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Samenvatting NL Het doel van de huidige deskstudie was om te onderzoeken in hoeverre extra energie en eiwit nodig zijn voor de immuunrespons bij melkvee, specifiek in de vroege lactatie. Het immuunsysteem heeft tijdens activatie relatief veel energie nodig, evenals specifieke aminozuren. Om de eiwitbehoefte te ondersteunen, wordt daartoe spiereiwit gemobiliseerd. Verder onderzoek is nodig om de nutritionele behoefte van een immuunrespons te bepalen en eventuele voedingssupplementen te identificeren die van belang kunnen zijn ter ondersteuning van koeien tijdens de vroege lactatie.

Summary UK The present desk study aimed to investigate to which extent extra energy and protein are required for the immune response in dairy cattle, especially in early lactation. The immune system requires a relatively large amount of energy during immune activation, as well as specific amino acids. To support protein requirements, muscle protein is mobilized. Further research is required to determine the actual nutritional requirements of an immune response and the potential nutritional supplements that may be of relevance to support dairy cows, especially during early lactation.

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Foreword

This study was conducted within the research programme "Feed4Foodure": a public-private partnership between the Dutch Ministry of Agriculture, Nature and Food Quality and a consortium of various organizations within the animal feed industry and the animal production chain. Feed4Foodure aims to contribute to sustainable and healthy livestock farming in the Netherlands, simultaneously strengthening our competitive position on the global market.

The present literature study entitled "*Nutrition and immune response in dairy cattle; a literature study into trade-offs in the transition period*" was written to investigate the current state of knowledge on the nutrient requirements of an immune response in dairy cattle. Main aim of this study is to gain insight in the dynamics and regulation of an immune response, how it can be simulated in an experimental animal model, and what the energy and protein requirements of an immune response might be. The focus will be on the peri-parturient and early lactation period in particular as this period is known to be most critical. Based on the information discussed in this report, gaps in knowledge are described and associated needs for further investigation in future experimental work.

The present literature study will further support our understanding of the immune system in dairy cattle which is necessary to support animals with specialized nutrition or supplements during immune challenges, especially during the metabolically challenging transition period and during negative energy balance.

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Summary

Optimal functioning of the immune function is an important factor in the context of livestock resilience. Seeking a maximum efficiency of production or feed conversion to reduce environmental emissions may compete with protein, energy and nutrients for the immune system, challenging the ability to cope with disease. The present desk study aims to investigate to which extent extra energy and protein are required for the immune response in dairy cattle. Extra attention was paid to the period of early lactation which is considered most critical for dairy cow performance and health. An activated immune system demands a large amount of energy, at least 0.66 g glucose/kg of $BW^{0.75}$/h in lactating cows. The protein requirements are less investigated in dairy cattle. The immune response results in an increased production of acute phase proteins, requiring specific amino acids and trace mineral co-factors. The cytokines and glucocorticoids released during an immune response will also affect the regulation of protein metabolism resulting in a catabolic effect on muscle protein. Compared to muscle protein, acute phase proteins are relatively rich in aromatic amino acids like phenylalanine, tyrosine and tryptophan. For 1 g of acute phase proteins synthesized by the liver, 1.5-2 g of muscle protein should be degraded. Other nutrients (fatty acids, vitamins) may also have a role in immune response regulation.

Different research models may be used to determine the effect of an immune challenge on energy and protein metabolism. A challenge with lipopolysaccharides is described most frequently; other options are a challenge with tumor necrosis facter α, complete Freund's adjuvant, an actual infection or a vaccination. Descriptive research for dairy cows during an immune challenge hardly involves studies on nutrient metabolism and the implications of nutrition, which is a gap in knowledge. It is of high interest to investigate how specific physiological or metabolic states, environmental conditions including nutrition management, or perhaps even historic metabolic or disease events, affect the responsiveness of the immune system to challenges as well as the consequences for nutrient utilization.

1 Introduction

The immune system is the natural defence system of an animal. Optimizing immune function should be an important factor in the context of improving livestock resilience. The immune system and animal production both require energy, protein and other nutrients. Seeking a maximum efficiency of production or feed conversion in terms of stimulating cow productivity and maximizing nitrogen and energy utilization may compete with an optimal functioning immune system which is able to resist immune challenges. However, controlled studies focussing on both aspects simultaneously are rare.

In the light of the recent verdict of the Dutch Council of State to adhere to the European Nitrogen policy, reduction of Nitrogen emission has been a major national issue. One of the possible routes to reduce Nitrogen emission is through reducing the amount of protein in livestock feed. This may affect a protein-consuming process such as immunity or the production response as a trade-off. Although Klasing (1998) suggested nutritional costs maintaining the immune system are minimal to a host compared to other costs, Lochmiller and Deerenberg (2000) argued otherwise. The latter authors reviewed literature on this topic and conclude, in contrast to Klasing (1998), that there are generally substantial nutritional and energetic costs associated with immunological stress and maintenance of the immune system. They state that '*the host protein metabolism during infection and the nitrogen-limited environments in which they reside may be the most important determinants driving life-history trade-offs among growth, reproduction, and host immunocompetence'* . Quantitative estimates of these costs are hardly available however, also not for dairy cows, nor would they apply to all conditions. It is likely however that the costs involved compare to those for reproduction and growth or performance. Lochmiller and Deerenberg (2000) conclude that '*while in terms of absolute mass the immune system appears to be a minor contributor to body mass, such a comparison fails to incorporate the plethora of costs associated with indirect effects of acute-phase responses, anorexia, cellular metabolic rates, and cellular-molecular turnover* '. The case remains unresolved however, in particularly for dairy cows, as long as information is lacking and no studies or analyses are performed with the specific design or aim to quantify these costs and the consequences of immune challenges. The present desk study was set up to explore what aspects need to be considered with focus on the relationship between nutrition (protein nutrition in particular) and immune response and resilience to immune challenges in dairy cattle. At the moment it is unclear to what extent reducing crude protein content of diets of high-yielding dairy cattle may or may not lead to impaired immune function. Impact on metabolic health is extensively investigated and is considered to maximize immune function as well. However, direct measurement of effects on immune function seems sparse.

1.1 Aim

The present desk study aims to investigate to which extent extra energy (glucose) and protein (specific amino acids; AA) are required for the immune response in dairy cattle. Although general aspects of an immune response will be discussed, it was attempted as well to specifically address the period of early lactation which is considered most critical for dairy cow performance and health. Research questions addressed in this desk study are:

- What is the AA composition of products of the immune response
- What are nutrient costs of an immune response (including associated metabolic processes) in terms of glucose, individual AA, and apparent costs in terms of metabolizable protein and energy
- To what extent does a successful immune response depend on the supply of metabolizable protein, individual AA and glucose
- To what extent does an immune response lead to a different prioritization in utilisation of metabolizable protein and/or glucose
- How does lactation stage of an animal affect this prioritization of nutrients for an immune response
- How can the negative impact of an immune response on milk synthesis be counteracted by nutritional measures

2 Immune response

The immune system is the defence system of an organism, and its task is to defend the organism against internal and external health threats. During the activation of the immune system, whether it is triggered by an infectious or non-infectious agent, a cascade of events is started which we consider an "immune response". The immune response can be a result of common farm issues like mastitis, metritis, ruminal acidosis, heat stress, and parturition. Although an immune response can be beneficial for livestock, for example on uterine recovery after parturition (Richards et al., 2008), an excessive immune response is considered detrimental for production (Bradford et al., 2015). The type and height of the response may depend on several factors, and may result in some degree of reduced feed intake, synthesis of acute phase proteins, and generally a redistribution of nutrients towards the immune system (Klasing and Johnstone, 1991; Spurlock, 1997; Colditz, 2002; Lochmiller and Deerenberg, 2000; Le Floc'h et al., 2004).

2.1 The immune system

In general, the immune system is the organ system to protect the cow from pathogens, remove foreign objects that may be harmful to the cows' health and assist in removing deteriorated cells as needed in e.g., the process of expulsion of the placenta and involution of the uterus. The immune system can be divided into two functional parts: the innate (natural) immune system and the acquired (adaptive) immune system. The innate immune system is considered to be non-specific and the acquired immune system specific, but the two systems are highly related and interact through signalling proteins and molecules.

2.1.1 Innate immune system

The innate immune system is the first barrier as an immediate response to any known and unknown pathogens. It consists of the physical barrier (skin and epithelial cell layers), acute phase proteins, the complement system and natural killer cells and mast cells as well as phagocytic cells such as macrophages, monocytes, dendritic cells and polymorphonuclear granulocytes (neutrophils, basophils and eosinophils). The response of the innate immune system to invading pathogens is very quick, its disadvantages on the other hand are its non-specificity and the lack of memory against agents.

2.1.1.1 Acute phase proteins

Acute phase proteins are proteins whose serum concentrations increase (positive acute phase proteins) or decrease (negative acute phase proteins) with more than 25% during an immune challenge. The general function of the response in production of the acute phase proteins is to defend the host. Some proteins directly protect the host in different ways, some stimulate parts of the immune system, and others have the ability to down-regulate the cytokine production probably as a feedback mechanism. Most acute phase proteins, including serum amyloid A, haptoglobin, ceruloplasmin and C-reactive protein are produced by the liver, at the cost of other proteins (such as albumin) (Ceciliani et al., 2012). Albumin is therefore considered a negative acute phase protein. During the immune response the serum concentration of albumin decreases (Jacobsen et al., 2004). Nevertheless, albumin is also considered a positive acute phase protein because it increases in the mammary gland during mastitis (Shamay et al., 2005). Acute phase proteins have various functions in the defence against foreign objects. Ceruloplasmin will bind copper while haptoglobin binds hemoglobin, to reduce Cu and Fe availability for pathogens; serum amyloid A recruits immune cells; and C-reactive protein binds to cells in apoptosis, to activate the complement system.

2.1.1.2 Complement system

The complement system is a group of proteins that can bind to microbes to enhance chemotaxis, support antibody binding and activate phagocytosis. Complement proteins can also directly eliminate Gram-negative bacteria and viruses by disturbing their membrane structure. Inactive precursors circulate in the blood until activation by acute phase proteins, foreign objects (e.g. pathogens) or tissue damage. Examples of complement proteins are C1-C6, C9 and B.

2.1.1.3 Natural killer cells

Natural killer cells are cytotoxic lymphocytes, comparable to the cytotoxic T lymphocytes of the acquired immune system. They are important in the response to viral infection by recognizing infected cells by stress signals, without antigen presentation.

2.1.1.4 Phagocytic cells

Phagocytes such as macrophages, monocytes and dendritic cells may present antigens of ingested pathogens to lymphocytes responsible for the acquired immune response; and also release cytokines (TNF-α, IL-1, and IL-6) that initiate the acute phase response as well as the acquired immune response.
Polymorphonuclear leukocytes (**PMNL**) or granulocytes will release the toxic contents of their granules such as reactive oxygen species, reactive nitrogen species and proteases. **Neutrophils** are the primary PMNL, with a short half-life and few mitochondria, utilizing little oxygen; most glucose is used to produce reducing equivalents required for phagocytosis or in anaerobic glycolysis producing lactate (Moyes, 2015).

2.1.2 Acquired immune system

If an infection cannot be eliminated from the body within a short period of time, the acquired immune system is activated. The acquired immune system consists of specific antigen recognition by T- and B-lymphocytes. An acquired response may take several days or weeks to develop; the response is much faster when an animal has encountered the same pathogen (antigen) before, by the support of memory lymphocytes. Because of this memory capability, the acquired immune system is very specific against microorganisms. Its major disadvantage is the prolonged time it takes to activate the acquired immune system.

2.1.2.1 T-lymphocytes

The T-lymphocytes may be divided in two main groups: CD4 and CD8 expressing T-lymphocytes. The CD4 T-lymphocytes are helper cells that produce cytokines such as IL-2, IL-4, IL-5, IL-13, INFγ and TNFα and help to activate macrophages and B-cells. The CD8 T-lymphocytes can be either cytotoxic (secreting cytotoxic enzymes to kill infected cells) or suppressor cells (producing IL-4, IL-5 and IL-10 to control the immune response) (Mehrzad et al., 2008).

2.1.2.2 B-lymphocytes

The number of B-lymphocytes (or CD21 lymphocytes) is much lower than T-lymphocytes but their function is as important. The B-lymphocytes produce cytokines, may present antigens to phagocytic cells and may differentiate into plasma cells that produce immunoglobulins. Immunoglobulins will stimulate the innate immune system, by activation of the complement system and opsonisation of infected cells and pathogens for phagocytosis (Mehrzad et al., 2008). After infection, B-lymphocytes may develop into B-memory cells that circulate in the blood and may be reactivated at reinfection.

2.2 The immune response

The immune response of an individual animal is influenced by a lot of factors. For instance type of challenge, genetic variation, nutritional state, earlier contact with pathogens, dose and duration of exposure to the immune challenge all contribute more or less to the individual immune response. This is the reason that individual animals can respond very differently to the same challenge.

2.2.1 Basic immune response

The immune response basically starts with the immune challenge. These challenges mostly originate from some form of infection injury or tissue trauma somewhere in the body leading to a local response. This local response is marked by the release of cytokines. Cytokines are intercellular signalling polypeptides which are released by activated cells in the locally challenged area. Cytokines have different targets and functions, and are the main stimulators for the production of acute phase proteins (Gauldi et al., 1987). The most important inflammation-associated cytokines include interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor α (TNFα). Most of these cytokines are secreted by activated monocytes and macrophages in the local challenged area. Within hours after infection plasma concentrations of cytokines will increase and lead to activation of the hepatocytes into producing the acute phase proteins (Webel et al., 1997).
At the same time plasma complement proteins are active. They have three major functions; (1) stimulation of inflammatory response, (2) opsonisation of pathogens through the stimulation of phagocytosis by macrophages or (3) cause cell lysis. Complement proteins interact with specific pathogens, and mark them for removal by phagocytic cells.
If this innate immunity fails to eliminate the pathogen, the acquired immune system will be activated. It can take days for the latter adaptive immune system to become activated because the pathogen specific B and T lymphocytes need to develop. Re-infection with the same pathogen will lead to a faster and stronger response of the acquired immune system because of the clonal expansion of immunological memory and antigen-specific effector cells.

In terms of defining or quantifying nutrient costs of the immune response and of the associated metabolic effects in the lactating cow, no information was found in literature and therefore it remains difficult to give estimates or compare the costs of the innate and acquired immune response. Nevertheless, reviews are available on the relative costs of the innate and acquired immune response in other species, such as that of McDade et al. (2016) for the immune system in humans. They conclude that the developmental costs of the acquired immunity are high, but the costs of maintenance and activation are relatively low. In contrast, the innate immunity imposes lower upfront developmental costs, but higher operating costs. Furthermore, the balance between investment in these costs is thought to be made during the conditions in early development. During the sensitive periods of immune development, nutritional abundance and high pathogen exposure are thought to favour relatively higher levels of investment in acquired immunity; undernutrition and low pathogen exposure favour innate immune defence. Applying this concept to lactating cows, one might hypothesize that the impact of developmental environment prior to the cow entering into lactation determines the nutrient investment and nutrient costs made for innate and acquired immunity, as well as their relative importance.

2.2.2 Clinical signs

The acute phase proteins produced by the liver and the cytokines released by immune cells will result in clinical signs of infection. General signs associated with infection in dairy cattle are reduced feed intake and milk yield, inflammation, metabolic effects, fever. Local signs of inflammation can be seen as well. The classic signs of inflammation include, swelling, redness, heat, pain and loss of function (Ceciliani et al., 2012).
Fever is the result of a changed setpoint of the hypothalamus and is caused by the release of prostaglandin E2 due to endogenous or exogenous pyogens. Examples of endogenous pyogens are cytokines like TNFα, IL-1, IL-6 or INF-γ, and exogenous pyogens for example are bacterial toxins like lipopolysaccharides (LPS) (Kluger, 1991).

2.2.3 Variation between individuals

Jacobsen et al. (2004) tested the dose dependency and individual variability of acute phase proteins specific blood serum proteins and biochemical responses after an immune challenge with LPS in cattle and found major differences between individuals. Indicating that the magnitude of the serum concentration of SAA and Hp was not only influenced by the dose of LPS but also by the ability of the individual to produce these acute phase proteins.

2.2.3.1 Type of response – intensity – duration

An experimental infection may induce different levels of immune response and severity of clinical signs between two individual animals; after inoculation with 30-50 CFU of E. coli, bacterial counts (directly related to clinical signs) may reach a 20-fold difference between cows (Kornalijnslijper et al., 2003). The height of the immune response and the effective time to bacterial clearance will depend on the infection level (CFU) as well as an individual's history with the specific pathogen. A repeated infection with the same antigens will result in a quick response of the adaptive immune system, thereby quickly reducing the number of pathogens. Other cow factors that may be involved in the difference in immune response may be related to genetic background, age, production level, energy balance or endocrine status.

2.2.3.2 Genetic background

Genetic differences in the immune system may be involved in the individual variation. Genetic variation in for example antigen receptor, cytokine or antibody production may result in a different height of the immune response. Breed (Holstein vs. Jersey) does not seem to be relevant in determining the clearance of pathogens after an experimental infection with E. coli (Bannerman et al., 2008).

2.2.3.3 Parity

The height of the immune response may also be affected by cow parity. Pluriparous cows may exhibit higher numbers of polymorphonuclear leukocytes and peripheral blood mononuclear cells (Bühler et al., 2018). Cows in second lactation, however, showed higher numbers of white blood cells and an increased percentage of lymphocytes compared to third or higher parity cows (Schäfers et al., 2018).

2.2.3.4 Production level

In a study with 18 high (11,443 kg/305d) vs. 18 low producing (7,727 kg/305d) dairy cows, an experimental *E. coli* mastitis in the fourth week postpartum resulted in large individual variation in clinical symptoms as well as bacterial counts; this was however not related to production level (Kornalijnslijper et al., 2003).

2.2.3.5 Energy balance

The energy balance may directly affect the immune system as well as an immune response may affect energy balance. During the negative energy balance ketosis may develop and ketone bodies may compromise immune function (Nonnecke et al. 1992; Franklin et al., 1991). Cows with higher plasma beta-hydroxybutyric (BHB) levels develop more severe disease after experimental *E. coli* mastitis (Kremer et al., 1993, Van Werven, 1999) which is directly related to a reduced migration capacity of leukocytes (Suriyasathaporn et al., 1999). Liver function can be compromised during the negative energy balance with TAG accumulation in the liver. In cows with hepatic lipidosis, humoral as well as cellular immune responses after immunization are reduced (Wentink et al., 1997). A humoral response is mediated by macromolecules that are found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. A cellular response is cell-mediated and does not involve antibodies, rather the activation of phagocytes, antigen-specific cytotoxic T-lymphocytes, and the release of various cytokines in response an antigen. Both the innate and the acquired immune response contain a humoral as well as cellular component.

2.2.3.6 Endocrine status

Hyperketonemia during an intramammary induction of mastitis by LPS challenge was demonstrated to influence the immune response. Infusion with LPS increased glucose, but less in cows with intravenous BHB infusion where gluconeogenesis was reduced, likely because BHB can replace glucose as an energy source (Zarrin et al., 2014). To test the effect of energy balance per se, ten mid-lactating cows were feed-restricted to 60% of calculated net energy requirements for 7 days and infected with Streptococcus uberis at day 5 to induce mastitis (Moyes et al., 2009). The feed-restricted animals experienced a negative energy balance comparable to early lactation, but differences in the response to inflammation with control animals (in positive energy balance) were minimal and could not be compared to the reduced response in transition cows (Moyes et al., 2009).

A compromised immune response during a negative energy balance experienced by cows in early lactation may be related to the increase of blood levels of ketone bodies and other associated metabolic effects or factors, but is not the consequence of a negative energy balance in itself. This interpretation needs to be confirmed by further research, however.

Total white blood cell count increases toward calving while the percentage of lymphocytes drops (Schäfers et al., 2018). Phagocytosis activity of immune cells decreases from 6 weeks before calving as well as the percentage of basal ROS producing polymorphonuclear leukocytes and reached initial values only at 28 days in milk again (Bühler et al., 2018). In the first days postpartum most dairy cows will experience some degree of subacute inflammation which is partially beneficial (to restore the uterus and prevent infection) but may also be uncontrolled, impairing lactation performance (Bradford et al., 2015).
In sheep, the immunity to parasites is reduced in the periparturient period (Houdijk et al., 2003). An increase in the supply of metabolizable protein from below requirements to a level above requirements resulted in an increase in milk production as well as a reduction of faecal egg counts; total worm burden only reduced with the last increment resulting in a level of metabolizable protein above requirements (Houdijk et al., 1993).

2.2.4 Measuring ruminant immune status

Nutrient availability is likely to influence several parts of the immune system. Production and the immune system are both dependent on the availability of these nutrients and may therefore influence each other. In order to assess the immune function in individuals, there are multiple and complex methods available in modern research. The availability of analytical facilities and the researchers interest influence the suitability of the method.

For the in vivo assessment of the immune function serum concentrations of acute phase proteins are often used. Acute phase proteins are sensitive markers for inflammation but their specificity is not very high and variate between species (Eckersall et al., 2010).

More specific information of the most important acute phase proteins for cattle follows here:
- C- reactive protein (CRP) does not give a convincing serum response after induction of immune response in cattle. It may be lactation associated (Murata 2004) and is therefore not considered an acute phase protein in cattle (Nakajima et al., 1993).
- Haptoglobin serum concentrations increases over 100-fold on immune stimulation in ruminants (Gånheim et al., 2003; Murata et al., 2004). After experimental induced inflammation, inflammatory or natural disease an increase in serum or plasma concentration of Hp was found in cattle (Plessers et al., 2015; Fernandes et al 2019). Serum concentration of Hp was correlated to the dose after induced inflammation (Jacobsen et al., 2005).
- Serum amyloid A (SAA) is considered a sensitive marker for inflammation. Serum concentration increase following experimental and natural inflammation (Gånheim et al., 2003; Carroll et al., 2009). The severity of the clinical signs correlate with the magnitude and duration of the serum SAA response (Gånheim et al., 2003).
- Horadagoda et al. (1999) concluded that the combination of serum Hp and SAA can also be used to distinguish between acute and chronic inflammation.

3 Energy and nutrient costs

3.1 Maintenance

First of all, the immune system has maintenance costs. Production of immune cells requires 1-2% of total maintenance energy costs. The energy needs appear relatively higher compared with those of lysine, for the diversification of the lymphocyte receptor repertoire. Furthermore, 0.5-2% of total maintenance requirements of lysine are required for leukocyte production and antibody secretion (Klasing, 2007). Mature lymphocytes are exceptionally long lived and have very low metabolism when at maintenance; heterophils are very short lived.

3.1.1 Suboptimal status

Restricted nutrient intake early postpartum alters the metabolic response to an intramammary LPS challenge, but effects on the inflammation response were limited (Pires et al., 2019). Energy restriction to 85% prepartum (3.7 kg DM/day less) did not affect the response to an injection with granulocyte colony-stimulating factor that is known to increase neutrophil count and function (McDougall et al., 2017). A higher feed intake might counteract an infection due to delivery of more nutrients; on the other hand a lower feed or protein intake may also be beneficial for the cow due to a lower production response and hence reduced requirement of certain nutrients. In general, stress preceding or during an infection induces hormonal changes, leading to a reduction in feed intake (Davis, 1998). The lowered feed intake may increase proliferation of lymphocytes and T-cell development and function (Pahlavani, 2000). Upon feed restriction, in mice inflammatory responses to LPS decreased (Matsuzaki et al., 2010), and in pigs energy restriction modified expression of immune genes (Lkhagvadorj et al., 2010). Although a reduction in feed intake may help to preserve homeostasis by (amongst others) lowering endogenous secretions and lowering nutrient supply to malign micro-organisms in the gut, the beneficial effect of feed restriction on health in farm animals may occur only for digestive diseases (Le Floc'h et al., 2014).

3.2 Energy

An activated immune system demands a large amount of energy (Johnson, 2012; Kvidera et al., 2016; Kvidera et al., 2017a). In case of an inflammatory response, the total energy costs increase 10-49% of total maintenance costs in sheep (Lochmiller and Deerenberg, 2000).
There is a large amount of glucose required to maintain euglycemia in steers with an activated immune system (Kvidera et al., 2016). Kvidera el al. (2017a) found that in reaction to a single dose of intravenous LPS administration, cows developed hyperglycaemia, which was followed by hypoglycaemia that developed on average 143 minutes after LPS injection. The hypoglycaemia then lasted until the end of the 12-hour trial. Kvidera et al. (2017a) estimated the glucose requirements of the immune system to be approximately 0.66 g/kg of $BW^{0.75}$/h in lactating cows and 1.0 g/kg of $BW^{0.75}$/h in steers (Kvidera et al., 2016), which is comparable with glucose requirements of the immune system in pigs (1.1 g/kg of $BW^{0.75}$/h) (Kvidera et al., 2017b). The estimation did not include the amount of glucose used by Immune cells during the hyperglycaemic phase, and may therefore be an underestimation. The hyperglycaemic phase is probably due to the endotoxemia, which is reported to induce the increase of hepatic gluconeogenesis and glycogenolysis in ruminants (Waldron et al., 2003). This hyperglycaemic phase is caused by the combination of peripheral insulin insensitivity and increased hepatic glucose production. The hypoglycaemic phase is caused by the inability of this mechanism to meet the glucose increasing demands of the immune system.
With a strong immune response, as can be mimicked for example with an LPS infusion protocol used by Kvidera et al. (2017a), glucose delivery may even fall short to sustain the most essential vital functions and cows may die (Baumgard, personal communication).

Glucose is essentially converted into lactate, as demonstrated by the elevated lactate levels in blood after a LPS challenge found by Kvidera et al. (2017a). Nevertheless, it appears conversion of this lactate to glucose again in the liver by gluconeogenesis is insufficient to keep up blood glucose levels and in severe cases glucose delivery collapses. This also makes it comprehensible that the cow, experiencing a strong negative energy balance post-calving with a high rate of mobilization of fat tissue, with a high glucose requirement, and with glucose levels falling short due to a simultaneous immune response, become susceptible to develop ketosis and impairment of other vital "maintenance" requirements perhaps. The vital functions to maintain include the strong development and homeostasis of visceral tissues (particularly epithelia of the gastrointestinal tract and the liver) as well as acid-base status (Bannink et al., 2018; Van Gastelen et al., 2020) with the onset of lactation that is accompanied with a rapid increase in feed intake and milk yield towards peak levels in a period of a few weeks.

3.3 Protein

With an immune response the liver increases in activity (and size) to produce acute phase proteins. Acute phase proteins require specific AA and trace mineral co-factors. The cytokines released during an immune response will affect the regulation of protein metabolism as shown in Figure 3.1. At the same time, increases in glucocorticoids would have a catabolic effect on muscle.
Protein synthesis does not keep pace with accelerated rates of protein loss from skeletal muscle, resulting in losses in total body protein of 20% in septic patients (Biolo et al., 1997).

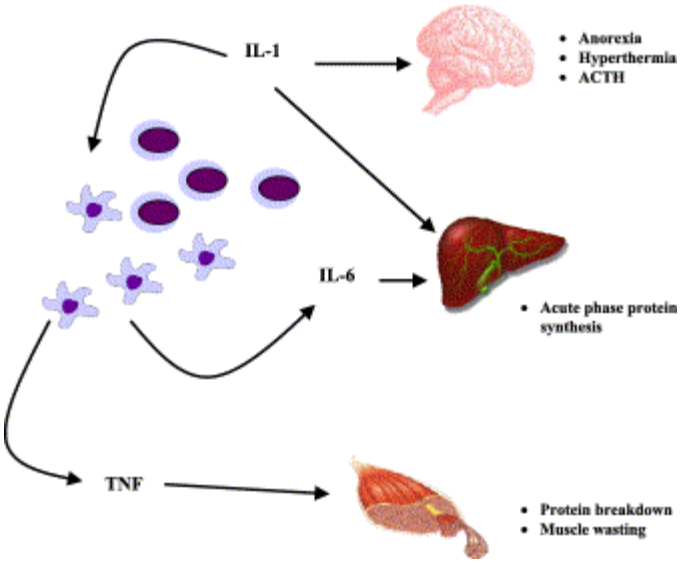

Figure 3.1 *Impact of cytokines on nitrogen metabolism in pigs (Le Floc'h et al., 2004).*

Results of the study of Waggoner et al. (2009) demonstrate that infusion with LPS alters concentration of several serum hormones. For example several plasma amino acids decreased, and immune response resulted in greater urinary N excretion and less N retention. The production of acute phase proteins, immune cells and glycogenic precursors require a higher protein availability to compensate for the altered intake and higher need for amino acids.
In a study with pigs fed a deficient dietary protein supply, blood concentrations of C-reactive protein and albumin decreased; after immune system activation, urinary N excretion was increased but no differences could be observed in acute phase protein levels between animals on a protein deficient vs., a control diet, suggesting a high priority for the immune system in growing pigs (Kampman-Van de Hoek et al., 2015).
In a study with lactating sheep artificially infected with gastrointestinal parasites, increasing the level of metabolizable protein supply in early lactation from 65, 80, 95, 110 to 125% of requirements (at an energy intake of 90% of requirements) showed a priority of scarce metabolizable protein allocation to milk production over immune function which was not absolute but gradual.

With the first incremental levels of dietary metabolizable protein milk production increased, whereas with further increment milk production appeared to maximize and a decrease in worm burden became prevalent (Houdijk et al., 2003). It is not demonstrated that the same concept holds for a lactating cow in early lactation experiencing a severe immune challenge. Nevertheless, it appears relevant to investigate whether gradual changes in prioritization between milk protein synthesis and immune response occur in high-yielding cows and how this is related to stage of lactation and nutritional supply of metabolizable protein.

Antibody production

The production of antibodies without an acute phase response does according to Van Eerden et al., (2004) not require an excess of nutrients in laying hens. On the other hand, protein is required for their synthesis, and as there will be a turnover of antibodies and this turnover of antibody protein is unlikely to be 100% efficient, protein costs must be involved. Costs may remain small of if the majority of amino acids becomes available for intermediary metabolism again with turnover; note though that such turnover will always demand energy to re-synthesise the protein. No information was found on this aspect in literature however.

Muscle breakdown during inflammation

TNFα may be one of the most important cytokines leading to inflammation induced cachexia. Another protein complex highly associated with regulating the atrophy of skeletal muscle is nuclear factor kappa B (NF-kB).

Under healthy conditions there is a balance between protein synthesis and protein degradation. During inflammation this balance is altered leading to net protein catabolism in muscle protein to compensate for the increased need of the liver for amino acids in order to produce acute phase proteins. The combination of inflammation associated muscle atrophy and the reduced feed intake due to cytokine production will lead to cachexia (Londhe and Guttridge, 2015). Like with acute inflammation, Mercer et al. (2002) demonstrated similar effects with chronic inflammation in mice induced with dextran feeding. Protein metabolism was stimulated in several splanchnic organs, and in muscle both protein synthesis and degradation were reduced. These changes appear to occur across species and may lead to insufficient amino acid supply to meet the increased amino acid requirements of these organs. AN insufficient amino acid supply hence does not have to be caused by a lack of metabolizable protein nutrition but can also be caused by an acute/chronic inflammation. Similar chronic inflammatory effects are suspected for dairy cattle with the development of acidosis and production of endotoxins of microbial origin in the hindgut (Plaizier et al., 2012; Li et al., 2012). Excessive starch feeding might hence cause similar changes in amino acid metabolism in dairy cows. Nevertheless, with moderate levels of abomasal starch infusion (to keep rumen fermentation unaffected) Van Gastelen et al. (2020) did not observe clear signs of an immune response, although hindgut acidosis did occur as evidence by low fecal pH, and nitrogen balance did become less positive. Figure 3.2 gives a schematic overview derived from Chua & Puthucheary (2019) of how various physiological states may be related to protein metabolism.

Changes in amino acid metabolism might have been investigated by the amino acid 3-methylhistidine in urine. The amino acid 3-methylhistidine is a component of actin and myosin and is released after muscle protein breakdown. It is believed not to be reutilized for protein synthesis after breakdown and is quantitatively excreted in the urine (Sheffield-Moore, 2014). This amino acid may hence serve as a proxy for measurement of rate of muscle protein breakdown and may be an interesting indicator of severe changes in protein metabolism.

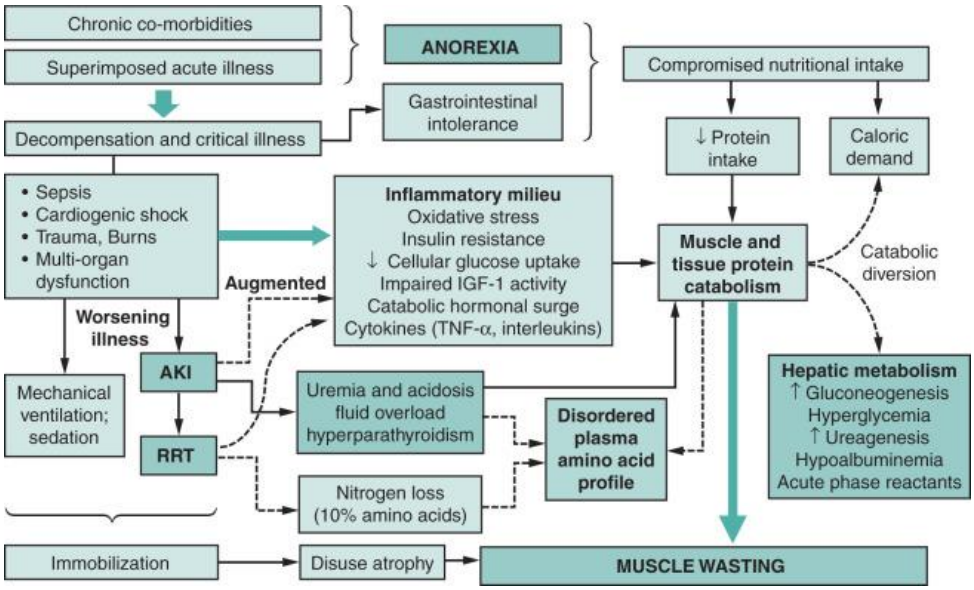

Figure 3.2 *Relationships between acute and chronic disease and amino acid supply and tissue protein metabolism (derived from Chua & Puthucheary, 2019).*

3.3.1 Amino acid composition

After an immune challenge, specific AA may be required for the production of acute phase proteins, glucose precursors, antibodies, plasma proteins, hormones, free-radical scavengers and metabolic cofactors (Li et al., 2007). It is not difficult to find the AA composition of inflammatory proteins, and of specific antibodies. From most of the proteins associated with an immune response the composition of AA is well known and free available online. It is much harder to make a quantified estimation model for the AA requirements of a full immune response, because of the broad range of inflammatory proteins involved and the large variety between individual and group responses. Furthermore, measurement of concentrations does not indicate their synthesis rate which also complicates any quantification of AA requirements or costs.

In a study with 20 Angus-cross steers, serum concentration of several AA (Met, Lys, Leu, Ile, Phe, Trp, Gly, Ser and Asn) decreased after injecting LPS, suggesting that the metabolic demands of these AA increase under immune challenged conditions (Waggoner et al., 2009).
Although a lot of research is conducted on the effects of supplementation of several single amino acids, Bounous et al. (1983) state that an important factor responsible for the immune system does not appear to be the availability or concentration of some essential amino acid, but rather the composite effect of the amino acid distribution in the proteins synthesized by the immune system. Compared to muscle protein, acute phase proteins are relatively rich in aromatic AA like **phenylalanine** (Phe), **tyrosine** (Tyr) and **tryptophan** (Trp). Phenylalanine (and because of its metabolism to tyrosine, also tyrosine itself) and tryptophan are considered so-called Group 1 essential amino acids whose uptake from blood and subsequent output in milk protein occurs at a 1:1 ratio as clearly demonstrated in previous work and the more recent work by Kelly Nichols et al. reviewed in the general discussion of the PhD thesis of Nichols (2019). The fact that some of the required essential amino acids involves Group 1 amino acids implies that for every gram of these specific amino acids used with an immune response a gram is lacking for their supply to the mammary gland for milk protein synthesis. This also implies that for 1 g of acute phase proteins synthesized by the liver, 1.5-2 g of muscle protein should be degraded.

Tryptophan may be an essential amino acid for the immune response as shown in pigs. Tryptophan is an important constituent of acute phase proteins and is catabolised to reduce its availability for pathogens or be transformed to the antioxidant kynurenine (Le Floc'h et al., 2012). Supplementation with tryptophan may improve growth during inflammation (Le Floc'h et al., 2009; Trevisi et al., 2009) while in other experiments, no differences in growth rate were found (Le Floc'h et al., 2010).

Threonine (Thr) is important in immunoglobulin synthesis as well as in mucin, implying a threonine deficient diet will impair the natural barrier function in the gut (Le Floc'h et al., 2004).

The immune system uses **glutamine** (Gln) for the production of cytokines and proliferation of lymphocytes. Lymphocytes, macrophages and neutrophils use glutamine for energy production, by converting it to glutamate to be oxidized in the tricarboxylic acid cycle (Moyes, 2015). Glutamine is a glucogenic amino acid and may enhance hepatic gluconeogenesis. Thus, although Gln is a non-essential amino acid, under situations of high metabolic demand (including physiological stress) Gln may be considered conditionally essential (Doepel et al., 2007). Glutamine and glutamate are important contributors in the production of casein and their availability may therefore affect milk production. However, abomasal infusion of Gln in dairy cattle did not reduce glucose utilization across the gut or increase liver gluconeogenesis or mammary Gln uptake to increase milk protein synthesis (Doepel et al, 2007).

Lysine (Lys) requirements may increase up to 10% of total maintenance requirements for lysine in growing broiler chicks (Klasing, 2007), comparable with a 9% increase in pigs (Johnson, 2012).

In an experiment with pigs, the irreversible loss rate of AA from the plasma pool was not correlated with acute phase protein indices, suggesting that the AA requirements for the synthesis of acute phase proteins is likely outweighed by a decrease in muscle protein synthesis in these growing animals. The increased N excretion often observed during sepsis probably occurs because of the imbalance between excess amino acids mobilized and the amino acids used for utilization of acute phase proteins. Nevertheless, in a review of experimental trials with growing pigs, Van der Peet-Schwering et al. (2019) concluded that maintenance requirement for individual amino acids may increase with 0 to 30% in pigs with an activated immune system; a low number of available studies showed that the maintenance requirement of lysine, methionine + cysteine, threonine may increase with up to 15, 23, and 5%, respectively, or were not affected for tryptophan and arginine; and immune system activation has no effect on the post-absorptive utilization of lysine, methionine + cysteine, threonine and arginine for protein deposition, but may reduce the efficiency of tryptophan utilization for protein deposition in pigs with 7%. Although no such information is available for dairy cows, it is probable that similar effects will occur in cows when the immune system is activated, particularly during early lactation.

3.4 Specific nutrients

A severe imbalance or malnutrition between of protein or energy or of their balance, are likely to also affect or alter the immune response. Although the Committee on Military Nutrition Research (1999) states this in particular for humans, there is perhaps even more reason to presume this would hold for a high-yielding cow having an energy requirement during early lactation that supersedes three times maintenance. Subclinical deficits may be associated with a catabolic response, impaired immune response and an altered risk of infection, and also for this conclusion there is no apparent reason why this would not hold for a high-yielding cow. In the table below nutrients are listed which are involved in one of the elements of the immune system function. More recently, Sordillo (2016) reported a similar conclusion for cows specifically, stating that '*the nutritional status of the dairy cow and the metabolism of specific nutrients are critical regulators of immune cell function. There is now a greater appreciation that certain mediators of the immune system can have a reciprocal effect on the metabolism of nutrients. Thus, any disturbances in nutritional or immunological homeostasis can provide deleterious feedback loops that can further enhance health disorders, increase production losses* '. It was concluded that relationship between nutrition and immunity is required to be able to design nutritional strategies that reduce disease susceptibility in early lactating cows. Harmful metabolic or hormonal responses for immune response have to be identified and known, before they can be prevented by nutrition and management.

Table 3.1 below gives an overview of nutrients involved with various elements of the immune response (derived from the Committee on Military Nutrition Research (1999).

The major challenge will be to determine when nutrient supply is short in requirements for immunity under various nutritional conditions of the dairy cow. This seems particularly relevant for early-lactating cows that are in negative energy (and protein) balance.

In addition to the discussion of amino acids in 3.3, two further nutrient types will be discussed here; fatty acids, and vitamins and minerals and trace elements. It is clear however that for important elements of the immune response, involving cellular synthesis and protein synthesis, various macronutrients are required which may impact on the energetic and proteinaceous nutrient requirements of the dairy cow.

Table 3.1 *Overview of nutrients involved (indicated by " X ") with various elements of the immune response (derived from the Committee on Military Nutrition Research, 1999).*

	Humoral Immunity	Surface Immunity	Cell-mediated Immunity	Antioxidant Activity	Cytokine Release & Eicosanoid Production
Multiple nutrients for cellular synthesis	X	X	X		X
Multiple nutrients for protein synthesis	X		X		X
Vitamine A	X	X	X(-)		
Vitamins C & E				X	
Thiamine		X			
Vitamine B6	X	X	X		X
Folate	X		X		
Iron	X		X(=)		X
Copper	X		X		X
Zinc	X	X	X(-)		X
Selenium	X		X	X	
PUFAs	X		X(-)		X
Arginine			X		
Glutamine		X	X		

As components of the hos"s defence mechanisms, humoral immunity involves the antigen-specific immune response mediated by B- and plasma-cell production of circulating or secretory antibodies (immunoglobulins). Surface immunity, or passive defence measures, include anatomical barriers and pathways (e.g., skin and mucous membranes), exogenous body secretions (e.g., mucin, saliva, bronchial fluids), and a host of physiologic factors. Cell-mediated immunity is the antigen-specific and nonspecific immunity provided by the direct localized cellular activity of T-lymphocytes and natural killer cells. Cytokines play a role in cellular communication, function as intercellular signals and mediators, and are active participants in nonspecific immune responses such as acute-phase reactions. PUFAs, polyunsaturated fatty acids; (-), excessive amounts can sometimes be immunosuppressive; (=), excessive amounts can sometimes increase severity of infections).

3.4.1 Fatty acids

Specific fatty acids are also involved in immune response regulation. Especially the long chain n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are known to suppress inflammatory pathways. Increasing n-6 relative to n-3 fatty acids may stimulate the activity of neutrophils but also increase the level of acute phase proteins (Silvestre et al., 2011); this approach requires further investigation to determine a potential use in transition dairy cows (Bradford et al., 2015).
Conjugated linoleic acid (CLA) may affect the local and systemic immune response after a challenge with intramammary LPS (50 µg LPS); CLA supplemented cows showed a higher body temperature but provided more glucose and preferentially used BHB as energy source (Gross et al., 2018).

3.4.2 Vitamins, minerals, trace elements

Dietary antioxidants such as selenium and vitamin E are important to neutralize ROS and attenuate inflammation and self-damage. Vitamin E may decrease monocyte percentages (Schäfers et al., 2018). Nicotinic acid supplementation around parturition may affect the phagocytosis capacity of blood leukocytes (Bühler et al., 2018). In a trial with dairy cows, an intramammary LPS challenge was performed to determine the supportive effect of supranormal levels of zinc (Zn), copper (Cu) and manganese (Mn) in either an inorganic or an organic form. However, no difference were found in clinical response to the challenge (Yasui et al., 2019).

3.5 Alteration in protein metabolism with tissue damage or infection

With injury, large tissue damage or infection, there is a strong metabolic response which in severe cases may lead to a loss in lean body mass (Wolfe, 1999). Response may differ for individual organs however, with one organ losing mass due to net protein degradation and another increasing due to net protein synthesis. The liver producing acute phase proteins increases metabolic rate as well as the immune system and repair mechanisms induced in damaged or infected tissues (sepsis). This may be accompanied by a catabolic state of skeletal muscle as a source of mobilizable protein. Time course of a response to infection or injury is important as when this is extended over various days or even weeks, muscle functioning may become impaired due to reduced muscle strength and mass (Wolfe, 1999, referring to Bams & Miranda, 1985). A negative protein balance of skeletal muscle results from a severe increase in protein breakdown, often offsetting the simultaneous increase in protein synthesis, thereby serving as a source of amino acid precursor elsewhere in the body. Extra supply of amino acid through nutrition may not be used by muscle tissue due to a supressed amino acid uptake (Wolfe, 1999). This means that once muscle tissue entered a strong catabolic state extra dietary supply of amino acids is expected not to be effective to turn muscle tissue into an anabolic state again. As this is due to a reduced amino acid uptake, it is also unlikely that the pattern of amino acid supply makes any difference to overcome such a metabolic state. Therefore, where a particular mix of essential amino acids is important for its efficient utilisation for milk protein synthesis (Nichols et al., 2019), this unlikely holds for catabolic muscle tissue which needs to regain its synthetic capacity.

Instead of extra dietary amino acid supply, an increase of the anabolic hormone insulin may have a more potent effect in this respect. For a study under a clinical setting with severely burned patients, Sakurai et al. (1995) described the effect of supplementation of insulin together with glucose (to maintain euglycemia) for 7 days with patients acting as their own control in a cross-over design. The insulin therapy appeared to reverse the negative amino acid balance across muscle tissue but also increased protein degradation. It was postulated that this might perhaps become converted into a net protein synthesis with an addition extra dietary supply of amino acids. This concept might apply to the early-lactating dairy cow as well, which is in a catabolic state with incidentally high protein requirements for tissue growth and repair and immune challenges, a state of insulin resistance and a high requirement for glucose.

It seems worth testing the hypothesis that cows in an extra negative protein balance due to an immune challenge, benefit from dietary strategies which raise insulin levels together with an increased supply of glucose, and simultaneously increase the supply of amino acids. A future area of investigation in post-parturient cow nutrition may hence be the interactive effect between protein catabolism during early lactation and cow's immune state on the one hand, and the hormonal and nutritional state on the other hand. Comparable to clinical work with severely injured humans, it is plausible that increasing amino acid supply in itself is insufficient to counteract the catabolic effect on body protein induced with severe or prolonged immune challenge, and to let the cow regain muscle strength, and regain functionality and performance (allowing feeding and milking). Impairment of the latter may create locomotion problems and cow dysfunction (and reduced feed intake, worsening the cow's condition) which eventually leads to replacement of the cow.

Finally, it is worth noting that considering protein as such does not always cover the underlying regulatory mechanisms and protein requirements. A review study by Wu (2010) on protein nutrition discusses so-called functional amino acids, which were defined as amino acids that regulate key metabolic pathways to improve health, survival, growth, development, lactation, and reproduction of organisms. It was concluded that both the non-essential as well as the essential amino acids have to be considered in the classic "ideal protein" concept, and that both should be considered with formulation of balanced diets to prevent chronic diseases and optimize immune function in all species. Together with the recent findings by Nichols (2019) on regulation of amino acids utilization for milk synthesis, this demonstrates the complexity of defining protein cost and requirement for immune function as well as maintenance and productive functions. Whether also non-essential amino acids have to be considered next to the essential ones has to be confirmed however for lactating cows as studies in relation to immune response are essentially lacking.

3.5.1 Stress factors and hormones

In an extensive review, the Committee on Military Nutrition Research (1999) documents that all of the hormones that regulate carbohydrate metabolism also participate in responses to infection by humans. These hormones include glucocorticoids from the adrenal cortex, the catecholamines from the adrenal medulla and the sympathetic nervous system, and glucagon which is a pancreatic hormone. When infusing these three families of stress hormones in normal volunteers the metabolic and immunologic responses were very similar to those described in injured and infected humans (Bessey et al., 1984; Watters et al., 1986). In analogy of this, it is likely to assume that also in cows stress hormones will affect the metabolic and immune response, and furthermore that changes in carbohydrate metabolism due to nutrition may also affect immune response. Figure 3.3. is derived from a review by Chen et al. (2015) and depicts which biological functions are affected through two axes of stress hormones.

In addition, Wilmore (1999) concludes protein catabolism may occur due to a decreased food intake, reduced exercise (no locomotion), and hormonal and cytokine signals that favour protein breakdown. This protein catabolism may benefit the animal if it fuels the required response to severe injury or infection, but it also may become deleterious when it impairs muscle strength or condition of the animal. If such impairment is already occurring, this response should actually be attenuated instead of stimulated to prevent dysfunction and subsequent cow replacement. The main stereotypic metabolic response to infection and injury or damage described by Committee on Military Nutrition Research (1999) is an increase in metabolic rate, increased rate of gluconeogenesis (utilizing protein), increased rate of fat oxidation, and a negative nitrogen balance. This response is beneficial and typical in the absence of diseases or dysfunctional organs and Willmore (1999) hence concludes that body protein is a structurally indispensable element for a well-functioning body. If also true for the (early) lactating dairy cow this is an important notion for cow management and nutrition , and it might be important for cow nutrient requirements as well. Stress factors and conditions that affect stress hormones as indicated in figure 3.3 affects or may modulate these mechanisms involved in protein catabolism. This explains why also stress factors and their hormonal effect are important targets when optimizing cow nutrition and farm management. Research in this area of cow metabolism and performance is sparse however.

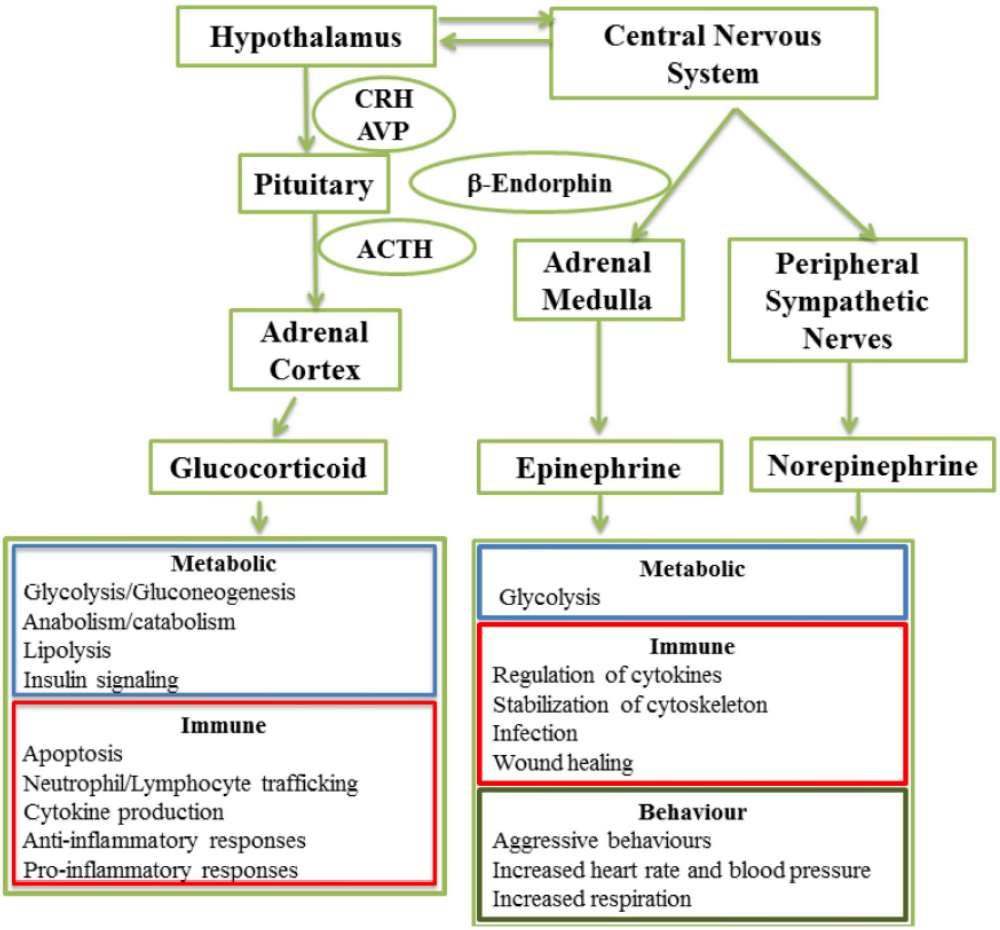

Figure 3.3 *Biological functions regulated by the two stress axes (derived from Chen et al, 2015).*

4 Model studies

4.1 Lipopolysaccharides (LPS)

Several studies use different models for the assessment of the immune response. A few models used to assess the immune system are an induced inflammatory response, induced inflammatory disease or natural disease. Listed below are the most important used models.
A lot of infective stressors result in increased LPS as a product of microbial breakdown, locally or circulating in the blood. Injecting LPS to induce an inflammatory response is therefore a commonly used method in scientific experiments, to reflect such infective stressors. Both intramammary and intravenous infusions of LPS are used to examine local and systemic responses.

4.1.1 Effects of LPS

An immune challenge with LPS will lead to clinical signs like an increased temperature, respiration rate, and heart rate, a decreased dry matter intake and milk yield and can cause cold extremities in cows (Waldron et al, 2003; Carroll et al, 2009; Dickson et al, 2019). Besides the clinical signs, infusion of LPS will induce an acute phase response leading to increased serum concentrations of cortisol, SAA, Hp, TNFα, LBP, IL-1β, IL-6 and INF-γ (Waldron et al., 2003; Carroll et al., 2009; Waggoner et al., 2009; Kvidera et al., 2017; Dickson et al., 2019; Fernandes et al., 2019). IL-1, IL-6 and TNF are associated with inhibition of muscle protein synthesis and increase of muscle protein wasting (Londhe and Guttridge, 2015).

4.1.2 Model studies with LPS

Intramammary
A local application of 100 µg of LPS by intramammary infusion will result in productive, clinical and physiological signs of moderate to severe inflammation, increasing plasma insulin and glucose and decreasing plasma NEFA and BHB (Waldron et al., 2006).
Intramammary infusion of 10 µg of LPS will also induce an inflammatory response peaking after 24h with decreased milk secretion, milk lactose, glucose and citrate, and increased infiltration of PMNL, increased milk malondialdehyde concentration and degradation of casein (Silanikove et al., 2016). The reduction of milk yield and lactose concentration indicated a reduction of glucose extraction from the blood; glucose was shifted to the pentose phosphate pathway and shifting cell metabolism to aerobic glycolysis at the expensive of mitochondrial activity (Silanikove et al., 2016).

Intravenously single dose
In a model study of Kvidera et al. (2016), LPS was administered intravenously to steers (1.5 µg/kg BW) to induce an acute phase response and glucose requirements were estimated over a 720 hour fasting period combined with dextrose infusion to maintain euglycemia. Body temperature increased (+0.5°C) and animals were hyperglycemic in the first three hours after the LPS bolus; then glucose decreased by 30% which required 516 g glucose to maintain euglycemia for 720 min. In lactating cows, the same LPS bolus was applied (1.5 µg/kg BW) after which cows were fasted and monitored for 720min (Kvidera et al., 2017a). This also resulted in an increased body temperature between 30-90 min post-infusion (+0.5°C), decreased NEFA, BHB and Ca and increased insuline, lactate (produced by leukocytes) and acute phase proteins (haptoglobin, serum amyloid A and LPS-binding protein). In 12 hours, 265 gram glucose was needed for euglycemia after the LPS challenge; moreover, milk production was reduced by 11 kg in the same period compared to the control cows, saving another 72 g × 11 kg = 793 gram glucose for the immune system.

Intravenously multiple dose
As a (single) LPS challenge results in a fast, well-described immune response with an equally fast return to the original homeostasis, a more continuous challenge may be of interest to simulate a natural infection. In a study with dairy cows, a continuous and exponentially increasing LPS infusion during 7 days (0.017 up to 0.148 µg/kg BW per hour) to mimic bacterial overgrowth during an infection (Horst et al., 2019). This resulted in chronic hyperinsulinemia during the infusion without a change in circulating glucose, and insulin resistance as measured by glucose tolerance test. Feed intake and milk production were however unaffected in the same cows suggesting the development of LPS tolerance (Dickson et al., 2019).

4.2 Tumor necrosis factor α

Yuan et al. (2013) used an intravenous injection with TNFα to induce an inflammatory response in early lactation cows. The injection resulted in 15-18% less milk yield. But the effect was not dose dependent. The treatment increased the plasma concentrations of Haptoglobin, but did not alter the plasma concentrations of insulin, 3-methylhistidine and BHBA.

An study with intramammary injection of TNFα was conducted in lactating cattle (Watanabe et al., 2000). An increase in somatic cell counts was observed. Total concentration of milk protein was not changed, but an decrease in caseins, α-lactalbumin and β-lactoglobulin was found. Also an increase in the serum concentration of Hp was found, although the increase was relatively small compared to increases reported in inflammatory studies.

4.3 Complete Freund's adjuvant

Another option to induce an immune response is by intravenous administration of complete Freund's adjuvant, a solution of inactivated and dried *Mycobacterium tuberculosis* (1 mg per mL). Injection (0.2 mL/kg BW) is used to induce a chronic lung inflammation in pigs, resulting in a granulomatous interstitial pneumonia (Kampman-Van de Hoek et al., 2015). Clinical signs include lethargy, fever and an increased respiration rate which decrease after two days (Le Floc'h et al., 2008).
In 1-2 months old calves, 0.5 mL/kg BW resulted in a strong immune response in the lungs with increased respiration and fever for approximately a week; 4 calves died of acute pulmonary edema (Lay and Slauson, 1982).

4.4 Infections

Infection models may also be used to induce an inflammatory response which can be of either bacterial, viral or parasitic origin.
An intramammary infusion of *E. coli* (50 cfu in 5 ml) in an udder quarter will result in clinical mastitis in dairy cow, including local signs (swelling of the udder, changes in the milk) and systemic signs (fever, loss of appetite); the severity of the signs may vary a lot between cows (Kornalijnslijper et al., 2003).

In sheep, an infection with gastrointestinal nematode *Teledorsagia circumcincta* (10,000 larvae, three times weekly) has been used to investigate the role of metabolizable protein supply in the immune response around parturition (Houdijk et al., 2003). The effect of treatment was monitored by faecal egg counts, plasma antibodies and indicators of parasitic damage, and final worm burdens after slaughter (Houdijk et al., 2003).

4.5 Vaccinations or boosters

Vaccinations are used as standard application in livestock farming. They are considered to be a mild challenge for the immune system. Vaccines realise different magnitudes of immune responses, but have found to be able to reduce the nitrogen retention in growing laying hens (Hentges et al., 1984). Although vaccinating cattle is not a common used technique to evaluate the requirements of an immune response some studies do report a decrease in milk yield post-vaccination (Bosch et al., 1997; Bergeron and Elsener, 2008). These results make vaccination an interesting possible method for assessment of the requirements of the immune system under practical conditions.

The commercially available pegbovigrastim is a granulocyte colony-stimulating factor, which can be injected intramuscularly resulting in an increased white blood cell count (especially lymphocytes, neutrophils and monocyte counts) as well as an enhanced neutrophil function (McDougall et al., 2017).

5 Future research

5.1 Knowledge gaps

Only a few studies have evaluated the nutrient requirement and magnitude of change in energy and protein balance during an immune challenge. Such knowledge is required to devise more effective dietary intervention strategies. Arguing from research in humans which indicates there are interactions between nutrition, hormonal state and immune response (depending on severity), it is plausible that a high-yielding cow consuming more than three time maintenance energy requirement may also have a trade-off between nutrient requirements for productive purposes, maintenance, immune response and repair mechanisms involved with injury or severe inflammations. Descriptive research for dairy cows hardly involves studies on nutrient metabolism and the implications of nutrition which hence must be considered a gap in knowledge. It is of high interest to investigate how specific physiological or metabolic states, environmental conditions including nutrition management, or perhaps even historic metabolic or disease events, affect the responsiveness of the immune system to challenges as well as the consequences for nutrient utilization.
When conducting trials to investigate the implications of an immune response choices need to be made with respect two aspects: the measurements to be performed and the setup of the trial.

5.2 Measurements

In order to get a total overview on the protein requirements of an immune response various measures need to be considered. In order to measure the magnitude of the immune response the serum concentration of produced acute phase proteins (SAA, Hp) are of interest. In case of a LPS-induced immune response, serum Lipopolysaccharide binding protein (LPB) should also be considered as an important acute phase protein.
The release of specific cytokines, such as IL-1B, IL-6 and TNFα, can be used to assess the immune response. Immune cells of interest are neutrophils and monocytes because of their role in the acute phase of an immune response.

It would be of interest to measure more appropriate markers (e.g., 3-methylhistidine, urine creatinine, or alike) to obtain an indication of the extent of muscle protein catabolism during immuno-activation in ruminants. Furthermore, immunity markers need to be studied in blood to evaluate whether the immuno-stimulation was successful and for how long. The following measurements of interest may be performed, based on what has been described in literature:

- Protein metabolism markers
  - BUN
  - AA
  - MUN
- Energy metabolism markers
  - Glucose
  - Insulin
  - NEFA
  - BHB
  - (L-lactate)
- Immune response markers
  - cytokines
  - immune cells
  - acute phase proteins
- Tissue damage parameters

- Specific parameters or indexes as indicative of organ functioning (often involving enzyme concentration or activity)
  - Parameters for liver function (including glycaemic index and insulin intolerance)
  - Parameters for respiration, gas exchange and acid-base regulation
  - Parameters for kidney function
  - Parameters for skeletal muscle activity.

5.3 Experimental design

Several designs and type of trials may be adopted to evaluate the impact of nutrition on cow performance and immune response, or the impact of immune response on nutrient requirement and energy and protein metabolism. The overall design of the project is organized as a series of pilots and experiments (within the maximum duration of a CCD project: 5 years). Results from each step are used to determine if the next step is still relevant and how to proceed regarding trial designs.

1. Immune challenge studies in climate-respiration chambers

This study design is meant to quantify the consequences of an immune response on nutrient utilization and energy and protein metabolism. The advantage of this design is utmost control on study conditions and on treatment control. Energy and protein metabolism can only be studied in detail with this design and it is proposed to start with trial to demonstrate the proof of principle that immune response impacts on energy and protein metabolism, and obtain an indication of energy and protein costs involved.

Using a block design with 4 cows per block, a combination of the factor immuno-activation and a nutritional factor (such as dietary DVE content) can be tested. The target animal may be early lactating cows or cows in mid-lactation. It is proposed to start experimenting with mid-lactation cows for demonstration of proof of principle.

As severity of immune response may have some temporal characteristics, and as a consequence also affect energy and protein metabolism as such, temporal response may be studied by measurements in time, for example by introducing three instead of a single balance period per measurement round.

Summarizing the specific research goals that suit this option:
- Evaluation of the height and duration of the immune response (through indicators in blood plasma) under controlled experimental conditions (i.e. maximal control, including environment and climate)
- Evaluation of the clinical response of the cow (cow performance in terms of feed intake, digestion, milk performance, excretion and welfare parameters)
- Comparing cow response in different metabolic or nutritional states (e.g. mid-lactating compared to early-lactating cows which are in negative energy / protein balance)
- Evaluation of changes in energy and protein metabolism and requirements due to an immune challenge by comparison pre- and post-challenge
- Evaluation of supplementing a diet (e.g. through infusions, or by feed supplements altering nutrient availability).

2. Immune challenge under practical conditions

Also under practical conditions the effect of an immune challenge can be tested. The challenge cannot be as severe and controlled as in tie stall or climate chamber conditions. Nevertheless, challenges that can be performed may involve controlled schedules of vaccination of groups of cows, as they are being performed on regular farms may. Effects of vaccinations on milk production have been reported and it therefore does appear feasible to test the effect of nutritional interventions on the consequence of vaccination.

In this case vaccination of the herd is hence taken as a model for an immune challenge, which may not comply with all types of immune responses such as infection or inflammation. Nevertheless, the advantage is that a much larger group of cows is available to study the consequences of the nutritional intervention. It is advised that although these studies are performed under practical farming conditions, as much as possible control is exerted on study design, timing of challenging and measurements, and dietary composition, and furthermore as much as possible information is gathered for individual cow performance (without uncertainty due to unknown feed losses, lacking registration of feed refusals, lacking data on variation in diet composition, etc.).

Summarizing the specific research goals that suit this option:

- Evaluation of challenges that regularly occur in practice (e.g. implementing regular vaccination or combinations of vaccination according to the experimental design)
- Evaluation of the effect of nutritional interventions under practical conditions
- Evaluation of consequences on performance on a larger group of cows

3. Immune challenge in tie-stalls

Studies in tie stalls are a design in between 1. and 2., using some of the befits of both but also having some of the disadvantages of both. Energy and protein metabolism cannot be studied in tie stalls. Also collection of excreta will not be as accurate as in option 1. The benefit of no need to have the cow housed in a climate chamber is according to the authors of marginal importance. Enclosing a cow in the climate chambers normally does not pose much stress on a cow, whereas the freedom to move comparable to that on a stand in the tie stall.

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To explore the potential of nature to improve the quality of life

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