times \text{treatment}} < 0.05$ ) and beta-hydroxybutyrate (BHB, lower panel,  $p_{\text{week} \times \text{treatment}} < 0.05$ ) levels in blood of cows with a higher BCS that were fed an energy-dense diet a.p. (60% concentrate feed proportion) and subjected to a decelerated concentrate feed proportion increase p.p. (○) in comparison to adequately fed cows with a lower BCS (●) (Drong et al., 2017).

environmental challenges includes much more molecular, metabolic, endocrine, immunological and other physiological adjustments than can be explained by only a few indicative parameters. Instead, integrative -omic approaches are required in the future to cover the complexity more comprehensively and deductively (Buescher and Driggers, 2016; Jha et al., 2015).

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