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Consequences of intensive fattening: Influence on claw health, blood metabolite profile and the insulin signaling cascade in tissues of Holstein fattening bulls

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ABBREVIATIONS

AA	Amino acid
AAA	Aromatic amino acid
AC	Acylcarnitine
ADG	Average daily gain
AMPK	AMP activated kinase
Arg	Arginine
AST	Aspartate-aminotransferase
BCAA	Branched chain amino acid
BHBA	β-Hydroxybutyrate
BW	Body weight
CP	Crude protein
DM	Dry matter
DM2	Diabetes mellitus, type 2
GLDH	Glutamate dehydrogenase
GLUT	Glucose transporter
HEP	High energy and protein
Ile	Isoleucine
InsR	Insulin receptor
IRS1	Insulin receptor substrate 1
LEP	Low energy and protein
Leu	Leucine
LPS	Lipopolysaccharide
Met	Methionine
mTOR	Mechanistic target of rapamycin
NEFA	Non-esterified fatty acids
PBS	Phosphate buffer saline
PC	Phosphatidylcholine
PI3K	Phosphoinositide 3-kinase
PKB	Protein kinase B
SCFA	Short chain fatty acids
SM	Sphingomyelin
TMR	Total mixed ration
TTN	Tail tip necrosis
Val	Valine

1 INTRODUCTION

Holstein cattle are a one purpose dairy breed, but male offspring is mainly used as fattening bulls. Intensive fattening includes starch- and protein-rich components in the diet to achieve the full growth potential of the animals as soon as possible. However, chronic excessive dietary nutrient intake is known to increase the risk of developing metabolic disorders, as well as it is observed in humans. Insulin resistance and dyslipidemia are frequent disorder examples in humans, commonly labeled as obesity-related morbidities or as clusters of the human metabolic syndrome (VOLEK & FEINMAN, 2005). In companion animals similar concepts are established for horses, i.e., the equine metabolic syndrome in relation to a starch-rich diet and sedentary lifestyle (FRANK et al., 2010). In farm animals, fattening lambs developed insulin resistance and metabolic disorders during the fattening period, particularly when fed restrictively during early life (FRUTOS et al., 2018). In fattening bulls it is also known that such feeding regimes are associated with metabolic disorders and diseases like rumen acidosis (LIU et al., 2020), liver abscesses (NAGARAJA & CHENGAPPA, 1998), tail tip necrosis (KROLL et al., 2014) and laminitis (MAGRIN et al., 2020a; GREENOUGH et al., 1990). Especially laminitis could reflect metabolic health of the animals, because it is a key symptom of metabolic disorders (BERGSTEN, 2003). Blood analyses of affected dairy cows show indications of inflammation and metabolic acidosis (THOEFNER et al., 2004; NOCEK, 1997), but do not provide more detailed insights into which pathways or metabolites are involved. The underlying (patho)physiological pathways are not clear.

Metabolomics approaches provide snapshots of the metabolic status by determining the concentrations of a wide array of small metabolites in a biological sample. As these metabolites are intermediary or end products of various metabolic pathways, a good indication of the current metabolic condition is obtained. Using the AbsoluteIDQ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria), new biomarkers and affected pathways were successfully found in metabolic disorders like human diabetes mellitus type 2 (DM2) (LOTTA et al., 2016), insulin dysregulated horses (KENÉZ et al., 2018) and retained placenta in dairy cows (DERVISHI et al., 2018). This kit was established to identify metabolites of several pathways associated with energy metabolism, amino acid metabolism, pro-inflammatory signaling, dysregulation of glucose and lipid metabolism, obesity-related disorders, insulin resistance and mitochondrial dysfunction.

Holstein bulls, which are genetically less prone to accrete protein, may provide a very special metabolic setting. A surplus of energy and protein in the diet may strongly affect metabolic conditions in this breed promoting an adverse situation and consequently, resulting in dysregulation of metabolic pathways.

Therefore, it is hypothesized that excessive intake of energy and protein provokes alterations of metabolism, which lead to a pro-inflammatory phenotype clinically apparent as laminitis.

Aims of this study were

1) to characterize changes in metabolite profiles of Holstein bulls fed a high energy and protein diet (HEP) versus bulls fed a low energy and protein diet (LEP),

2) to elucidate alterations in insulin signaling pathway, reflected by expression and phosphorylation of insulin receptor (InsR), Protein kinase B (PKB), mechanistic target of rapamycin (mTOR) and AMP activated kinase (AMPK) in liver, muscle and retroperitoneal adipose tissue of these bulls

and finally

3) to combine health, performance and metabolic data by integrative analyses to generate new hypotheses of (patho)physiological relationships between chronic excessive energy intake and laminitis.

2 LITERATURE OVERVIEW

2.1 Fattening of Holstein bulls

In May 2020, there were about 857,000 bulls aged between one and two years in Germany and about 144,000 of them were Holstein bulls (DESTATIS, 2020). There are three main management systems for fattening bulls. Firstly, extensive fattening on pasture, secondly, management in stables with feeding of silage and by-products, and thirdly, management in feedlot-like systems with a high proportion of grain feed components. The second type is most common in Germany. The animals are fed at least 30 % silage or other basic feedstuffs and the feed is largely produced on the own farm (DAVIER et al., 2020).

Holstein cattle are a dairy breed and thus genetically show less potential for meat production than meat or dual-purpose breeds. In comparison to meat breeds, Holstein bulls have lower birth weights and lower daily gains and appear to be less suitable for fattening and meat production (DAVIER et al., 2020). At specialized dairy farms reproduction is necessary in order to begin a new lactation. Holstein bull calves therefore incur as "by-products". In combination, this situation leads to very low prices for these bull calves and results in an often reduced willingness of the farmers to rear these animals in the same way as the more valuable female offspring. In a large study in Germany, 7.2 % of the farm managers in Northern Germany admitted to take less care of male calves compared to female calves. This unequal treatment could lead to higher mortality rates observed in male calves (HOEDEMARKER et al., 2020). Studies in France and New Zealand also reported higher male than female mortality rates (RABOISSON et al., 2013; CUTTANCE et al., 2017). Male Holstein bulls are usually sold through cattle dealers and calf markets at the age of only two weeks, weighing about 45 kg. As the demand for these animals in Germany is relatively low, many animals are also sold abroad (DAVIER et al., 2020).

It should be emphasized that Holstein bulls compared to the beef breed “Charolais” have lower daily gains and slaughter yields, but express better characteristics in terms of meat quality. Meat of Holstein bulls has greater marbling, darker color and greater water binding capacity compared to meat of Charolais bulls (PFUHL et al., 2007).

2.2 Health of fattening bulls

Most diseases are caused by multiple factors, so in addition to possible infectious factors, many other factors such as genetics, feeding, husbandry, management and environmental climate conditions play an important role. Furthermore, studies have shown that the personality of the farm manager has a significant influence on the prevalence of diseases and mortality (ADLER et al., 2019). Respiratory diseases are most prevalent in younger fattening bulls. Injuries of the skin or locomotor system are often seen in heavy animals at final fattening. These damages are discussed to be caused by housing environment and are called technopathies. They include skin lesions and swellings, bursitis, changes in the tip of the tail and injuries of the musculoskeletal system (TROXLER, 2012; KIELLAND et al., 2009; COOK et al., 2016). The following is an overview of the most important diseases of fattening bulls described in the literature.

2.2.1 Respiratory diseases

Diseases of the respiratory system are the most important disorders in young fattening bulls (BRSCIC et al., 2012; FERTNER et al., 2016; SCHNYDER et al., 2019; MARCHESINI et al., 2018). In a study of 4,014 veal calves on 43 farms in Switzerland, 81.1 % of the animals were treated because of respiratory disorders. The average mortality was 5.1 %, maximal mortality of affected calves on individual farms was 15.6 %. Risk factors for more than 3 % mortality were purchase of calves, no examination at arrival or quarantine upon arrival, as well as bad hygiene of feeder and more than 10 calves per drinking nipple (SCHNYDER et al., 2019). In a larger study based on data of 174 farms in the Netherlands, France or Italy postmortem inspected lungs of veal calves showed signs of pneumonia by 21.6 % and 21.4 % had pleuritis. Prevalence of *in vivo* observed signs of respiratory disorders, like coughing, nasal discharge and hampered respiration was always below 7 % in all fattening stages. Calves were on lower risk to develop coughing, if individual separators (baby boxes) and troughs as milk delivery system were used, as well as if bulls were visited at a high frequency by a veterinarian per fattening period (BRSCIC et al., 2012). Similar results were obtained in a study by GARDNER et al. (1999), revealing that 33 % of 204 slaughtered finishing steers showed lung lesions and had lower final weights and carcass performance than animals without lung lesions. Interestingly, lung lesions were found in animals which were grouped alive as ‘treated, diseased’ as well as in ‘untreated, healthy’ animals, so observed clinical symptoms correlated badly with real status of lungs (GARDNER et al., 1999). As respiratory diseases are often associated with lower daily weight gains, early detection of diseases is also of economic interest. Reduction of motion activity and rumination activity were already observed one to

three days before visible respiratory symptoms (MARCHESINI et al., 2018) therefore, monitoring those activities could be used as early warning system.

2.2.2 Locomotor system disorders

Besides diseases of respiratory system, diseases of the locomotor system play an important role in fattening bulls. Antibiotic usage in Danish veal calves and young bulls was mainly for treatment of respiratory diseases, secondly for disorders of joints, limbs or the central nervous system (FERTNER et al., 2016). Intensively fed beef bulls were also treated mainly because of respiratory disease and locomotor disorders. Limousine bulls on concrete floor (n = 57) compared to slatted floor covered with rubber (n = 58) were treated because of locomotor disorders by 15.8 % and 1.72 %, respectively. Early culling due to lameness or traumatic events occurred at 6 % of 116 Charolais bulls kept on concrete floor (MAGRIN et al., 2019). Floor conditions had a great influence on the occurrence of alopecia, skin lesions and carpal and tarsal bursitis. Lesions were lowest in bulls bedded on straw and highest on concrete floor, positively correlated with body weight (BW) and fattening time (SCHULZE WESTERATH et al., 2007). Bulls fattened on fully slatted floor covered with rubber mats showed less skin lesions, swelling and bursitis than on concrete floor (PLATZ et al., 2007; COZZI et al., 2013; BRSCIC et al., 2015; MAGRIN et al., 2019). However, especially older, heavier animals are often kept on a fully slatted floor. The use of rubber mats instead of pure concrete shows advantages for animal health and welfare (PLATZ et al., 2007; BRSCIC et al., 2015; MAGRIN et al., 2019), reducing the risk for early culling due to lameness or locomotor disorders (BRSCIC et al., 2015).

2.2.3 Digestive and metabolic disorders

Gastrointestinal disorders were the third most common reason for antibiotic usage in Danish veal calves (FERTNER et al., 2016). Ruminal acidosis and ruminal bloat are the most common digestive diseases in feedlot cattle (MEYER & BRYANT, 2017; NAGARAJA et al., 1998). Fattening based on large amounts of easily digestible carbohydrates, such as starch- and sugar-rich components combined with low fiber content, exposes animals to a high risk of digestive and metabolic disorders (OWENS et al., 1998; KLEEN et al., 2003; KHAFIPOUR et al., 2009; KHIAOSA-ARD & ZEBELI, 2018). The adaptation of the microbial flora and also the adaptation of the rumen epithelium to increase capacity of short chain fatty acids (SCFA) uptake are critical factors to prevent digestive disorders (GÄBEL et al., 2002).

Early studies focused on experimental induction of ruminal acidosis by different amounts of acids, combined with different feedstuffs (PERKINS & MONROE, 1933). DUNLOP & HAMMOND (1965) described that all starchy and sugary feed components trigger rumen acidosis and alterations in the microbial population have a great impact on the occurrence of D-lactate acidosis in blood. Cattle with acute rumen acidosis show pathological alterations of the rumen wall, like sloughing, disruption and swelling of the mucosa. Furthermore, reduced intestinal motility, damage to rumen, liver and other tissues occur (SLYTER, 1976). Associated alterations in blood are dehydration and a rise in lactate (DUNLOP & HAMMOND, 1965). The symptoms vary between mild forms of inappetence to sudden death, due to cardiovascular and respiratory failures (HUBER, 1976). Management aspects trigger occurrence of acidosis, emphasizing the influence of feed changes, weather effects and feeding after withdrawal (ELAM, 1976; DONOVAN et al., 2004; RABAZA et al., 2020).

Principal pathogenetic mechanisms of ruminal acidosis were well described in a review of NOCEK (1997). There is an imbalance of production, utilization and absorption of organic acids. Easily digestible carbohydrates are rapidly degraded to SCFA by microorganisms in the rumen and rumen pH decreases. Lactate producing bacteria grow more pronounced, the lactic acid concentration increases and pH decreases further on. This leads to a circle of increased growth of lactate builders, whereas the number of lactate utilizer decreases and pH shifts further down. Rumen motility, blood flow and absorption of organics decrease. Osmolarity increases and a hemoconcentration and dehydration follows, besides a decrease of bicarbonate and calcium concentration in blood. Ruminitis develops and epithelium gets leaky, so pathogens flow to liver, provoking liver abscesses (NOCEK, 1997). Rumen epithelium showed a reduction in depth, a decline of cellular junctions, increased sloughing of the stratum corneum and undifferentiated cells near the stratum corneum in cows with induced subacute ruminal acidosis (STEELE et al., 2011). Calves fed with concentrate had more morphological abnormalities of the rumen epithelium than hay-fed calves. Although the epithelial lining was heavier and the animals had more mucosa in relation to the muscle layer, the number of papillae per area and length was greater in the hay-fed animals (NOCEK et al., 1984).

NAGARAJA & TITGEMEYER (2007) emphasized the different degrees and courses of this disease and described the knowledge about the changes in the microbial flora. They defined subacute ruminal acidosis as ruminal pH below 5.6, with slight or no clinical signs and no mortality. Total organic acid concentration in rumen is increased by high concentrations of SCFA. Lactic acid producers and utilizers are both increased and until pH does not fall below 5 (for undetermined time), lactate is converted to SCFA. But if pH falls below 5, normal

bacterial flora gets destructed. There is a decrease in gram-negative bacteria and lactic acid utilizers. On the opposite, there is an increase in gram-positive and lactic acid producers, which is pushing further to an imbalance towards lactic acid and acidosis. Ciliated protozoa are absent or decreased in acute and subacute acidotic situations (NAGARAJA & TITGEMEYER, 2007).

Trials with oligofructose overload (17 g/kg BW orally) resulted in acute rumen and systemic acidosis, with diarrhea, dehydration, depression and anorexia. In addition, within 30 hours lameness due to laminitis and joint effusion developed (DANSCHER et al., 2009). Aseptic synovitis was also observed 24 hours after oligofructose overload (13 g/kg BW) in heifers (HIDALGO et al., 2019).

Potentially toxic products of microbes, especially histamine and endotoxins (lipopolysaccharide, LPS), were increased in rumen of grain-fed animals and LPS were detected in blood after induction of acidosis (ANDERSEN et al., 1994; EMMANUEL et al., 2007; KHAFIPOUR et al., 2009; YANG et al., 2018; HUMER et al., 2018, 2018), promoting systemic inflammation reflected by an increase of proinflammatory cytokines and acute phase proteins (LIU et al., 2020; KHAFIPOUR et al., 2009). An overview of some recent studies investigating the influence of diet on parameters in rumen and blood is given in Table 1. Interestingly, LPS permeates through mucosal tissue independently of pH, but permeability increases rapidly if pH drops below 4.5 in rumen and 5.5 in colon tissues (EMMANUEL et al., 2007). The increase of endotoxins in rumen is associated with a decrease of gram-negative bacteria. However, in vitro studies could not identify a direct correlation between the decrease in gram-negative bacteria and the increase of endotoxins (NAGARAJA et al., 1978).

Other blood parameters are only slightly altered in subacute ruminal acidosis, but sequelae are the same. Associated diseases are rumenitis, laminitis, polioencephalomalacia and liver abscesses (NAGARAJA & TITGEMEYER, 2007). This could indicate that not only acute systemic inflammation occurs, but also low-grade inflammation, which is difficult to measure. KHIAOSA-ARD & ZEBELI (2018) have hypothesized that LPS from the gastrointestinal tract leads to metabolic endotoxemia and low-grade systemic inflammation, caused by small local inflammations. This could activate the immune system chronically and promote metabolic disturbances, limiting growth and performance. They described three ways for LPS to cross the epithelial barrier. Firstly, paracellular through impaired tight junctions; secondly, transcellular through receptor mediated transcytosis and thirdly, via lipoprotein pathway transporting lipids. The passage of LPS into the tissues occurs throughout the gastrointestinal tract, especially if

starch flows into the hind gut, because it was not digested sufficiently in the rumen (KHIAOSA-ARD & ZEBELI, 2018).

Mechanisms, symptoms, diagnosis and management principals to prevent SARA in dairy herds has also been reviewed by KLEEN et al. (2003). It was emphasized that there is no standardized threshold for the presence of subacute or acute rumen acidosis. This is on the one hand due to the fact that different sampling procedures are used. Oral samples, taken with a stomach tube are considered to be contaminated with saliva and therefore, have higher pH values compared to samples taken directly from the rumen via rumenocentesis. On the other hand, time of sampling relative to feeding and duration of decreased ruminal pH are also important factors (PLAIZIER et al., 2009). For example, a threshold for subacute ruminal acidosis is a rumen pH depression between pH 5.2 and 5.6 for at least 3 h/day, measured directly in rumen (GOZHO et al., 2005).

The technology of metabolomics gives new insights in metabolic changes in rumen and blood of affected animals. In a feeding trial with diets consisting of 40 % or 60 % concentrate, 144 metabolites in rumen and 56 plasma metabolites differed between the feeding groups (YANG et al., 2018). In dairy cows with induced subacute rumen acidosis, an increase of LPS and biogenic amines, especially histamine, ethanolamine and pyrrolidine were observed in the rumen fluid. The most important differences in blood of SARA affected cows in comparison to healthy control cows were an increase in hexoses, glycine, serine, aspartate aminotransferase (AST) and glutamate-dehydrogenase (GLDH), and a decrease in cholesterol, arginine (Arg), PCs, lyso-PCs, sphingomyelins (SMs), citrulline, isoleucine (Ile) and lysine. The concentration of lactate and non-esterified fatty acids (NEFA) were unaffected (HUMER et al., 2018)

There is also an ongoing discussion about the comparability of different models to induce acidosis, distinguishing between challenges where the carbohydrates are given directly into the rumen or experimental diets, which have to be eaten by the animals. Furthermore, animals are normally kept in groups and therefore, larger fluctuations in feed intake are possible due to competition between the animals. Consequently, knowledge established in studies often differ to observations in practice (NAGARAJA & TITGEMEYER, 2007).

In this context, PLAIZIER et al. (2009) concluded that the triggered inflammatory response cannot be solely dependent on rumen pH and the amount of endotoxins, as studies show limited reproducible results in severity of this disease. For example, even if induction of subacute rumen acidosis was successful, endotoxins and systemic inflammation is not always detectable (RODRÍGUEZ-LECOMPTE et al., 2014). This could be due to the fact that endotoxins from

different bacteria have different bioactivities and therefore pH value or endotoxin quantity alone is not sufficiently indicative (KHIAOSA-ARD & ZEBELI, 2018). Detailed knowledge of the toxicity of LPS from various bacteria could lead to new feeding strategies in the future, promoting the growth of bacteria which have low toxicity LPS and causing little or no inflammation (MONTEIRO & FACIOLA, 2020).

Table 1. Overview of studies investigating the influence of diet on parameters in rumen and blood.

Author	Animals in trial	Feeding[1]	Rumen alterations[2]	Blood alterations[3]
GOZHO et al. (2005)	Jersey steers n = 3	4-6 kg/d concentrate and alfalfa hay	pH < 5.6 (day 4 187 min/d; day 5: 174 min/d); LPS ↑ day 2-5	Hp ↑ day 3, 5; SAA ↑ day 2-5
KHAFIPOUR et al. (2009)	Holstein cows n = 8	Control time: C50:50F week 1-5; SARA time: C60:40F week 6	Control time: pH < 5.6 118 min/d, LPS ↑; SARA time: 279 min/d, LPS ↑↑ SCFA ↑ ammonia ↑ lactate n.s.	SARA time: glucose ↑ SAA ↑ LBP ↑ Hp ↑ lactate n.s.
AMETAJ et al. (2010)	Holstein cows n = 2	TMR with 0, 15, 30, 45% of DM barley grain	35 and 45%: glucose ↑ maltose ↑ ethanol ↑ SCFA ↑ Ala ↑ Lys ↑ Leu ↑ Val ↑ glycerol ↑ fumarate ↑ uracil ↑ xanthine ↑ nicotinate ↑ methylamine ↑ phenylacetate ↑ dimethylamine ↑ N-nitroso- dimethylamine ↑ phenylacetylglycine ↑	
HUMER et al., (2018)	Holstein cows n = 8	First week C0:100F Seconde week: gradually increase of concentrate day SARA time: C65:35F	SARA time: LPS ↑ biogenic amines ↑ (histamine ↑↑)	SARA time: AST ↑ GGT ↑ GLDH ↑ glucose ↑ cholesterol ↓ PCs ↓ lyso-PCs ↓ SMs ↓ (AAs ↓) n.s.: lactate, NEFA
YANG et al. (2018)	Beef bulls n = 7	Low group C40:60F vs High group C60:40F	High group: pH ↓ LPS ↑ carbohydrate metabolites ↑ AA ↑	High group: LPS ↑ carbohydrate metabolites ↑ AA ↑ tyramine ↑
LIU et al. (2020)	Beef bulls n = 6	Low group C40:60F vs High group 60:40	High group: pH ↓ LPS ↑	High group : LBP ↑ SAA ↑ CRP ↑ Hp ↑ TNF-α ↑ IL-1β ↑ IL-6 ↑ IL-8 ↑

[1]total mixed ration (TMR), dry matter (DM), ratio of concentrate to forage based on DM (Cxx:xxF); subacute rumen acidosis (SARA);

[2] and [3] ↑ increased concentrations; ↓ decreased concentrations; not significant (n.s.); lipopolysaccharide (LPS); short chain fatty acids (SCFA); amino acids (AA); haptoglobin (Hp); serum amyloid A (SAA); lipopolysaccharide-binding protein (LBP); tumor necrosis factor α (TNF-α); interleukin (IL); C-reactive protein (CRP), aspartate-aminotransferase (AST), γ-glutamyltransferase (GGT), glutamate-dehydrogenase (GLDH), phosphatidylcholine (PC), sphingomyelin (SM), non-esterified fatty acids (NEFA).

2.2.3.1 Liver abscesses

Liver abscesses are a great problem in feedlots. Incidences of 20 % to 26 % (GREENOUGH et al., 1990) or 32 % to 77 % of the herd (BRINK et al., 1990) are reported, but differences between feedlots and fattening period are great and variations in the incidence of liver abscesses range from only a few percent of affected animals to almost the entire herd (NAGARAJA & CHENGAPPA, 1998). The occurrence depends on several factors, but the feed is most crucial. The more starch and sugar-rich concentrate is used and the fewer fiber components are included, the more likely it is that severe rumen acidosis with liver abscesses occurs. It depends not only the proportion, but also on the nature of the individual used components. Very long fibers are selected more easily and the more the cereal is grounded and preprocessed, the more likely it is that liver abscesses will occur (NAGARAJA & CHENGAPPA, 1998). High grain feeding leads to ruminal acidosis and ruminitis, therefore bacteria enter through altered rumen wall into portal blood, causing abscesses in the ruminal wall and liver (NAGARAJA & CHENGAPPA, 1998). Affected animals show reduced feed intake, daily weight gains and final BWs. This connection was mainly observed if there were one or more large and active abscesses (BRINK et al., 1990). There was a higher incidence observed in Holstein bulls, due to higher feed intake and longer fattening period compared to beef bulls of the same starting weight (NAGARAJA & CHENGAPPA, 1998). The bacteria identified in the abscesses were mainly *Fusobacterium necrophorum* subspecies and *Actinomyces pyogenes* (current nomenclature: *Truperella pyogenes*). Therefore, preventive methods recommended were not only adapted feeding, but also the extended use of antibiotics and vaccination of the animals against the leukotoxoid of *Fusobacterium necrophorum* (NAGARAJA & CHENGAPPA, 1998).

2.2.3.2 Tail tip necroses

Similar applies to the tail tip necrosis (TTN), which is a multifactorial disease. It occurs in different degrees of severity. Macroscopically, in the mildest form, hairless areas are seen on the tip of the tail, as well as keratinization and increased hardness of the skin at the tip of the tail. In more severe cases, necrosis and clear inflammatory changes start at the tail tip and ascend along the tail (DROLIA et al., 1991; SCHRADER et al., 2001; KORDOWITZKI, 2015). Failure or delay of treatment leads to bacterial spread to other organs and then results in lameness, depressed general well-being and death of the affected animals by multi-organ failure (THOMSON et al., 2009). The TTN is a common disease in fattening animals, especially in those kept on slatted floors. In southern Ontario, 96 % of feedlots with slatted floors reported TTN as a stock problem (DROLIA et al., 1991). KROLL et al. (2014) observed TTN in 60 %

to 76 % of animals in a feedlot with slatted concrete floor. In a big feedlot (80,000 animals) in Nebraska, 1 % of the animals showed severe cases of TTN, typically after 120 to 150 days on feed, 30 % of these animals died or were culled early (THOMSON et al., 2009).

The occurrence of TTN is associated with increasing BW, less space availability and is also influenced by season. It is hypothesized that trampling initially causes bruising and injuries, then humid and dirty conditions further support the severity of TTN (MADSEN & NIELSEN, 1985; DROLIA et al., 1991; SCHRADER et al., 2001). Bulls bedded on straw show less TTN and more importantly, less severe cases of TTN than bulls on fully slatted concrete (SCHRADER et al., 2001). However, there are also indications that metabolic changes could be a causal factor, because bulls kept on straw also showed signs of TTN (SCHRADER et al., 2001). DROLIA et al. (1991) found evidence of edema of the blood vessel walls in histological examinations of macroscopically inconspicuous tails. Zinc and Vitamin A deficiency or mycotoxins are also assumed to be primary causes of TTN (SALIB & FARGHALI, 2016). Furthermore, there was a correlation of low rumen pH and the occurrence of TTN, as well as significantly higher concentrations of total protein and quantity of leukocytes in bulls with moderate loss of hair, reduced elasticity of the skin, with fissures and crusts of the tip tail (KORDOWITZKI, 2015).

2.2.4 Claw disorders

Health of the claws mirrors metabolic health due to the fact, that metabolic disorders are associated with severe claw disease (LISCHER et al., 2000). Claw health, claw size, horn growth quantity and quality depend on various factors. Identified intrinsic factors like breed, age, individual metabolic status and BW play an important role, but also extrinsic factors like season, housing, feeding and management conditions are relevant (ANDERSSON & LUNDSTRÖM, 1981; VERMUNT & GREENOUGH, 1995; DERVISHI et al., 2020).

Individual genetic background have a great impact on feet and leg conformation, but only minor effect on occurrence of hoof disorders (HÄGGMAN & JUGA, 2013). Only the occurrence of dermatitis digitalis have a considerable heritability (SMIT et al., 1986; HÄGGMAN & JUGA, 2013). Length of the dorsal wall, length and breadth of sole are positive correlated with age and BW of the animal (ANDERSSON & LUNDSTRÖM, 1981). Seasonal effects have also influence on horn growth and digital diseases. Low temperature was associated with low hoof growth in sheep (WHEELER et al., 1972). Foot rot in feedlot cattle occurred most likely in spring and summer, whereas lameness with no visible swelling was seen mostly in fall (DAVIS-

UNGER et al., 2019). In a study of MAGRIN et al. (2020a) intensively finished beef cattle had the highest risk to develop a white line abscess when slaughtered in winter.

The type of floor plays an important role for horn growth and occurrence of diseases. A rubberized surface of slatted floors has been proven to be beneficial for claw health (VANEGAS et al., 2006; MAGRIN et al., 2020b; MAGRIN et al., 2019). The softer surface reduces bruising of the dermis as well as abrasion of the claw horn compared to concrete slatted floors (PLATZ et al., 2007). Fattening bulls on rubber mats showed longer dorsal walls of the claws (MAGRIN et al., 2019; PLATZ et al., 2007). The suspected low wear of the claws on rubber mats in cows resulted in less horn growth compared to cows kept on concrete. In addition, fewer treatments of the claws were needed for cows kept on rubber mats (VANEGAS et al., 2006).

There is also a great influence of dietary factors on horn growth and claw health. High content of fiber in diet is protective, whereas high amounts of easily digestible carbohydrates are a risk factor for digital disorders (MAGRIN et al., 2020a). Cows fed a diet low in fiber and high in starch have a high risk to develop clinical laminitis and sole ulcers (LIVESEY & FLEMING, 1984). Greater amounts of concentrate (7 kg vs. 11 kg) lead to more cases of lameness in dairy cows, and also severity and duration of lameness is greater in animals fed high concentrate diet (MANSON & LEAVER, 1988a). Furthermore, high protein content in diet has also an influence on claw health and leads to a increased prevalence of lameness in dairy cows (MANSON & LEAVER, 1988b).

The supplementation of selenium to the diet had no effect on claw health (RÄBER et al., 2008), but source of organic zinc showed an effect in a nine months lasting feeding trial with crossbreeds of Red Holstein x Simmental calves. Zinc polysaccharide supplementation showed an improvement of claw status at the end of the trial in comparison to claw status at the beginning (KESSLER et al., 2003).

Finally, there are some general management factors that influence hoof health and thus lead to farm-specific high prevalence of lameness (DAVIS-UNGER et al., 2019). In dairy cows, hoof trimming reduces prevalence of lameness the occurrence of digital disorders (MANSON & LEAVER, 1988b). However, this factor is not considered much in fattening bulls. There is no specific data on the influence of claw trimming on the claw health of fattening bulls.

2.2.4.1 Laminitis

Some aspects of laminitis have already been mentioned in previous chapters, but due to its importance, occurrence, clinical aspects and possible pathogeneses will be presented here in more detail. Laminitis or *Pododermatitis aseptica diffusa* is an aseptic inflammation of the corium. It is highly associated with feeding high grain diets, as local manifestation of systemic diseases and is commonly observed in animals used for meat as well as for milk production (GREENOUGH et al., 1990; LANGOVA et al., 2020).

Etiology of laminitis is multifactorial. Table 2 gives an overview about relevant studies related to laminitis in cattle. Factors identified are nutrition, genetics, perinatal period (LISCHER & OSSENT, 1994), but also housing and management factors have an influence. Soft floor and bedding, as well as feeding high fiber diet are preventive factors. Abrupt changes in the amount of exercise, great reduction or over exercise, due to transport or rehousing, are assumed to be risk factors for severity of laminitis (VERMUNT & GREENOUGH, 1994), as well as concreted slots (MAGRIN et al., 2020a). LONGOVA et al. (2020) summarized that feeding additives containing copper, zinc or biotin reduce severity of laminitis. The incidence and severity of laminitis differed between breeds (GREENOUGH & GACEK, 1987), although observed heritability in Finnish Holstein cattle was only 0.02 for chronic laminitis (HÄGGMAN & JUGA, 2013). Furthermore, there were age-dependent expressions of laminitis observed in calves compared to yearlings (GREENOUGH et al., 1990) and in heifers compared to older cows (VERMUNT & GREENOUGH, 1994).

The most used model to induce laminitis is by fructose overload. Clinical symptoms of ruminal and metabolic acidosis disappeared after approximately two days and lameness due to laminitis occurred (THOEFNER et al., 2004). In further experiments positive reactions in hoof testing started 30 hours after oligofructose overload and reached a maximum on day seven and nine, where 12 of 28 reactions were marked positive. In this study, dairy heifers with good body condition and grass feeding before induction, were observed to be predisposed to a more severe systemic affection (DANSCHER et al., 2009). It was summarized that experimental high concentrations of histamine, endotoxin and lactate, individually or together trigger laminitis (LEAN et al., 2013).

Possible mechanisms provoking laminitis were summarized by NOCEK (1997). A systemic metabolic insult due to ruminal and systemic acidosis, supported by endotoxins and histamine could lead to impaired vascularization of the corium. Unphysiological arteriovenous shunts increase blood pressure in the corium and cause seepage through vessel walls until vessels are

damaged and edema, thrombosis and hemorrhage occur (NOCEK, 1997). This condition causes severe pain and local mechanical damage follows by a cycling of edema and ischemia, causing hypoxemia and hypoxia of epidermal cells. Less supply of nutrients and oxygen promotes degeneration of the corium and dermal-epidermal junctions. It further comes to a separation of the laminae and mechanical stabilization of the pedal bone is no longer possible. The bone "sinks" and soft tissues are compressed between bone and sole. This again leads to hemorrhage, thrombosis, edema and necrosis of the tissue, resulting in scar tissue and poor claw horn quality (NOCEK, 1997).

Acute laminitis is characterized by intense pain, swelling and higher temperature above the coronary band (BARGAI et al., 1992). The affected animals are systemically ill, and vessel seepage and edema occur inside the claw capsule; thus, claw conformation is initially unaffected (NOCEK, 1997). Later on, inflammation results in red and yellow discolorations of the sole, which appear within five days (BOOSMAN et al., 1991). Subclinical laminitis is dependent on persistence of low-grade metabolic insults, resulting in insidious changes of horn quality and conformation of the claw. Horn gets softer, yellowish colorations and hemorrhages occur in the solar area, as well as ridges of the dorsal wall, white line separation and increased occurrence of double soles and sole ulcers, due to ischemia, hypoxia and epidermal damage inside occur (NOCEK, 1997). In the stage of chronic laminitis epidermal cell destruction, separation of dermal-epidermal junctions has continued so far that the damage is irreversible. Claws become more flattened, elongated and broadened, with prominent ridges and a concave dorsal claw wall. In several cases, the pedal bone protrudes through the corium and the horn capsule (NOCEK, 1997) or the pedal bone can be affected by osteolysis and osteitis (KOFLER & STANEK, 1990). Chronic inflammation progresses six to eight weeks post initial metabolic insult, until associated lesions become evident. There is an interaction of inflammation and repair, resulting in extended, deformed claws and bad horn quality (UWIERA et al., 2017).

Histopathological findings in acute laminitis are hyperemia, edema, thrombosis and hemorrhages (ANDERSSON & BERGMAN, 1980), as well as extended epidermal lamellae, pointed tips and more cuboidal formed epidermal basal cells than normal (THOEFNER et al., 2004; DANSCHER et al., 2010). However, assumed weakening of the suspensory tissue of the claw could not be proven 24 or 72 hours after induction of acute laminitis (DANSCHER et al., 2010). In chronic stage, arteriosclerosis, chronic thrombi and granulation tissue, besides of hyper- and parakeratosis are observed (ANDERSSON & BERGMAN, 1980). Macroscopic and arteriographic appearance of the claws are positively correlated in cattle affected by chronic laminitis (BOOSMAN et al., 1989).

Especially in subclinical courses, an early diagnosis is difficult. The temperature of the claws measured by infrared scanners, appear to be of limited use in mild courses and diagnosis was only confirmed when external changes of the claw became visible after months (MOMCILOVIC et al., 2000). In acute cases, the claws and coronary band heat up sufficiently so that a diagnosis is possible by infrared thermography, in addition to hoof pain testing and lameness scoring (SOUSA et al., 2020).

Although research on this disease has been conducted for a long time, biomarkers or laminitis-specific blood alterations are rare. Associated blood changes are inconsistent; an overview of some relevant studies is given in Table 3. Increased blood concentrations of histamine appear to be characteristic, especially in animals with chronic laminitis (MACLEAN, 1966). As laminitis is a multifactorial disease, it is difficult to establish reproducible study results or causal relationships (BERGSTEN, 2003). UWIERA et al. (2017) highlighted that the animal as a whole should be considered and expected that the interaction of the new "-omics" approaches (genomics, proteomics, metabolomics) could provide new insights into the pathophysiology of laminitis.

Laminitis is also an important disease in horses and it was summarized that there are three main causes triggering laminitis in horses. One cause is inflammatory laminitis, induced by starch or oligofructose overload, or systemic illness. A second cause is endocrinopathic laminitis induced by insulin and the third cause is laminitis due to mechanical overload (KATZ & BAILEY, 2012). It is assumed that certain biological principles have similar consequences in several species, due to similarities in the occurrence of laminitis in cattle and horses (BOOSMAN et al., 1991). Hyperinsulinemia alone was identified to trigger laminitis in horses (ASPLIN et al., 2010; LAAT et al., 2010a; KARIKOSKI et al., 2015). Studies investigating endocrinopathic laminitis in cattle are rare. In a recent study laminitis was induced by a diet high in cassava. There was no effect on dry matter (DM) intake, body temperature, heart and respiratory rate, but diet provoked diarrhea and ruminal acidosis in all animals. Three of five animals showed signs of acute laminitis. Comparing insulin concentrations within the experimental feeding group, animals which developed laminitis had almost twice as high basal insulin concentrations and reduced insulin sensitivity in an oral glucose test compared to non-diseased animals from the same feeding group (PILACHAI et al., 2019).

Table 2. Overview of described incidences of laminitis related claw disorders.

Author	Animals in trial	Feeding[1]	Claw health
LIVESEY & FLEMING (1984)	Friesian dairy cows n = 25	Control group: low starch, high fiber High group: high starch, low fiber	Control group: 8 % clinical laminitis, 8 % sole ulcer High group: 68 % clinical laminitis, 64 % sole ulcer;
GREENOUGH & GACEK (1987)	Bulls, various breeds n = 65	C50:50F, 13.5-15.5 % CP	92% ridges of the dorsal claw wall, hemorrhages, erythema and edema of the coronet or peri-coronal epidermis
MANSON & LEAVER (1988a)	British Friesian cows n = 24	LowCon group: C44:56F, 157 MJ/day ME, 2.4 kg/day CP HighCon group: C58:42F, 184 MJ/day ME, 2.8 kg/day CP	Diagnosis of solar problems (Pododermatitis circumscripta and aseptica traumatica) LowCon group: 7/10 lameness cases; HighCon group: 28/37 lameness cases
MANSON & LEAVER (1988b)	British Friesian cows n = 24	C60:40F CP kg/day LowP group: 2.67 kg/day CP, 204 MJ/day ME HighP group: 3.21 kg/day CP, 197 MJ/day ME	Diagnosis of solar problems (Pododermatitis circumscripta and aseptica traumatica) LowP group: 13/16 lameness cases; HighP group: 32/38 lameness cases
GREENOUGH et al. (1990)	Charolais cross steer calves n = 74	12 feeding groups: two levels of energy: 73.5% or 78.5 % of total digestible nutrients, six levels of CP: 11, 13, 15, 16, 17, 19 % of DM Calf group: 8 months old Yearling group: 13.5 months old	Calf group: Osteopathy of distal phalanx 44 %, rotation of distal phalanx 6%, ridging of dorsal claw wall 19 % Yearling group: Osteopathy of distal phalanx 12 %, rotation of distal phalanx 58 %, ridging of dorsal claw wall 72 %; high energy diet: hemorrhages at toe and heel ↑ no effect of protein content
SOUSA et al. (2020)	Nelore heifers n = 29	Day 1-3: 0.765 g/kg BW oligofructose twice daily; day 4: 10.71 g/kg BW oligofructose	27/29 positive pain reactions, lameness, increased temperature of claws and coronary band
MAGRIN et al. (2020a)	Beef cattle, various breeds n = 1305	Risk factors: diets with ↑ water soluble carbohydrates, ↓ neutral detergent fiber	Hemorrhages: 57 % of feet/batch (ranged from 6 to 100 %); white line abscesses: 10 % of feet/batch at batch (ranged from 0 – 53 %)

[1] dry matter (DM); ratio of concentrate to forage based on DM (Cxx:xxF); body weight (BW); crude protein (CP); metabolizable energy (ME)

Table 3. Overview of blood alterations associated with laminitis.

Author	Animals in trial	Feeding[1]	Blood alterations	Claw health
CHEW (1972)	Holstein cows n = 50	9 kg grain mixture, 4.5 kg corn silage per cow and in summer pasture, in winter hay at free choice	Hypoalbuminemia, hyperglobulinemia	4 cows yellow-waxy horn, hemorrhages, swelling of coronary band; 9 cows sole ulcers
BARGAI et al. (1992)	Dairy calves n = 600 (4-6 months old)	18 % digestible protein in the diet	Protein ↑ CK ↑ GGT ↑	6 % laminitis, dilatated PIII vascular channels, PIII rotation, hemorrhages in the laminar section of the claws
BRANDEJSKY et al. (1994)	Braunvieh cows n = 50		Calcium-thromboplastin, partial thromboplastin time, thrombin time, antithrombin III	No correlation of blood coagulation factors and claw scoring
LISCHER et al. (2000)	Dairy cows with sole ulcers n = 160	Pasture in summer, at least 50 % hay in winter	Urea ↑ Mg ↓ (n.s.: Bilirubin, CK, GGT, AST, iron)	106 had subclinical or clinical laminitis
MOMCILOVIC et al. (2000)	Dairy bull calves n = 4 17 weeks old	4 feeding groups: energy level: 71 or 81 % TDN, protein level: 15 or 20 % of DM	High energy level: D-lactate ↑ L-lactate n.s.	No symptoms of acute laminitis, 3 and 7 months later: hardship grooves in nearly all animals, hemorrhages, erosions and double soles only few cases, no group effects
THOEFNER et al. (2004)	dairy heifers n = 2	3 oligofructose dosages: 13, 17, 21 g/kg BW	Base excess ↓ packed cell volume ↑	Pain reaction 5/6, lameness 4/6 heifers, no effect of dosage
DANSCHER et al. (2009)	Danish Holstein heifers n = 8	Oligofructose overload 17 g/kg BW	Base excess ↓ packed cell volume ↑	Lameness, pain reaction, distension of tarso-crural joint
DANSCHER et al. (2010)	Danish Holstein heifers n = 10	Oligofructose overload 17 g/kg BW	Base excess ↓ packed cell volume ↑	Moderate lameness (100%), weight shifting (35%), pain reaction (50%), polysynovitis (100%),

Author	Animals in trial	Feeding[1]	Blood alterations	Claw health
PILACHAI et al. (2019)	Heifers n = 5	Low group: NSC 328 g/kg DM CP 151 g/kg DM High group: NSC 523 g/kg DM CP low: 161 g/kg DM	High group: insulin ↑ glucose ↑	Weight shifting and swelling of coronary band: Low group 0/5; High group 3/5 heifers
ZHANG et al. (2020)	Dairy cows n = 20		Subclinical laminitis: His ↑ IL-6 ↑ LPS ↑ TNFα ↑ Chronic laminitis: His ↑ IL-6 ↑ LPS ↑ TNF α ↑ iNOS ↑ TBX2 ↑ COX-2 ↑; n.s.: IL-1 β, PGI-2, 5-HT, ET-1	Subclinical and chronic laminitis

1dry matter (DM); ratio of concentrate to forage based on DM (Cxx:xxF); subacute rumen acidosis (SARA); Crude protein (CP); Non-structural carbohydrates (NSC); Total digestible nutrients (TDN)

[2] and [3] ↑ increased concentrations; ↓ decreased concentrations; not significant (n.s.); lipopolysaccharide (LPS); aminoacids (AA); haptoglobin (Hp); serum amyloid A (SAA); lipopolysaccharide-binding protein (LBP); tumor necrosis factor α (TNF-α); interleukin (IL); C-reactive protein (CRP); creatine kinase (CK); γ-glutamyl-transferase (GGT); aspartate-aminotransferase (AST); magnesium (Mg); histamine (His); lipopolysaccharide (LPS); inducible nitric oxide synthase (iNOS); thromboxane 2 (TBX2); Cyclooxygenase-2 (COX-2); prostacyclin-2 (PGI-2); 5-Hydroxytryptamine (5-HT); Endothelin-1 (ET-1)

2.3 Examination of metabolic disorders

There are several possibilities to investigate changes in metabolism. Blood analyses are useful to evaluate the overall metabolic status of an animal. To get a detailed insight in the metabolic situation, new methods such as the metabolomics approach is used to analyze a large number of metabolites in a small sample quantity. This method will be presented in more detail in the following chapter 2.3.2.

To measure alterations of specific pathways in tissues, analyses of protein expressions and phosphorylation by the western blot analysis provide semiquantitative information about capacity and proposed activity of the target pathway. This method is well established and was used in various studies of cattle and other species (KENÉZ et al., 2019; KINOSHITA et al., 2016; WARNKEN et al., 2017). The effects of the insulin signaling cascade are of essential importance for the entire metabolism. The most important disturbance, namely insulin resistance, is one of the most common diseases in humans, especially in connection with obesity and a sedentary lifestyle (SCHENK et al., 2008; ARAÚJO et al., 2019). In horses, insulin dysregulation is associated with obesity and laminitis (LAAT et al., 2010b). Knowledge about insulin sensitivity in cattle and in particular in fattening bulls is rare. Important aspects of the physiology of insulin signaling cascade and knowledge of related literature in cattle will be briefly explained.

2.3.1 Expression and phosphorylation of the insulin signaling cascade

Figure 1 gives an overview of important components, functions and interactions of the insulin signaling cascade, focusing on insulin, the InsR, PKB, mTOR and AMPK. Insulin signaling transduction on molecular level is not well established in ruminants. However, in general events appear to be very similar to those in other species (SASAKI, 2002).

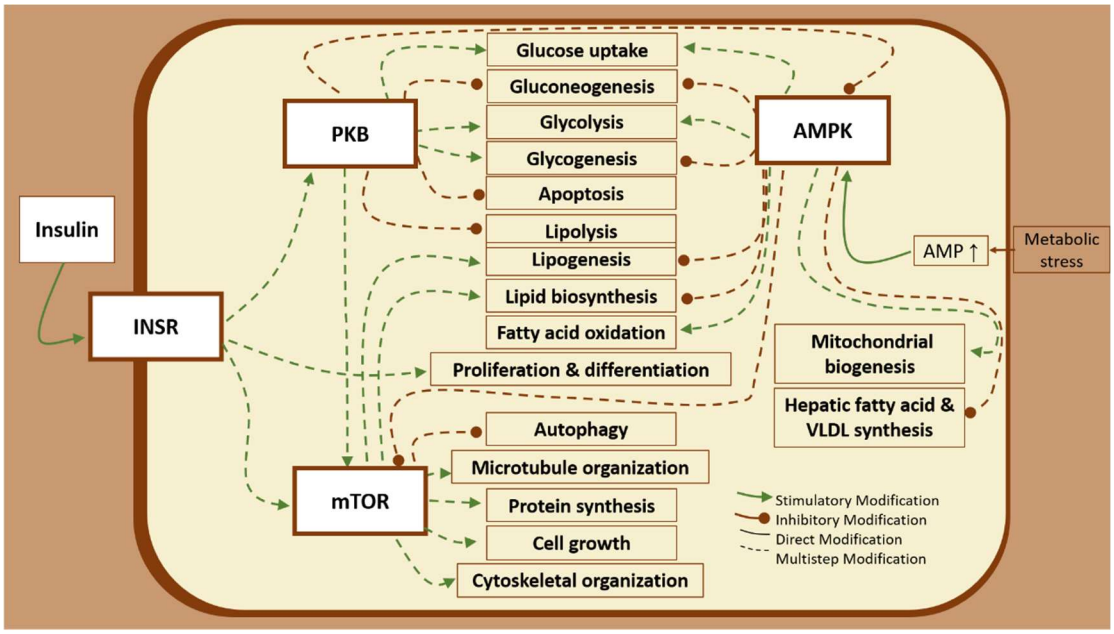

Figure 1. Scheme of insulin signaling cascade simplified to some main target components, demonstrating interactions and main functions influenced by activation of the components. Insulin receptor (INSR), protein kinase B (PKB), mechanistic target of rapamycin (mTOR), AMP-activated kinase (AMPK).

Scheme is based on: http://www.genome.jp/kegg/pathway.html.

Insulin is one of the most important metabolic hormones to maintain energy homeostasis. It is produced in and secreted by the beta-cells of the islets of Langerhans in the pancreas (MÖSTL, 2010). Biosynthesis is induced by glucose and includes several steps. Firstly, pre/pro-insulin is built, and after separation of a signaling molecule the retaining molecule is called proinsulin. In the last steps the connection of the double chain peptide is separated and disulfide bridges are built. Insulin is stored in the beta cells until stimulus of secretion. It is degraded by the liver (MÖSTL, 2010).

Human and bovine insulin is mostly structured in the same way; only three amino acids (AAs) are different. Main stimulus for insulin secretion in monogastric species is the glucose concentration in the blood. In ruminants, due to the forestomach system, plasma concentration of the SCFA, especially propionate, instead of glucose are the most important stimulus for the secretion of insulin (MÖSTL, 2010; HORNIO et al., 1968; TRENKLE, 1970).

Effects of insulin were summarized by KOSTER & OPSOMER (2013) as follows: anabolic effects of insulin are the uptake of glucose and AAs in muscle and adipose tissue and stimulation of cell proliferation and differentiation. It stimulates glycolysis, protein synthesis and glycogenesis, whereas protein degradation and glycogenolysis is inhibited. In muscles, the use of ketone bodies is supported and in adipose tissues, lipogenesis is stimulated and lipolysis

is inhibited. Main precursor for lipogenesis in cattle is acetate and not glucose, so glucose is mainly taken up by muscle tissue in cattle. In the liver, insulin suppresses gluconeogenesis and stimulates triglyceride synthesis (KOSTER & OPSOMER, 2013).

Insulin resistance is defined as a decreased biological response in insulin sensitive tissues induced by insulin. Resistance is based on reduced sensitivity or reduced responsiveness of the tissues (KAHN, 1978). Insulin sensitivity is defined as insulin concentration for half-maximal response, which is related to alterations prior to interaction of insulin and its receptor, for example reduced affinity or number of InsRs. Responsiveness is defined as maximal effect of insulin. Reduction of responsiveness is related to post receptor alterations of the insulin signaling cascade (KAHN, 1978).

In ruminants insulin reaches a lower responsiveness than in omnivorous monogastric species (DUHLMEIER et al., 2005; KASKE et al., 2001), probably due to impaired insulin stimulated glucose transport via GLUT4. In cattle, there is a predomination of insulin-independent glucose transporters (GLUT1) in glycolytic muscles (DUHLMEIER et al., 2005).

Insulin concentration in blood is influenced by age and concentration increased during growth in beef steers aged between two weeks and 17 months (RONGE & BLUM, 1989; RÖPKE et al., 1994; VERDE & TRENKLE, 1987). It is also influenced by sex (RÖPKE et al., 1994) and breed of the animals (BEEBY et al., 1988; VERDE & TRENKLE, 1987). Feeding and feeding level influence insulin concentration (SANO et al., 1999). A higher plane of nutrition results in higher concentrations of insulin (RÖPKE et al., 1994).

Tissue responsiveness is not influenced by feeding cycle (SANO et al., 1990), but glucose utilization is increased by dietary energy level, although mainly based on non-insulin mediated glucose transporters (JANES et al., 1985). Insulin sensitivity was not influenced by energy level of diets fed for five weeks (STERNBAUER & LUTHMAN, 2002). However, basal insulin concentrations were higher in obese heifers compared to lean ones and reduced insulin sensitivity but good responsiveness was detected (MCCANN & REIMERS, 1985). Insulin sensitivity was correlated positively with type I muscle fibers in calves and negatively correlated with mean muscle fiber area (STERNBAUER & ESSÉN-GUSTAVSSON, 2002).

The InsR is an oligotetramer located in transmembrane. It consists of two α- and β-subunits. Binding of insulin at α-subunits induces autophosphorylation of the β-subunits, beginning at Tyr^{1146} and $Tyr^{1150/1151}$. Kinase activity is stimulated for about an hour (WHITE et al., 1985). It results in tyrosine phosphorylation of cytosolic substrates, especially insulin receptor substrate-

1 (IRS-1). Phosphorylation of IRS-1 induces its association with other proteins, activating phosphoinositide 3-kinase (PI3K). PI3K further activates 3-phosphoinositide-dependent protein kinase 1, which activates PKB (Figure 1). The InsR also induces activation of mitogen activated tyrosine kinase, but this pathway appears to play a limited role in glucose metabolism (SASAKI, 2002).

The PKB is a serine/threonine kinase, activated by phosphorylation of Thr^{308} and Ser^{473}. It promotes the translocation of GLUT4 from intracellular vesicles into cell membrane (SASAKI, 2002). It also promotes cell surviving and inhibits apoptosis by influencing transcription factors and inactivating several targets (CARDONE et al., 1998). It is involved in cell cycle regulation (DIEHL et al., 1998) and inhibits glycogen synthase kinase, which inhibits activation of glycogen synthase (CROSS et al., 1995). It directly activates mTOR by its phosphorylation and inactivating of tuberin/TSC2, which is an inhibitor of mTOR (NAVÉ et al., 1999).

The mTOR is a serine/threonine protein kinase which is critical for protein synthesis, cell growth and regulation of the cell cycle (BROWN et al., 1994). It is activated by phosphorylation at Ser^{2448} (NAVÉ et al., 1999) and is auto-phosphorylated at Ser^{2481} (PETERSON et al., 2000). It exists in two complexes, termed as mTOR complex 1 (mTORC1) and mTORC2 (SAXTON & SABATINI, 2017). The mTORC1 is a sensor for ATP and AAs, balancing nutrient availability and cell growth (DENNIS et al., 2001). The AA leucine (Leu) directly stimulates protein synthesis via mTOR pathway (WOLFSON et al., 2016). Activated mTORC1 promotes anabolic processes, including biosynthesis of proteins, lipids and nucleotides (Figure 1) and inhibition of autophagy (BEN-SAHRA & MANNING, 2017). The mTORC2 influences proliferation, cytoskeletal organization and cell survival (SAXTON & SABATINI, 2017).

The AMPK is an intracellular energy sensor, maintaining energy balance within the cell by influencing whole body energy metabolism. It is heterotrimeric, consisting of a catalytic α- and regulatory β- and γ-subunit (CARLING, 2004). It is activated by metabolic stress, which elevates AMP/ ATP ratio, like hypoxia and ischemia. It inhibits energy (ATP) consuming biosynthetic pathways and regulates up ATP producing pathways (Figure 1). It partly deactivates protein synthesis and cell growth via activation of tuberin/TSC2, which inhibits mTORC1 (HARDIE, 2004).

2.3.2 Metabolomics and plasma metabolite profile

Metabolomics is a relatively new method to investigate metabolic pathways in more detail. It opens up new perspectives and could provide new insights in pathophysiology of diseases (UWIERA et al., 2017), in an attempt to understand mechanisms better and to generate new hypotheses.

Metabolomics is a study of cell metabolism, quantifying small water-soluble substrates, intermediate and end products of the cells. It is the newest in the field of "omics" technologies. Metabolites are all detectable small molecules, which are less than 1.5 kDa. The term of metabolite profiling is the quantitative description of all metabolites in a biological sample. Whereas the genome only reveals what might happen, the metabolome indicates what is happening (WISHART, 2019). It is the final product of multiple intracellular actors, starting with genes and transcriptional activators, over RNA transcripts, protein transporters and enzymes (FIEHN, 2002). This results in a high number of possible metabolites. For example in humans, there are about 20.000 genes consisting out of four different nucleotide bases, but it is expected that there are about one million different metabolites, related to over 3.000 chemical classes (WISHART, 2019). This technology provides a snapshot of all the processes that took place in cells (tissue sample) or organism (blood sample) at the time the sample was taken. The individual metabolite profile depends on physiological parameters like gender, age, genetics and time of day and is further influenced by dietary pattern, environmental exposures and gut microbiome functions (KIM et al., 2014). This sensitivity towards internal and external variables on the one hand and on the other hand the fact that metabolite profiles are highly conserved across an entire organism and across species, this technology opens up many opportunities for research (PEREGRÍN-ALVAREZ et al., 2009).

The analysis of metabolite profiles is based on liquid or gas chromatography as separation method and mass spectrometry or nuclear magnetic resonance spectrometry as detection method (ROBERTS et al., 2012). There are basically two ways to examine parts of the metabolome. The untargeted metabolite profiling tries to find as many (up to 10,000) metabolites as possible in a biological sample (WISHART, 2019). The metabolites are characterized and assigned to substance classes by comparing them with databases, but quantification and reproducibility is difficult (JONG & BEECHER, 2012). The targeted metabolomic approach searches for specific, previously characterized metabolites. Internal standards and quality controls enable precise quantification of the metabolites and ensure reproducibility (ROBERTS et al., 2012).

2.3.2.1 Absolute IDQ p180 Kit

The AbsoluteIDQ® p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria) is a targeted metabolomic approach. With this kit, up to 188 metabolites are quantified. It identifies metabolites of several pathways associated with changes in amino acid and energy metabolism, pro-inflammatory signaling, mitochondrial dysfunction, as well as dysregulation of lipid and glucose metabolism. It quantifies 21 AAs, 21 biogenic amines, 1 hexose, 40 acylcarnitines (ACs), 15 sphingolipids (SMs), 76 phosphatidylcholines (PCs) and 14 lyso-PCs. The kit was originally developed for human research, to investigate obesity-related disorders, insulin resistance and inflammation in more detail. It was successfully used to identify new biomarkers and affected pathways in human DM2 (LOTTA et al., 2016), in insulin dysregulated horses (KENÉZ et al., 2018) and also in various studies of cattle (HAILEMARIAM et al., 2014; HUBER et al., 2016; KENÉZ et al., 2016; DERVISHI et al., 2018; HUMER et al., 2018; GHAFFARI et al., 2019). For example, long-chain ACs were identified as a possible indicator for extended productive life span of dairy cows (HUBER et al., 2016) and cows with retained placenta already had changes in their metabolite profiles 8 weeks before calving, compared to later healthy animals (DERVISHI et al., 2018).

3 ANIMALS, MATERIALS and METHODS

3.1 Feeding trial

3.1.1 Rearing of the animals

The animal experiment was conducted from December 2017 to July 2018 at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, Germany. All experimental procedures were approved by the Animal Ethics Committee of the Department for Animal Welfare Affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany) in agreement with the German Animal Welfare Act (permit number: G-17-20-070).

Thirty-two Holstein Friesian bulls intended for beef production, were raised at the Institute of Animal Nutrition, Federal Research Institute for Animal Health (Friedrich-Loeffler-Institute, Braunschweig, Germany). All animals received 3 L of colostrum within 2 h after birth. They were kept in straw-bedded single hutches for the first week and were fed twice a day with 3 L of pooled herd milk until day three post natum. Starting at the age of three days, milk replacer (MR) (NOLAC GmbH, Zeven, Germany) was mixed with the pooled herd milk, with gradually increasing amounts from 0.3 kg MR powder/d (day 3 of life) to 0.9 kg MR/d (day 5 of life), until the maximum of 6 L liquid feed with a concentration of 150 g/L MR was reached. At an age of eight to ten days, the calves were moved into straw-bedded stables in groups, with MR and concentrate self-feeding systems (Förster-Technik GmbH, Engen, Baden-Württemberg, Germany). From day 16 to 20, amount of milk replacer was gradually increased from 6 to 9 L per day until day 31 post natum. From day 32 to 43, MR was gradually decreased from 9 L and calves were weaned. Calves were offered a mix of concentrate for calves, hay and water ad libitum before weaning. After weaning, calves received a mix of grass silage and corn silage, with a moderate amount of concentrate.

At the age of six months, bulls were relocated to the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle (Müchweiler a.d. Alsenz, Germany), where they were further raised with a total mixed ration (TMR) consisting of grass silage and corn silage and 15 % concentrate of DM, until beginning of the trial.

3.1.2 Experimental design, feeding and husbandry

Bulls were randomly assigned to a HEP or a LEP nutritional regimen, at an age of 13 months and an average BW of 500 kg (mean ± SD; HEP 506 ± 35 kg, LEP 499 ± 35 kg). They were housed on slatted floor with rubber mats, in groups of four bulls with a space offer of 2.9 m^2/animal. The TMRs were mixed every two days and bulls were fed at 7.30 am. Individual mean feed intake was calculated by weighing and documenting remained and provided feed group-wise. The nutrient composition of the TMRs was analyzed by an accredited external laboratory (Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany) according to the protocols of the Association of German Agricultural Analytic and Research Institutes (VDLUFA, 2007). Bulls remained on their assigned nutritional regimen for the rest of the study period, seven months, until slaughter. An overview of the experimental design is given in Figure 2.

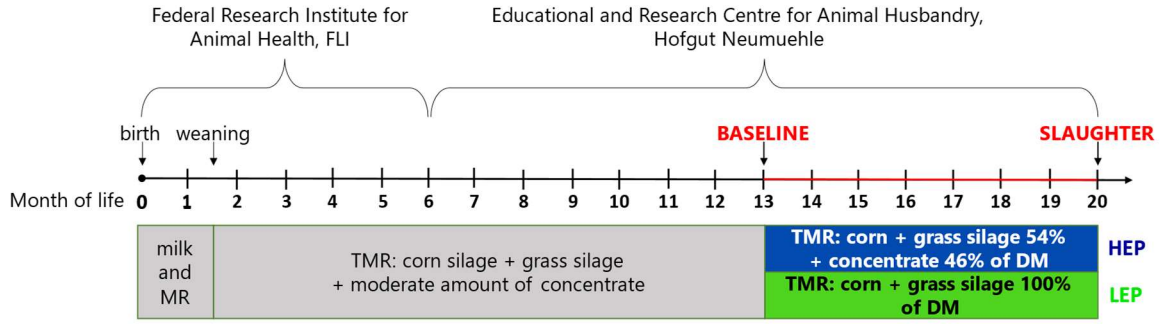

Figure 2. Experimental design of the feeding trial, consisting out of the two feeding levels: high energy and protein diet (HEP) and low energy and protein diet (LEP). Sample and data collection was at the beginning (baseline) and at the end (slaughter) of the feeding trial. Milk replacer (MR), total mixed ration (TMR).

The diets of the feeding groups were formulated according to Bavarian State Research Center for Agriculture (Bayerische Landesanstalt für Landwirtschaft (LfL), 2014), including metabolizable energy (ME) and crude protein (CP) recommendations for Simmental fattening bulls. The HEP diet was formulated according to the highest ME and CP recommendations, resulting in calculated 6 kg concentrate/animal/day in HEP TMR or more specifically, DM of HEP TMR consisted of 54 % grass- and corn silage and 46 % concentrate. In contrast, LEP diet was formulated only based on forage to generate strong differences between the feeding groups. The concentrate feed consisted of ground corn, rapeseed meal, ground wheat, palm kernel meal, wheat bran, molasses and soybean meal, accounting for an elevated total sugar, total starch and crude protein, as well as a decreased fiber content of the HEP diet, relative to the LEP diet. The

exact ingredients and chemical composition of the TMR are given in Table 4 and Table 5. The experimental design of the feeding trial is also described in BÄßLER et al. (2021).

Table 4. Ingredients of total mixed rations (TMRs) fed during the experimental period.

Ingredients of TMR[1]		
Component, % of DM	HEP	LEP
Grass silage	40.33	69.99
Corn silage	13.68	29.92
Concentrate feed (including minerals)	45.99	0
Salt	0	0.09

[1]dry matter (DM); high energy and protein diet (HEP); low energy and protein diet (LEP)
Data have already been published in BÄßLER et al. (2021).

Table 5. Chemical composition of total mixed rations (TMRs) fed during the experimental period.

Chemical composition of TMR[1]		
Item, g/kg of DM unless noted otherwise	HEP	LEP
DM, g/kg of FM	510	369
ME, MJ/kg of DM	11.4	10.2
Crude protein	155.0	110.0
Crude fat	40.0	37.0
Crude fiber	171.0	239.0
aNDFom	356.0	428.0
ADFom	174.0	232.0
Ash	82.0	85.0
Total sugar	31.0	20.0
Total starch	297.0	123.0
Ca	6.6	5.2
P	3.6	2.7
Na	1.4	1.0
K	18.8	24.1
Mg	2.2	2.1

[1]fresh matter (FM); dry matter (DM); metabolizable energy (ME); neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash (aNDFom); acid detergent fibre expressed exclusive of residual ash, (ADFom); high energy and protein diet (HEP); low energy and protein diet (LEP)

Data have already been published in BÄßLER et al. (2021).

3.1.3 Collection of samples and data

Bulls were weighed monthly. At the beginning (baseline) and end (slaughter) of the feeding trial (Figure 2) blood samples were taken and size and health of the claws were documented. In addition, tissue samples of muscle, adipose tissue and liver were taken at slaughter and conformation and fat class were documented.

3.1.3.1 Body weight, average daily gain

For monthly weighing a mechanical scale for cattle was used, which weighed to nearest kilogram. Average daily weight gain (ADG) was calculated with exact days between weighing.

3.1.3.2 Scoring of claws

At baseline, bulls were captured in a hoof trimming chute and size and health of the claws were documented by trained personnel, using a scoring system according to SOHRT (1999). The same protocol was used at slaughter, regarding the claws post mortem. Length of the dorsal claw wall and thickness of the sole horn of all digits were measured. Relation of height of the inner and outer digit of the claws were evaluated and scored as equal, higher inner or higher outer digit. Signs of the following disease were documented for all four claws of each bull: laminitis, heel horn erosion, dermatitis digitalis and interdigitalis, sole ulcers at typical and atypical localizations, digital phlegmon and tyloma. Alterations of the claws were classified per digit or per claw in case of dermatitis interdigitalis, phlegmon and tyloma according to the scoring system shown in Table 6. Score points of all digits and claws were summarized to one value per animal and disease.

Table 6. Scoring system for sole ulcer, heel horn erosion, laminitis and dermatitis interdigitalis, per digit and claw, respectively.

Disease		Score points				
		Absent	Discoloration	Superficial	Deep	Perforating
Sole ulcer, atypical		0	1	2	3	4
Sole ulcer, typical		0	1	2	3	4
		Absent	Low grade	High grade		
Heel horn erosion		0	1	2		
Laminitis	Concave dorsal claw wall	0	1	2		
	Wall defects	0	1	2		
		Absent	Present			
	Ridging on the dorsal wall	0	1			
	Double sole	0	1			
		Absent	Yellowish	Reddish	Black-red	
	Discolorations of the sole horn	0	1	2	3	
		Absent	Superficial	Deep		
Dermatitis interdigitalis		0	1	2		

3.1.3.3 Blood sample collection

Blood samples were collected at two points of time, baseline and slaughter, between 10 am and 2 pm by jugular venipuncture into plain tubes for serum and EDTA tubes and NaF tubes (S-Monovette®, Sarstedt AG, Nümbrecht, Germany). EDTA and NaF samples were centrifuged immediately after sampling at 3000 x *g* for 15 min. Serum samples were allowed to clot for 45 min at 20°C and then centrifuged at 3000 x *g* for 10 min. Plasma and serum samples were frozen at -80°C within 20 min after centrifugation and then continuously stored at -80°C until analysis.

3.1.3.4 Collection of tissue samples

At slaughter, tissue samples of liver and retroperitoneal adipose tissue were collected immediately after gutting and were put in ice cooled physiological saline solution. Samples of

liver tissue were taken from the facies diaphragmatica, in the lower third of the liver from the middle of the tissue, without liver capsule or major bile ducts. Muscle tissue was collected from Musculus longissimus dorsi, located at the level of the scapula. Retroperitoneal adipose tissue samples were taken from adipose tissue near the kidney. The tissue samples were cut in 0.5 x 0.5 x 0.5 cm^3 pieces and were frozen in liquid nitrogen. Frozen pieces were collected in Cryotubes (Sarstedt AG) and stored at -80 °C until further usage.

3.1.3.5 Conformation and fat class

Conformation and fat class was classified by a trained and experienced member of the slaughterhouse according to the European beef carcass classification system EUROP (Official Journal of the European Union, 2013), characterizing muscle development as follows:

E Excellent muscle development
U Very Good muscle development
R Good muscle development
O Fair muscle development
P Poor muscle development

Fat class was differentiated into 5 classes, characterized as follows:

1 No to very low fat coverage
2 Slight fat coverage; musculature visible almost everywhere
3 Musculature covered with fat almost everywhere; slight fat deposits in the thoracic cavity
4 Musculature covered with fat; clear fat deposits in the thoracic cavity
5 Carcass completely covered with fat; strong fat deposits in the thoracic cavity

3.2 Laboratory analyses

All samples were stored at -80 °C until analyzing or preparation. Blood chemistry was measured at the faculty of veterinary medicine, Clinic for Ruminants with Ambulatory and Herd Health Services of the Ludwig-Maximilians-Universität in Munich. The measurement of insulin concentration, metabolite profile and western blot analysis was done at the Department of Functional Anatomy of Livestock, University of Hohenheim, in Stuttgart.

3.2.1.1 Blood chemistry

Glucose and L-lactate in NaF plasma and urea, creatinine, total protein, albumin, aspartate aminotransferase, gamma-glutamyl transferase, GLDH, creatine kinase, phosphate, beta-hydroxybutyrate (BHBA) and NEFA in serum were analyzed with UV or visible colorimetric spectroscopy based on enzymatic assays on an automated analyzer (cobas c311 Analyzer, ROCHE, Mannheim, Germany), only at slaughter.

3.2.1.2 Insulin concentration

Insulin concentration was measured in serum at baseline and slaughter, by a sandwich-ELISA (Bovine Insulin ELISA, 10-1201-01, Mercodia AB, Uppsala, Sweden), according to the manufacturer's protocol.

3.2.1.3 Metabolite profile

Metabolite profiles were analyzed in EDTA plasma at baseline and slaughter, using the AbsoluteIDQ p180 Kit (Biocrates Life Science AG, Innsbruck, Austria), according to the manufacturer´s protocol, but with modifications which are pointed out below and are based on previous measurements described in KENÉZ et al. (2016); they are also described in detail in BÄßLER et al. (2021). The AbsoluteIDQ p180 Kit identifies and quantifies up to 188 metabolites from five compound classes: proteinogenic and modified AAs, biogenic amines, ACs, glycerophospho- and sphingolipids and a hexose. The AAs and biogenic amines were analyzed by liquid chromatography – mass spectrometry (LC–MS/MS) and the substance classes of ACs, PCs (including lyso-PCs), SMs and the hexose were analyzed by flow injection analysis – mass spectrometry (FIA-MS/MS) at the Core Facility of the University of Hohenheim (Stuttgart, Germany). Internal standard, PBS (phosphate buffer saline), calibration standards ("Cal 0.25", "Cal 0.5", Cal 1-6), quality control samples (QC 1-3) and EDTA plasma samples (10 µL) were applied and dried onto the matrix of the multititer plate provided in the kit under nitrogen flow (nitrogen evaporator 96 well plate, VLM GmbH, Bielefeld, Germany) for 30 minutes. "Cal 0.25" and "Cal 0.5" were 4× and 2× dilutions of the lowest calibrator solution included in the kit, respectively. These were used to enhance accuracy in the lower detection range, according to consultation with the manufacturer. Dried samples were derivatized with 5 % phenylisothiocyanate (PITC) for 20 min at room temperature and subsequently dried for another 60 min under nitrogen flow. Samples were extracted in 300 µL of extraction solvent (5 mM ammonium acetate in methanol) with shaking at 450 rpm for 30 min at room temperature. Eluted extracts were 10× diluted in 40 % HPLC grade methanol for LC–MS analysis and 50× diluted in the provided mobile phase solvent for FIA-MS/MS analysis. Both types of measurements were performed on a QTRAP mass spectrometer

applying electrospray ionization (ESI) (ABI Sciex API 5500Q-TRAP). The MS was coupled to an ultra-performance liquid chromatography (UPLC) (Agilent 1290, Agilent, Waldbronn, Germany). In case of LC–MS the metabolites were separated by a hyphenated reverse phase column (Waters, ACQUITY BEH C18, 2.1 x 75 mm, 1.7 µm; Waters, Milford, United States) preceded with a precolumn (Security Guard, Phenomenex, C18, 4 9 3mm; Phenomenex, Aschaffenburg, Germany) applying a gradient of solvent A (formic acid 0.2 % in water) and solvent B (formic acid 0.2 % in acetonitrile) over 7.3 min (0.45 min 0 % B, 3.3 min 15 % B, 5.9 min 70 % B, 0.15 min 70 % B, 0.5 min 0 % B) at a flow rate of 800 µL/min. The oven temperature was 50 °C. For LC-MS analysis 5 µL, and for FIA 20 µl were subjected for measurements in both, positive and negative modes. Identification and quantification were achieved by multiple reaction monitoring (MRM) standardized by applying spiked-in isotopically labelled standards in both positive and negative mode. A calibrator mix consisting of eight different concentrations was used for calibration. Quality controls deriving from lyophilized human plasma samples were included for three different concentration levels. For FIA an isocratic method was used (100 % organic running solvent) with varying flow conditions (0 min, 30 µL/min; 1.6 min 30 µL/min; 2.4 min, 200 µL/min; 2.8 min, 200 µL/min; 3 min 30 µL/min). The MS settings were as follow: scan time 0.5 s, IS voltage for positive mode 5500 V, for negative mode −4500 V, source temperature 200 °C and nitrogen as collision gas medium. The corresponding parameters for LC–MS were: scan time 0.5 s, source temperature 500 °C, nitrogen as collision gas medium. All reagents used in the processing and analysis were of LC-MS grade, unless otherwise stated. Milli-Q Water ultrapure was used fresh after being prepared by the high-purity water system by Merck KGaA (Darmstadt, Germany). LC-MS grade acetonitrile (83640.3201, VWR), Water (83645.320, VWR) and methanol (1.000971.500), as well as pyridine for analysis (1.09728.0100) and formic acid (98–100 %; 1.000263.1000) were purchased by Merck KGaA. PITC (P10034-10) and ammoniumacetate (81.7838-50) were purchased by Sigma Aldrich Chemie GmbH (Steinheim, Germany). Raw data of the Analyst software (AB Sciex, Framingham, MA, USA) were processed by the MetIDQ software which is an integrated part of the p180 Kit (Biocrates Life Sciences AG). This streamlines data analysis by automated calculation of metabolite concentrations provides quality measures and quantification. Before integrating LC-MS data in MetIDQ, it was validated in Analyst software. For fully quantitative measurements of the p180 Kit, the lower limit of quantification (LLOQ) was determined in plasma experimentally by the manufacturer.

3.2.1.4 Western Blot

Western blot analysis were done based on previous measurements, described in WARNKEN et al. (2017) with modifications pointed out below.

For western blot analysis, tissue samples had to be homogenized first, using FastPrep (MP Biomedicals, Eschwege, Germany) for protein extraction. About 100 to 150 mg of the tissues was homogenized in 600 µL of prechilled lysis buffer containing 50-mM HEPES (Carl Roth GmbH, Karlsruhe, Germany), 4-mM ethylene glycol-bis(2-aminoethylether)-N,N,N0,N'-tetraacetic acid (Sigma-Aldrich, St. Louis, MO, USA), 10-mM EDTA (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), 100-mM glycerol phosphate (Sigma-Aldrich), 15-mM sodium pyrophosphate (Sigma-Aldrich), 5-mM sodium orthovanadate (Sigma-Aldrich), 2.5-mM sodium fluoride (Sigma-Aldrich), a protease inhibitor cocktail (CompleteMini, Roche Diagnostics GmbH, Mannheim, Germany), and a phosphatase inhibitor cocktail (PhosStop, Roche Diagnostics GmbH), in two cycles (6 m/s, 40 sec, with a cooling break of 2 min on ice). The homogenates were centrifuged at 10,000 g for 10 min at 4 °C and were stored at -80 °C until electrophoresis. Protein concentrations of the homogenates were measured using Bradford reagent (Serva Electrophoresis GmbH, Heidelberg, Germany). Samples in loading buffer (50 mmol/L of Tris-HCL [SigmaAldrich], 10 % glycerol [Sigma-Aldrich], 2 % SDS [Serva Electrophoresis GmbH], 0.1 % bromophenol blue [SigmaAldrich], and 25-mM 1,4-dithiothreitol [DTT; Carl Roth GmbH]) were denatured by heating them for 5 min at 95 °C before loading 20 µg (liver and muscle), 60 µg (muscle p-mTOR, InsR, p-AMPK) and 20 µL of fat homogenate per lane onto a 5 % stacking and 8.1 % separation gel. Electrophoresis was carried out according to LAEMMLI (1970). Detection of specific proteins was performed after blocking membranes in a PBS-based solution containing 0.1 % Tween 20 (Sigma-Aldrich) and 5% fat-free milk powder (Carl Roth GmbH) or 5 % bovine serum albumin for 2 h at room temperature (Table 7). Membranes were incubated overnight at 4°C in PBS-based solution containing 0.1 % Tween 20 and 5 % fat-free milk powder or 5 % bovine serum albumin, with primary antibodies (Table 7). Detection of the primary antibodies and their phosphorylated forms was performed using secondary goat anti-rabbit-HRP antibodies for 60 min at room temperature. After washing three times in PBST and once in PBS for 5 min each, immunodetection was performed by incubating the membranes with Pierce West Dura chemiluminescence substrate (Thermo Scientific, Braunschweig, Germany) or LumiGLO reagent (Cell Signaling Technology Inc), and chemiluminescence was detected by a ChemiDoc XRS + system (Bio-Rad Laboratories GmbH, Munich, Germany). The bands were quantified by densitometry using Image Lab 5.2 software (Bio-Rad Laboratories GmbH).

Chemiluminescence signals were measured with at least five consecutive exposure times to determine the linear range of signal intensity of each antibody to ensure that quantitative data were obtained. Values of exposure times within the linear range were used for quantification and further analyses. Finally, membranes were stained with Indian ink (Pelikan PBS, Peine, Germany). Specific band signals were normalized to Indian ink signal as an internal standard. Two standard linker samples from pooled bovine tissue samples were blotted on each membrane to adjust signals from different membranes. This allowed the comparison of all the membranes of one tissue type.

Table 7. Primary and secondary antibodies used for Western blot analyses in bovine tissues.

Target	Antibody	Dilution	Company[1]	Blocked with[2]	Incubated in[2]
Insulin receptor β (InsR)	Rabbit anti InsR-β (4B8)	1:2,000	Cell Signaling	5 % milk	5 % BSA
Phosphorylated insulin receptor- β (Try1162/1163) (p-InsR)	Rabbit anti p-InsR-β (Try1150/115 ; 19H7)	1:500	Cell Signaling	5 % BSA	5 % BSA
Mechanistic target of rapamycin (mTOR)	Rabbit anti mTOR (7C10)	1:1,000	Cell Signaling	5 % milk	5 % BSA
Phosphorylated mechanistic target of rapamycin (Ser2448) (p-mTOR)	Rabbit anti p-mTOR (Ser2448)	1:500	Cell Signaling	5 % BSA	5 % BSA
Protein kinase b (PKB)	Rabbit anti AKT	1:2,000	Cell Signaling	5 % milk	5 % BSA
Phosphorylated protein kinase b (Ser473) (p-PKB)	Rabbit anti p-AKT (Ser473) (D9E) XP	1:2,000	Cell Signaling	5 % milk	5 % BSA
5’ adenosine monophosphate-activated protein kinase α (AMPK- α)	Rabbit anti AMPK α	1:4,000	Bethyl	5 % milk	5 % milk
Phosphorylated 5’ adenosine monophosphate-activated protein kinase α (Thr172) (p-AMPK- α)	Rabbit anti p-AMPKα (Thr172) (40H9)	1:1,000	Cell Signaling	5 % milk	5 % milk
Rabbit IgG	Goat anti rabbit IgG	1:2,000	Cell Signaling	5 % milk	5 % milk

[1] Cell Signaling Technology Inc (Cell Signaling), Bethyl Laboratories (Bethyl)

[2] Bovine serum albumin (BSA), fat-free milk powder (milk)

3.3 Statistical data analysis and evaluation

Metabolites were excluded from the analyses if ≥ 70 % of samples were 0 µmol/L. The following metabolites were excluded: spermine, phenylethylamine, dopamine, dihydroxyphenylalanine, cis-4-hydroxyproline and SM 22:3. Data of the remaining 182 plasma metabolites (absolute concentrations [µmol/L] of compounds) were analyzed in MetaboAnalyst 4.0 (CHONG et al., 2019) after normalization by log transformation and Pareto scaling. A heatmap was created to visualize differences between the dietary groups at slaughter. In addition, JMP.Pro 15 (SAS Institute, Cary, NC, USA) was used to detect significant differences between the groups by unpaired Student's t-test of fat class, conformation class, blood biochemistry, metabolites (including glucose and glucose-to-insulin [G:I] ratio) and expression and phosphorylation of proteins of insulin signaling cascade at slaughter. The G:I ratio was calculated according to LEGRO et al. (1998). The sum of branched chain amino acids (BCAAs), valine (Val), Ile and Leu and the sum of ACs C3 and C5 was built. Repeated measure two-way ANOVA was performed to detect effects of time, diet and interaction of time*diet on concentration of insulin and score points of laminitis, length, thickness and height relation of inner and outer digit of the claws, feed intake, BW and ADG. Significant interactions were afterwards checked by HSD Tukey post-hoc test. Data of laminitis and heel horn erosion were normalized by log transformation before performing the ANOVA. Linear regressions were performed to identify significant correlations in the data set. The values of all animals were used (n = 15) with two exceptions. In the correlation of the expression of mTOR in fat and PC ae C30:1, as well as in the correlation of the sum of BCAAs with the sum of ACs C3 and C5, the values of one HEP animal each were excluded, because they were identified as outliers, having higher values than the sum of mean plus twofold standard deviation. Nominal logistic regression was used to detect effect of time, group and time x group on prevalence of claw diseases, tested by Likelihood ratio tests. Differences in prevalence of claw diseases between groups and points of time were tested by Fisher's exact test. The level of significance was set at $P < 0.05$. Important findings were visualized with bar charts, scatter plot and box plots, also performed in JMP.Pro 15.

4 RESULTS

4.1 Feed intake

Individual feed intake of DM per day is shown in Figure 3. There was an effect of time ($P < 0.001$), diet ($P < 0.001$) and also an interaction of time*diet ($P < 0.001$). The HEP group had higher feed intakes than LEP. The mean feed intakes were 14.8 ± 1.1 kg DM/day (mean ± SD) of HEP and 9.2 ± 1.1 kg DM/day of LEP bulls ($P < 0.001$). Both feeding groups had increased feed intakes in the second month of the feeding trial, compared to the first month. The HEP bulls had the greatest feed intake from 15 to 17 month of life. Afterwards the feed intake decreased continuously until the end of the feeding trial. Bulls of LEP group had similar feed intakes from the second month until the end of the feeding trial. The ME intake was 169 ± 13 MJ/day and 93 ± 11 MJ/day, total starch intake was 4.4 ± 0.3 kg/d and 1.1 ± 0.1 kg/d, CP intake was 2.3 ± 0.2 kg/day and 1.0 ± 0.1 kg/day of HEP and LEP bulls, respectively (calculated means ± SD, $P < 0.001$).

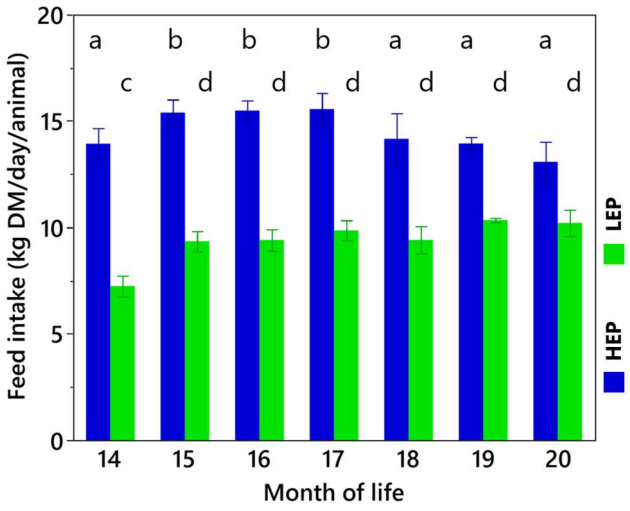

Figure 3. Calculated individual mean feed intake of dry matter (DM) per day (mean ± SD), of bulls fed a high energy and protein diet (HEP) or a low energy and protein (LEP). Different letters indicate significance with at least P < 0.05, n = 15. Data have already been published in a different form in BÄßLER et al. (2021).

4.2 Body weight

The BW and ADG were influenced by time ($P < 0.001$), diet ($P < 0.001$) and also showed an interaction of time*diet (BW $P < 0.01$; ADG $P < 0.001$). The results are shown in Figure 4. Experimental feeding led to different body weights between the feeding groups from 15 month of life until the end of the trial (Figure 4 a). The HEP bulls had higher final BWs than LEP, 807

± 9.4 kg and 712 ± 11.5 kg respectively (P < 0.001). Mean of ADG was higher in HEP than in LEP, 1474 ± 465 g/d versus 957 ± 542 g/d (P < 0.001). The greatest ADG of HEP bulls was 1883 ± 353 g/d at the age of 15 months and 1573 ± 372 g/d of LEP bulls at the age of 19 months (Figure 4 b). The mean of ADG was similar at the end of the feeding trial, it differed no longer significantly in the last three months of the trial (Figure 4 b).

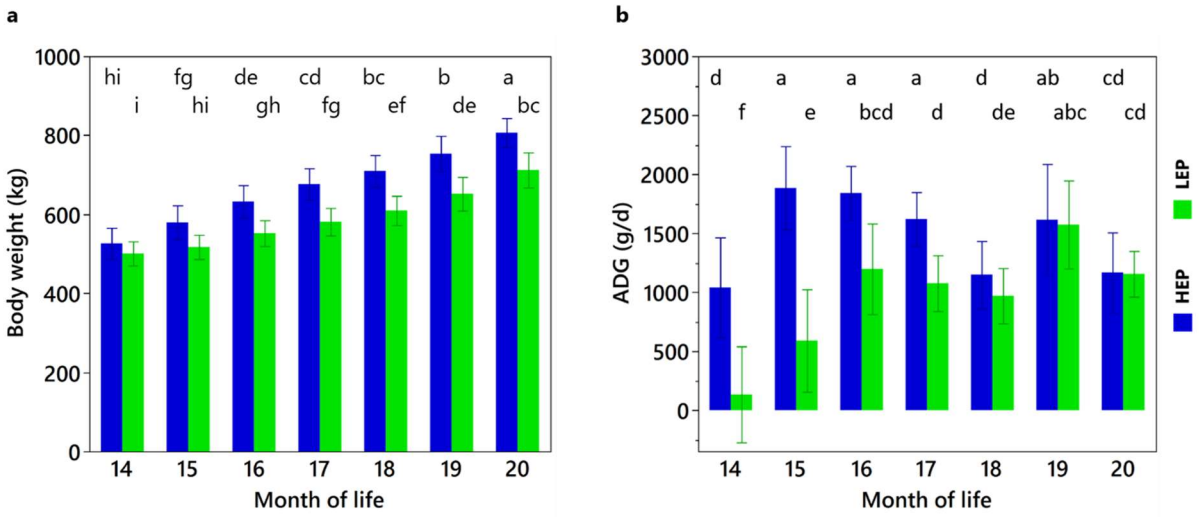

Figure 4. Body weight (mean ± SD) of bulls fed a high energy and protein diet (HEP) or a low energy and protein diet (LEP). Different letters indicate significance with at least P < 0.05, n = 15. Data have already been published in a different form in BÄßLER et al. (2021).

4.3 Conformation class and fat class

Experimental feeding had an effect on conformation and fat class at slaughter and is presented in Figure 5 a. Animals of HEP got higher scores for fat class than animals of LEP (P < 0.001). Mean of fat class score of HEP was 3.8 ± 0.9, for LEP 2.5 ± 0.4 (mean ± SD). All animals were scored in the two lowest categories of conformation class, O and P (Figure 5 b). Mean score of HEP was higher than of LEP (P < 0.05), 1.7 ± 0.5 and LEP 1.2 ± 0.4, respectively.

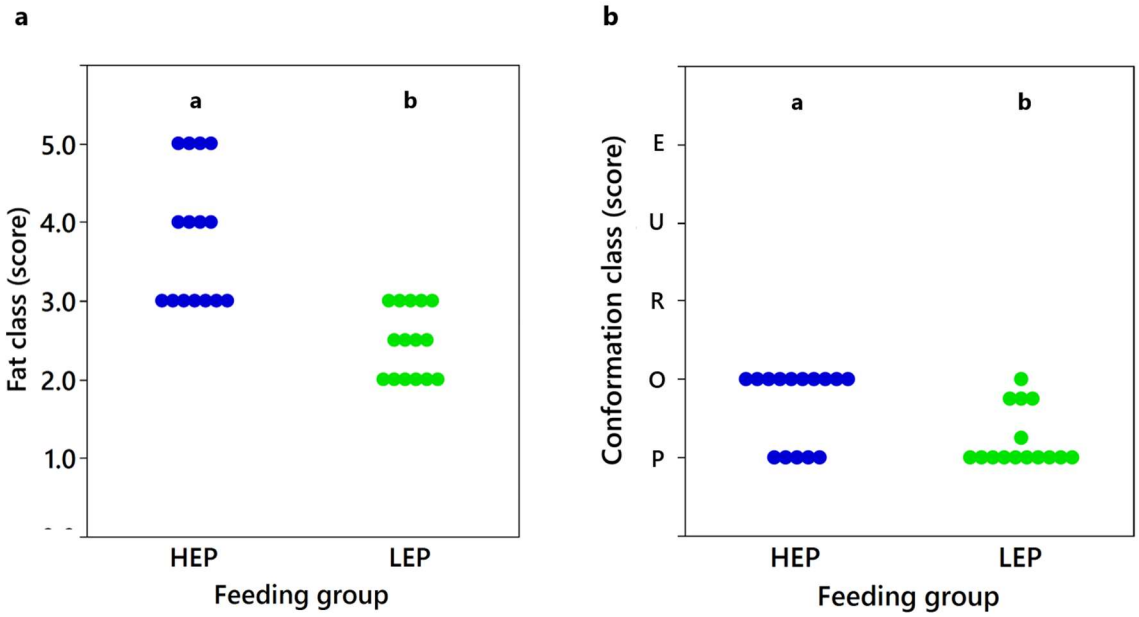

Figure 5. Score of a) fat class and b) conformation class at slaughter, of bulls fed a high energy and protein diet (HEP) or a low energy and protein diet (LEP). Different letters indicate significance with at least $P < 0.05$, $n = 15$. Data have already been published in a different form in BÄßLER et al. (2021).

4.4 Health of the claws

4.4.1 Conformation of the claws

Conformation of the claws differed between baseline and slaughter, the results are shown in Table 8. The length of the dorsal claw wall was mainly influenced by diet and a time*diet interaction was shown, whereas thickness of sole horn was only affected by time. Regarding the relation of inner and outer digit, demonstrated in Figure 6, scoring of the claws revealed an effect of diet at the claws of the hind legs only. Front legs had equal heights of the digits at baseline and also at slaughter in most of the bulls in both feeding groups. There was no effect of time, diet or time*diet interaction. Digits of the hind legs of LEP at slaughter were equal high or had higher inner digits. In contrast, most animals of HEP had higher outer digits at slaughter. Relation of height of the digits at the hind legs was influenced by an effect of time, diet and also a time*diet interaction.

Table 8. Length of the dorsal claw wall and thickness of the sole horn at the beginning of the trial (baseline, 13 months of age) and at slaughter (20 months of age).

Time point	Baseline		Slaughter		Effect summary P values		
Dietary treatment group	HEP	LEP	HEP	LEP	Time	Diet	T*D[3]
Length of the digit cranial medial[1]	8.1 ± 0.7b	8.3 ± 0.7ab	8.9 ± 0.9a	7.7 ± 0.6b	0.650	< 0.05	< 0.001
Length of the digit cranial lateral[1]	8.2 ± 0.6ab	8.2 ± 0.5ab	8.7 ± 0.7a	7.8 ± 0.8b	0.922	< 0.01	< 0.05
Length of the digit caudal medial[1]	8.3 ± 0.6ab	8.3 ± 0.5ab	8.6 ± 1.0a	7.7 ± 0.7b	0.340	< 0.05	< 0.05
Length of the digit caudal lateral[1]	7.9 ± 0.5b	8.0 ± 0.6ab	8.5 ± 0.8a	7.5 ± 0.6b	0.801	< 0.01	< 0.001
Thickness of the sole horn[1 2]	0.7 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.2	< 0.05	0.611	0.279

[1]Mean of all digits, Mean ± SD, n = 15; [a-c] levels not connected by the same letter are significantly different (P < 0.05), cm; data have already been published in BÄßLER et al. (2021).
[2]Mean of all digits
[3]time*diet interaction (T*D)

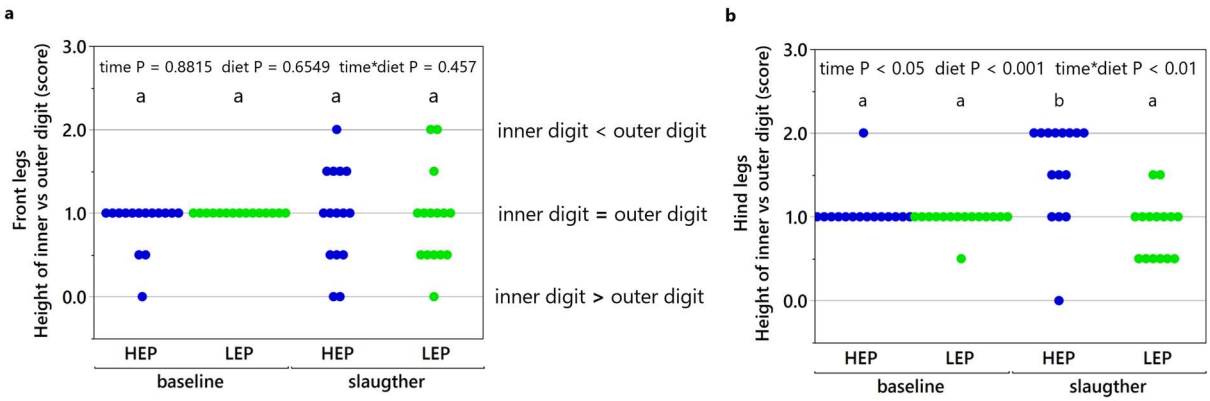

Figure 6. Relation of height of inner and outer digit at a) front and b) hind legs of bulls fed a high energy and protein diet (HEP) or a low energy and protein diet (LEP), at the beginning (baseline) and the end (slaughter) of the feeding trial. Different letters indicate significance with at least P < 0.05, n = 15, score 0: inner digit > outer digit, score 1: inner digit = outer digit, score 3: inner digit < outer digit.

4.4.2 Claw diseases

The occurrence of claw diseases at beginning and end of the feeding trial is presented in Table 9. Dermatitis digitalis and tyloma were never observed during the feeding trial, sole ulcer at typical and atypical location did not occur at slaughter or occurred less frequently than at baseline. Dermatitis interdigitalis was only observed in mild forms in some animals at slaughter. Heel horn erosions were observed in all animals at slaughter, thus the occurrence increased over time.

Table 9. Claw diseases at baseline (13 months of age) and at slaughter (20 months of age).

	Baseline		Slaughter		Effect summary P values		
Dietary treatment group	HEP	LEP	HEP	LEP	Time	Diet	T*D[2]
Sole ulcer, atypical[1]	33[a]	40[a]	0[b]	0[b]	< 0.001	1.000	1.000
Sole ulcer, typical[1]	47[a]	60[a]	7[b]	0[b]	< 0.001	0.347	0.175
Laminitis[1]	40[a]	13[ab]	100[c]	0[b]	1.000	< 0.001	< 0.001
Heel horn erosion[1]	67[a]	60[a]	100[b]	100[b]	< 0.001	1.000	1.000
Dermatitis interdigitalis[1]	0[a]	0[a]	27[ab]	47[b]	< 0.001	1.000	1.000
Dermatitis digitalis[1]	0	0	0	0			
Tyloma[1]	0	0	0	0			
Interdigital phlegmon[1]	0	0	0	0			

[a-c] levels not connected by the same letter are significantly different (P < 0.05). Data have already been published in BÄßLER et al. (2021).

[1]Affected animals per group %, n = 15

[2]time*diet interaction (T*D)

Effects of diet and a time*diet interaction were observed in the occurrence of laminitis. All animals of the HEP group had signs of laminitis at slaughter, none of the LEP animals. Score points representing severity of laminitis are presented in Figure 7. Severity of laminitis increased over time in HEP, but overall intensity was low. The most affected animals had 32 score points of possible 72 per animal.

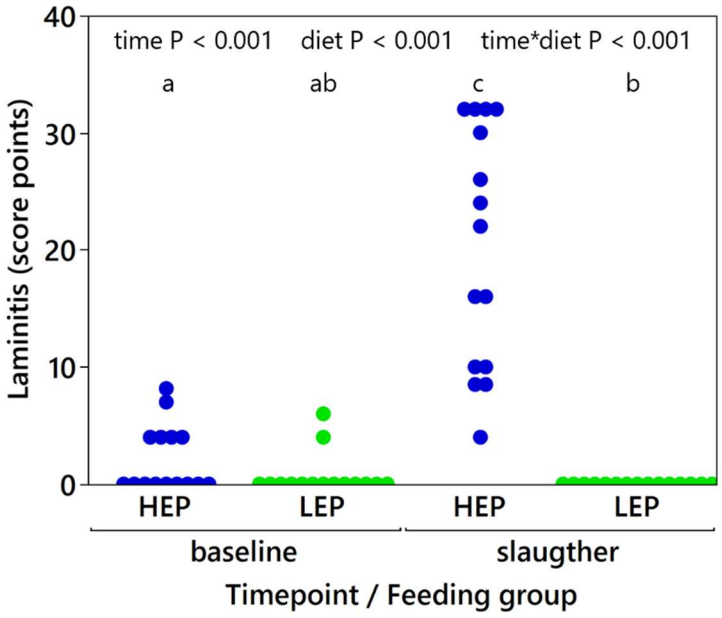

Figure 7. Severity score of laminitis of bulls fed a high energy and protein diet (HEP) or a low energy and protein diet (LEP). Different letters indicate significance, P < 0.05, n = 15. Data have already been published in a different form in BÄßLER et al. (2021).

4.5 Insulin concentration in blood

Serum insulin concentrations at baseline and slaughter are presented in Figure 8. They were influenced by the diet and an interaction of time*diet. There was no difference between the feeding groups at baseline, but at slaughter HEP bulls had two times greater insulin concentrations than LEP. Glucose concentration in blood at slaughter did not differ between the feeding groups; the mean concentration was 4.21 ± 0.84 and 4.18 ± 0.26, respectively (mean ± SD P = 0.7935). Calculated glucose-to-insulin ratio at slaughter was consequently lower in HEP bulls, 1.22 ± 0.08 mmol/10-3 g, 2.95 ± 0.31 mmol/10-3 g respectively (mean ± SEM; P < 0.001).

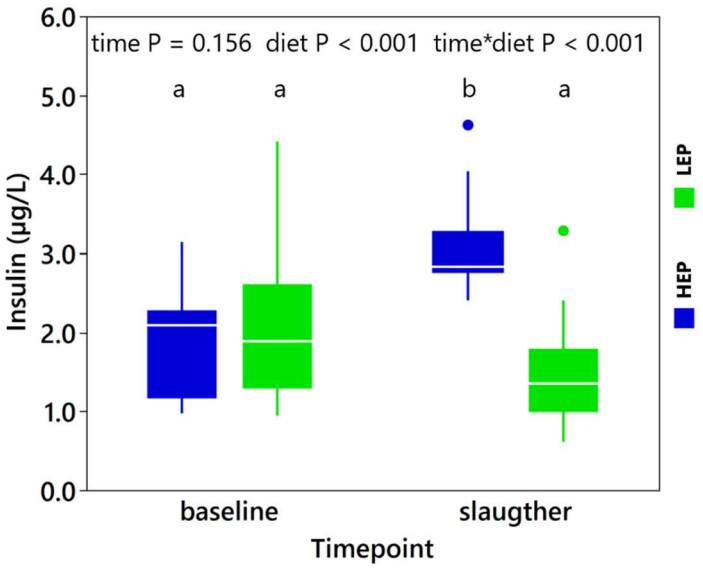

Figure 8. Insulin concentration at the beginning of the feeding trial (baseline) and at slaughter, of bulls fed a high energy and protein (HEP) diet and a low energy and protein (LEP) diet. Outlier Box Plot with interquartile range, n = 15, different letters indicate significance (P < 0.05). Data have already been published in a different form in BÄßLER et al. (2021).

4.6 Metabolite profile in blood

Blood biochemistry showed a diet effect only in five analyzed parameters. Concentrations of urea, BHBA, phosphate and activity of GLDH were greater in HEP bulls. Concentration of Crea was lower in HEP compared to LEP. The results of blood biochemistry are presented in Table 10.

Table 10. Blood biochemistry of bulls fed high energy and protein diet (HEP) and low energy and protein diet (LEP) at slaughter.

	HEP[1]	LEP[1]	P value
Glucose[2]	4.1 ± 0.2	4.2 ± 0.1	0.7935
L-Laktate[2]	1.5 ± 0.3	2.2 ± 0.5	0.2446
Urea[2]	5.0 ± 0.2	2.8 ± 0.2	< 0.001
Creatinine[3]	73.5 ± 2.2	86.9 ± 2.2	< 0.001
Total protein[4]	69.9 ± 3.1	68.8 ± 0.4	0.2007
Albumin[4]	39.8 ± 0.4	39.3 ± 0.4	0.3331
AST[5]	86.8 ± 3.1	83.1 ± 2.1	0.3281

	HEP[1]	LEP[1]	P value
GGT[5]	16.4 ± 1.4	14.5 ± 1.0	0.2695
GLDH[5]	16.1 ± 2.3	10.1 ± 0.9	< 0.05
CK[5]	230.8 ± 50.2	176.6 ± 7.5	0.2951
PHOS[2]	2.3 ± 0.0	2.2 ± 0.0	< 0.05
BHBA[2]	0.5 ± 0.0	0.4 ± 0.0	< 0.05
NEFA[2]	0.1 ± 0.0	0.1 ± 0.0	0.9046

[1] mean ± SEM, n = 15; [2] mmol/L; [3] µmol/L; [4] g/L; [5] U/L

Data have already been published in a different form in BÄßLER et al. (2021). Aspartate-aminotransferase (AST), γ-glutamyltransferase (GGT), glutamate-dehydrogenase (GLDH), creatine kinase (CK), phosphorus (Phos), β-hydroxybutyrate (BHBA), non-esterified fatty acids (NEFA).

Figure 9 presents all significantly different metabolites between feeding groups in the blood, clustered by Euclidean distance analysis and visualized by a heatmap. The samples were ordered according to their laminitis scoring; by grouping HEP bulls with the highest, intermedium and lowest laminitis scoring, in order to identify laminitis associated patterns. Every column represents the metabolite profile of one animal; a row represents relative concentration of one metabolite. Metabolite profiles in blood were strongly influenced by the diet, 91 of 188 detectable metabolites were significantly different between the feeding groups. A complete list of the concentrations of all analyzed metabolites is given in the appendix (Chapter 10.3). Feeding groups expressed a specific metabolite profile with high inter-individual variation. Especially the class of glycerophospholipids showed a very homogenous pattern. Three lysoPCs and PC aa C34:2 were higher concentrated in HEP, 59 diacyl (aa) and acyl-alkyl (ae) PCs of different length were all lower concentrated in HEP (Figure 9). Also, concentrations of seven SMs and five short-chain ACs were lower in HEP, only dodecenoylcarnitine (C12:1) was greater in HEP. The BCAAs, Leu, Val and Ile; as well as aromatic amino acids (AAA) phenylalanine, tyrosine and tryptophan; and Arg, asparagine, lysine, threonine and the non-proteinogenic amino acid ornithine were higher concentrated in HEP. Three amino acid derivates, namely Crea, trans-4-hydroxyprolin and acetyl-ornithine, were lower concentrated in HEP bulls. Comparing metabolite profiles within HEP, clustering by laminitis score did not reveal specific patterns of metabolites correlating with laminitis score.

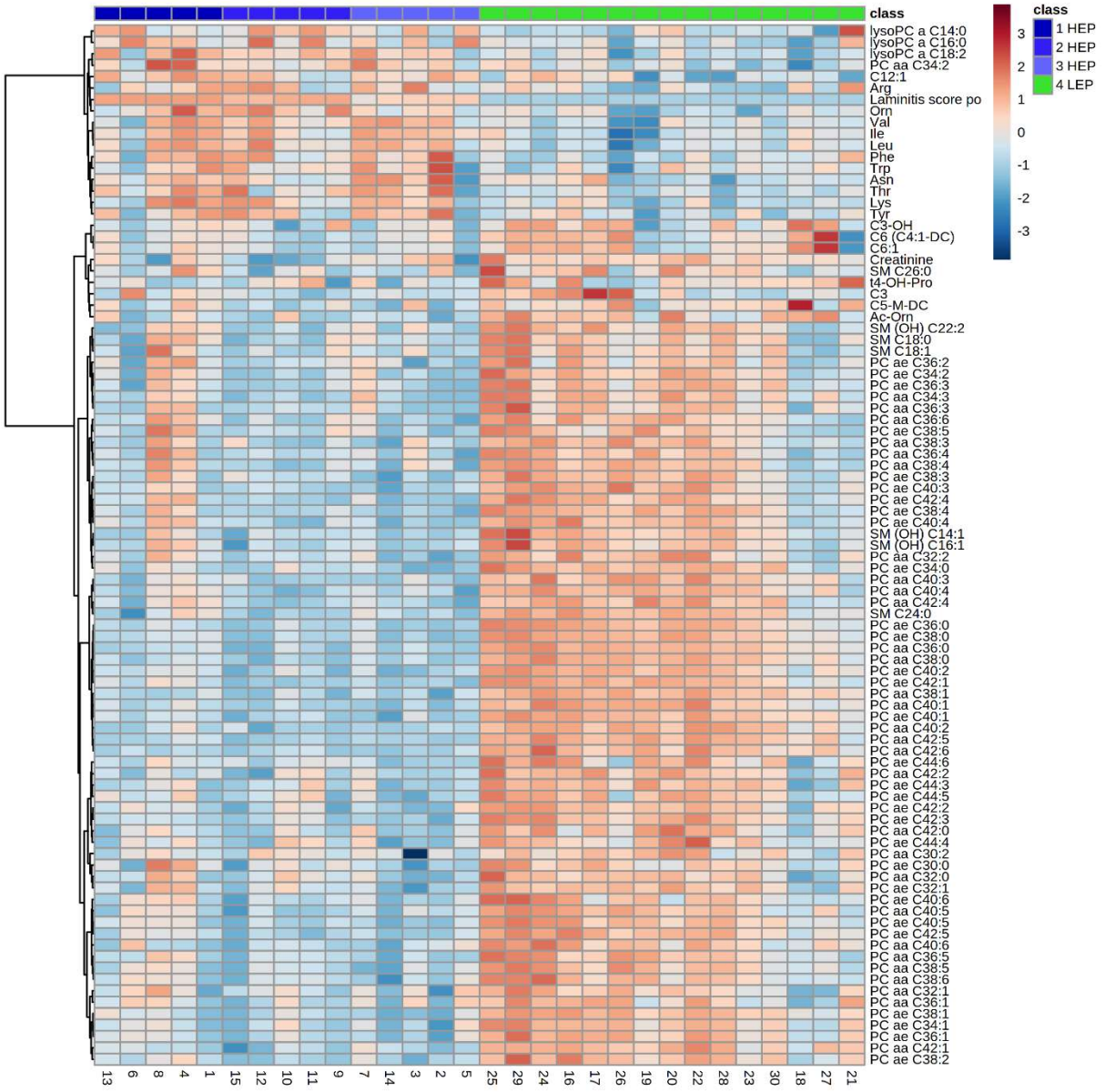

Figure 9. Heatmap visualizing all differentiating metabolites at slaughter of bulls fed a high energy and protein diet (HEP) and a low energy and protein diet (LEP). Samples were ordered by laminitis scoring, highest laminitis scoring (1 HEP), intermedium laminitis scoring (2 HEP), lowest laminitis scoring within HEP (3 HEP). All listed metabolites were significantly different between the feeding groups, P < 0.05, n = 15, acylcarnitine (C x), diacyl (aa), acyl-akyl (ae), phosphatidylcholine (PC), sphingomyelin (SM), arginine (Arg), acetyl (Ac), ornithine (Orn), valine (Val), isoleucine (Ile), leucine (Leu), phenylalanine (Phe), tryptophan (Trp), asparagine (Asn), threonine (Thr), lysine (Lys), tyrosine (Tyr). Data have already been published in a different form in BÄßLER et al. (2021).

Linear regression showed positive correlation of sum of BCAAs and insulin, presented in Figure 10 a. There was no correlation between BCAAs and sum of AC C3 + C5 among both groups. However, when one animal in the HEP group with much higher AC concentrations than the group mean (higher than the sum of mean plus twofold standard deviation) was excluded, a positive correlation was shown between BCAAs and AC C3 + C5 in HEP group. Details are

shown in Figure 10 b. Furthermore, mean ADG during the feeding trial was positive correlated with the concentrations of urea and lyso-PC a C18:2, demonstrated in Figure 10 c.

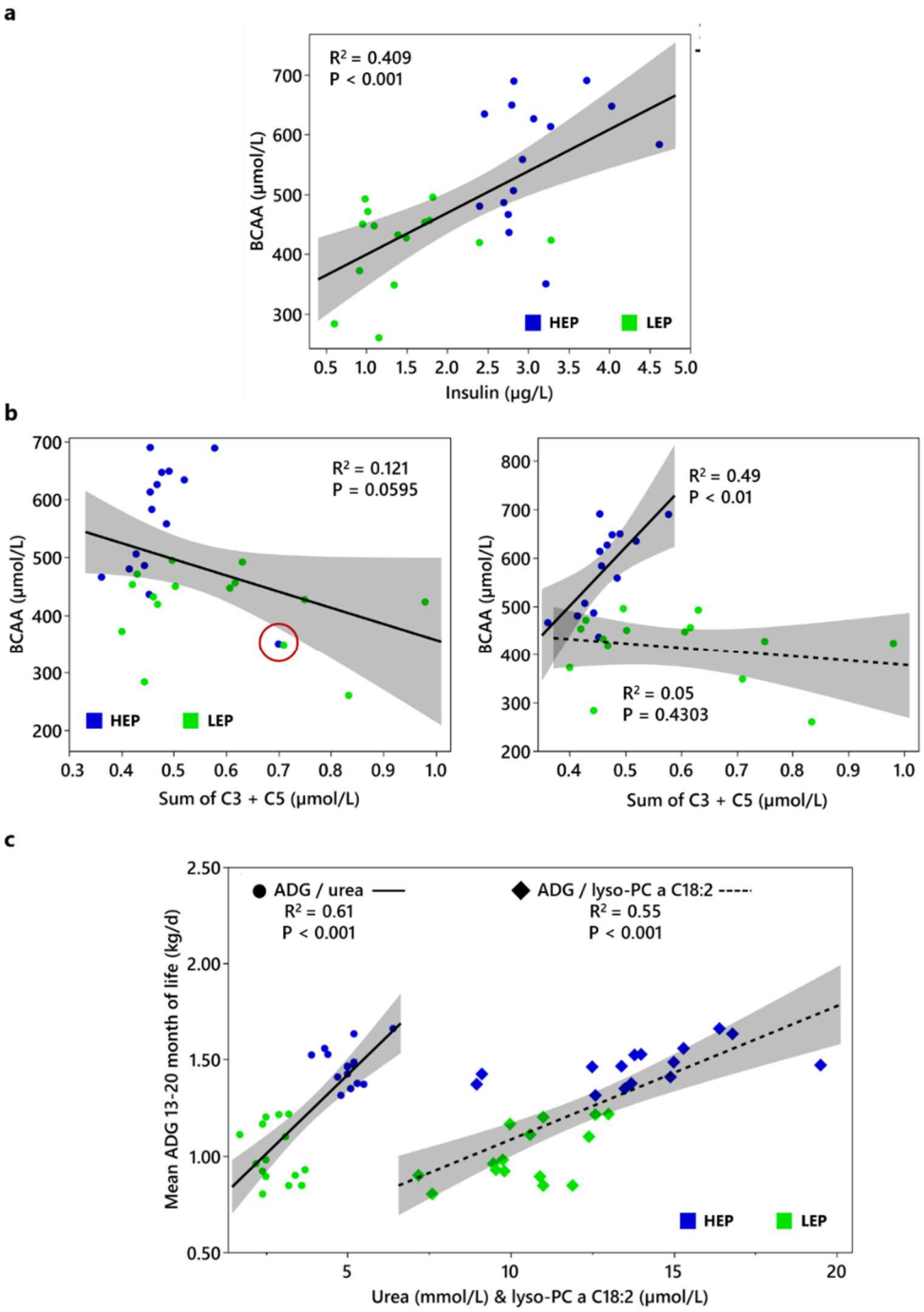

Figure 10. Correlation analysis by linear regression of a) BCAAs and insulin concentration b) BCAAs and sum of short chain acylcarnitines C3 and C5 (n = 14) and c) mean average daily gain (ADG) during trial and urea, and lyso-phosphatidylcholine (lysoPC) a C18:2, respectively. Bulls fed a high energy and protein diet (HEP), bulls fed low energy and protein diet (LEP), n = 15.

4.7 Insulin signaling cascade

Components of the insulin signaling cascade were semi-quantified by Western Blot analysis. The extent of phosphorylation of InsR could not be detected, in any tissue of both feeding groups. In order to verify the used antibody, samples of cows and horses were applied next to a pool sample of the bulls. All samples were prepared in the same way. The functionality of the used antibody was not reduced. Figure 11 shows the image of protein bands identified by the antibody against p InsR.

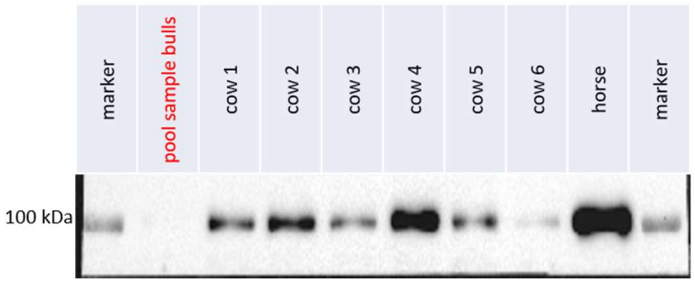

Figure 11. Image of protein bands of a western blot of liver samples from fattening bulls, dairy cows and a horse, identified with antibodies against phosphorylated insulin receptor. Bands were detected at the same level as the 100 kDa band of the protein marker, which was applied on the first and last position of the used membrane.

4.7.1 Liver

Insulin signaling cascade in the liver was strongly influenced by the diet. Expression of PKB, mTOR and AMPK was higher in HEP than in LEP animals. Extent of phosphorylation of mTOR was also higher in HEP, details are shown in Table 11. Correlations between proteins of the signaling cascade and all other described data is shown in Figure 12. Proteins within the cascade are positively correlated with each other; for example, extent of phosphorylation of mTOR is positively correlated with both, the amount of InsR and PKB. The combination of insulin and InsR results in an even stronger correlation to p-mTOR.

Table 11. Expression and extent of phosphorylation of key proteins of insulin signaling cascade in the liver of bulls fed a high energy and protein diet (HEP) and a low energy and protein diet (LEP).

Liver[1]	HEP	LEP	P value
InsR	2.4 ± 2.0	2.1 ± 1.1	0.5693
PKB	8.4 ± 3.2	5.2 ± 1.2	< 0.01
p-PKB	4.2 ± 1.3	4.4 ± 2.2	0.7906
mTOR	3.3 ± 1.5	1.8 ± 1.2	< 0.01
p-mTOR	8.5 ± 7.3	3.7 ± 2.6	< 0.05
AMPK	2.0 ± 0.7	1.4 ± 0.5	< 0.01
p-AMPK	2.2 ± 3.0	0.6 ± 1.1	0.0592

[1] arbitrary unit, means ± SEM, n = 15, insulin receptor (InsR), protein kinase B (PKB), mechanistic target of rapamycin (mTOR), AMP-kinase (AMPK), phosphorylated (p).

Furthermore, the protein expressions and extents of phosphorylation of proteins in the liver were positively correlated with plasma AAs and urea, for example mTOR with urea and p mTOR with Leu. Negative correlations included the expression of InsR L and lyso PC a C18:1.

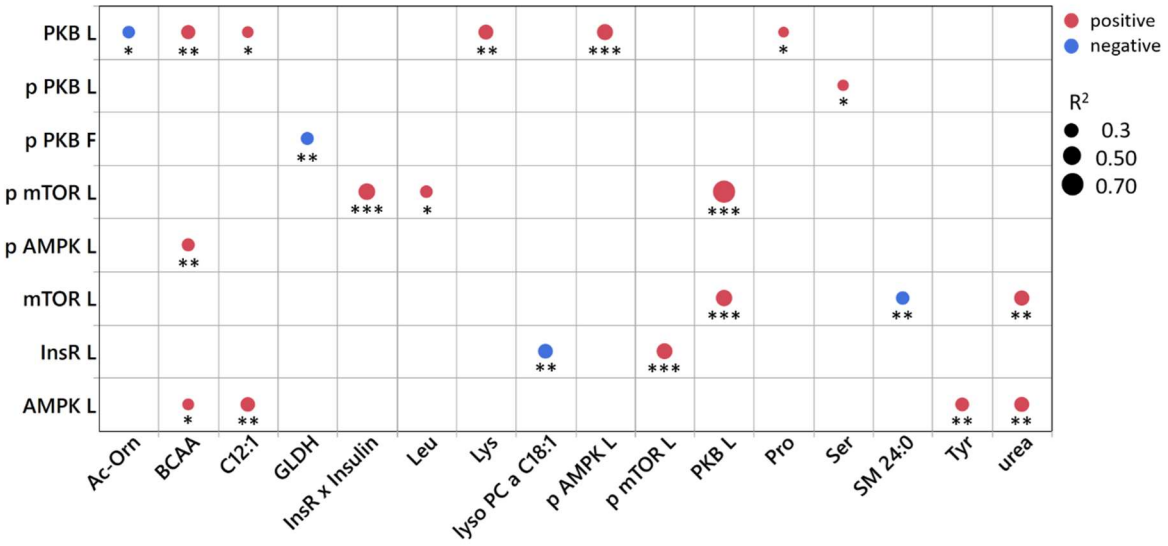

Figure 12. Overview of results of linear regression of items correlated with proteins of insulin signaling cascade in the liver in both feeding groups, n =15, *P < 0.05, **P < 0.01, ***P < 0.001. Protein kinase B (PKB), mechanistic target of rapamycin (mTOR), AMP activated-kinase (AMPK), insulin receptor (InsR), phosphorylated (p), liver tissue (L), fat tissue (F), acetylornithine (Ac-Orn), branched chain amino acids (BCAAs), dodecenoylcarnitine (C12:1), glutamate-dehydrogenase (GLDH), leucine (Leu), lysine (Lys), lyso-phosphatidylcholine (lyso PC), proline (Pro), serine (Ser), sphingomyelin (SM), tyrosine (Tyr).

4.7.2 Muscle

Differences between the feeding groups in the insulin signaling cascade of muscle were not significant (Table 12). There were great inter-individual variations, especially in the expression of InsR M in LEP and mTOR in HEP. The extent of phosphorylation of mTOR was not detectable in all muscle samples. The InsR in muscle was negative correlated with plasma insulin ($R^2 = 0.187$, $P < 0.05$), BHBA ($R^2 = 0.218$, $P < 0.01$) and lysoPC a C16:0 ($R^2 = 0.145$, $P < 0.05$).

Table 12. Expression and extent of phosphorylation of key proteins of insulin signaling cascade in the muscle of bulls fed a high energy and protein diet (HEP) and a low energy and protein diet (LEP).

Muscle[1]	HEP	LEP	P value
InsR	2.9 ± 3.9	10.8 ± 16.2	0.0782
PKB	10.3 ± 8.0	13.3 ± 11.2	0.4088
p-PKB	1.7 ± 0.94	2.0 ± 0.9	0.3529
mTOR	7.0 ± 12.2	3.5 ± 2.6	0.2819
p-mTOR	< LOD	< LOD	
AMPK	4.2 ± 2.4	4.5 ± 2.6	0.7199
p-AMPK	3.6 ± 4.4	9.1 ± 9.8	0.0581

[1] arbitrary unit, means ± SEM, n = 15, insulin receptor (InsR), protein kinase B (PKB), mechanistic target of rapamycin (mTOR), AMP-kinase (AMPK), phosphorylated (p).

4.7.3 Retroperitoneal adipose tissue

The results of expression and extent of phosphorylation of proteins in adipose tissue are presented in Table 13. Only the expression of InsR was significantly different between the feeding groups, LEP expressed more InsR than HEP in adipose tissue. There were negative correlations between InsR and inter alia BHBA, Arg, Val, insulin and four lysoPCs, details are presented in Figure 13.

Table 13. Expression and extent of phosphorylation of main proteins of insulin signaling cascade in the retroperitoneal adipose tissue of bulls fed a high energy and protein diet (HEP) and a low energy and protein diet (LEP).

Adipose tissue[1]	HEP	LEP	P value
InsR	5.6 ± 5.0	16.1 ± 9.3	< 0.001
PKB	1.5 ± 2.2	1.5 ± 1.2	0.92
p-PKB	9.0 ± 3.4	9.6 ± 3.3	0.5877
mTOR	1.3 ± 1.4	1.5 ± 1.5	0.6746
p-mTOR	1.4 ± 1.2	1.6 ± 1.4	0.6736
AMPK	1.1 ± 0.3	1.0 ± 0.3	0.2315
p-AMPK	5.6 ± 3.5	5.1 ± 3.4	0.6846

[1] arbitrary unit, means ± SEM, n = 15, insulin receptor (InsR), protein kinase B (PKB), mechanistic target of rapamycin (mTOR), AMP-kinase (AMPK), phosphorylated (p).

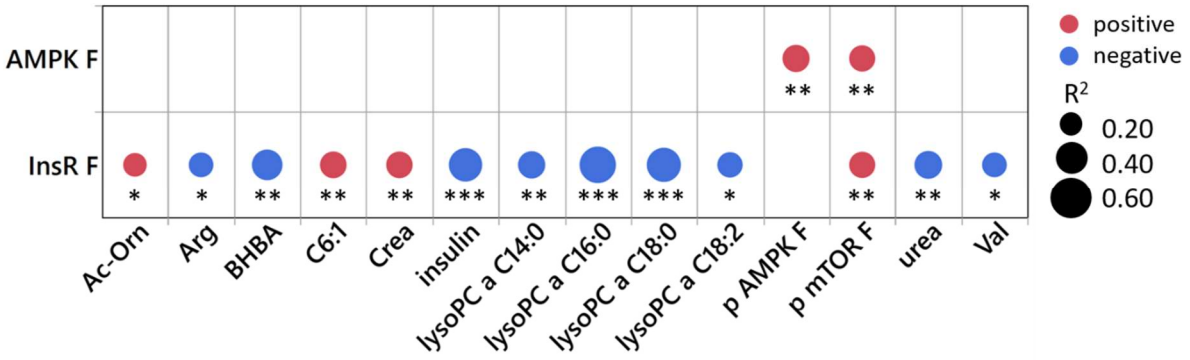

Figure 13. Overview of results of linear regression of items correlated with proteins of insulin signaling cascade in the retroperitoneal tissue, in both feeding groups, n =15, *P < 0.05, **P < 0.001, ***P < 0.001. Acetylornithine (Ac-Orn), arginine (Arg), β-hydroxybutyrate (BHBA), Hexenoylcarnitine (C 6:1), creatinine (Crea), lyso-phosphatidylcholine (lyso PC), phosphorylated (p), AMP activated kinase (AMPK), mechanistic target of rapamycin (mTOR), insulin receptor (InsR), fat tissue (F), valine (Val).

4.8 Correlations within the feeding groups

Some correlations were only seen in one feeding group, details per group are demonstrated in Figure 14. Especially birth weight was correlated negatively with concentration of insulin in both groups separately. The concentration of NEFA was correlated with the expression of mTOR in the liver in both groups; however, it was correlated positively in LEP and negatively in HEP. Only in LEP, expression of InsR in fat was negative correlated with lysoPC a C16:1.

Laminitis score of HEP correlated positively with albumin and expression of mTOR in the liver and negatively with AST, conformation class and p-mTOR in fat. The fat class correlated negatively with InsR in fat, p-PKB in the liver, PC aa C24:0, Met, C14:2-OH and C18:2.

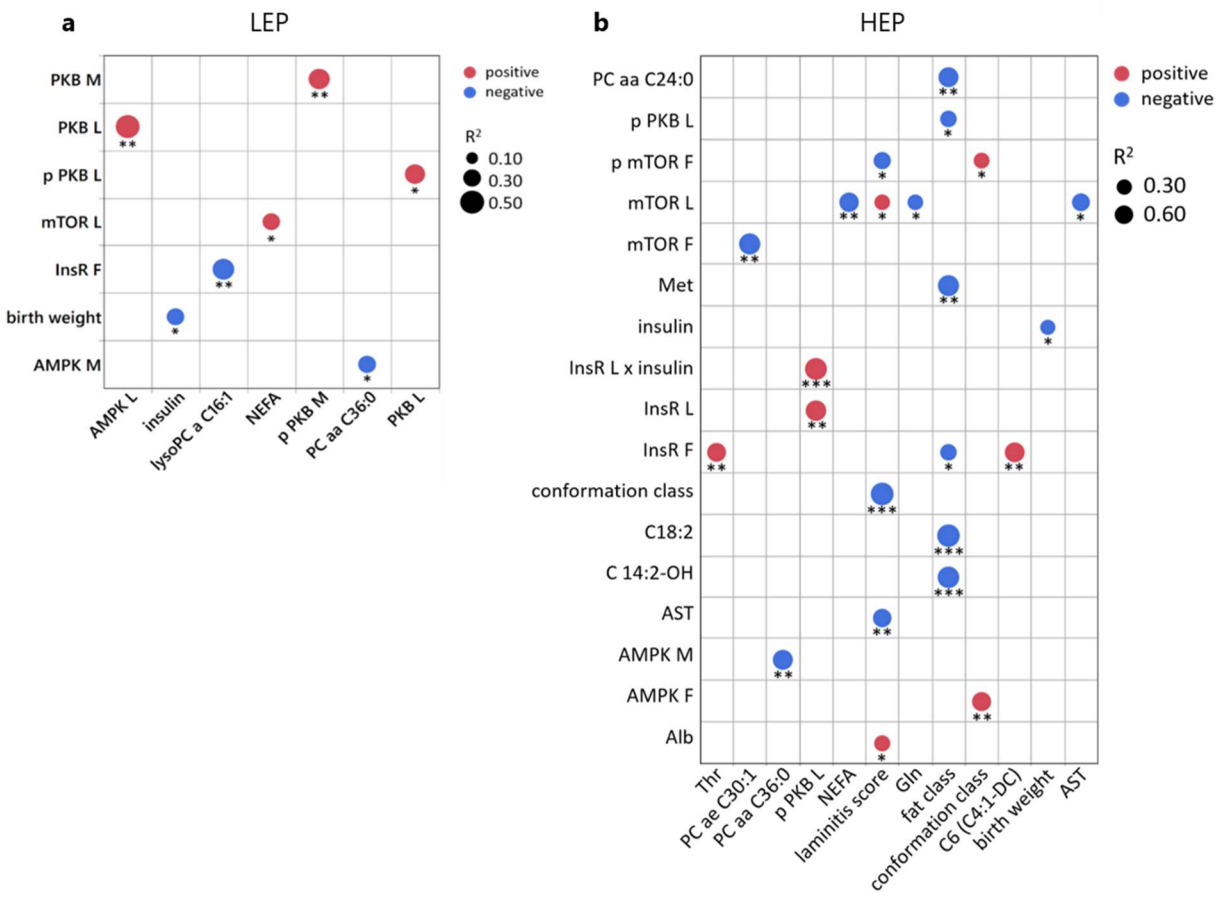

Figure 14. Overview of results of linear regression of items correlated with proteins of insulin signaling cascade a) in bulls fed a low energy and protein diet (LEP), b) in bulls fed a high energy and protein diet (HEP), n = 15, with exception of mTOR F – PC ae C30:1 n = 14; *P < 0.05, **P < 0.001, ***P < 0.001. Protein kinase B (PKB), mechanistic target of rapamycin (mTOR), AMP activated-kinase (AMPK), insulin receptor (InsR), phosphorylated (p), liver tissue (L), muscle tissue (M), fat tissue (F), phosphatidylcholine (PC), diacyl (aa), acyl-alkyl (ae), lyso-phosphatidylcholine (lysoPC), non-esterified fatty acids (NEFA), methionine (Met), octadecadienylcarnitine (C18:2), hydroxytetradecadienylcarnitine (C14:2-OH), aspartate-aminotransferase (AST), albumin (Alb), threonine (Thr), glutamine (Gln), hexanoylcarnitine-fumarylcarnitine (C6 (C4:1-DC)).

5 DISCUSSION

Results of this study support the hypothesis, that an excessive intake of starch- and protein-rich diet provokes alterations of metabolism, leading to a pro-inflammatory phenotype, clinically apparent as laminitis. It was demonstrated that such experimental feeding induced massive changes in conformation and health of the claws, the metabolite profiles and in the insulin signaling cascade.

5.1 Influence of diet on claw conformation and health

All HEP bulls had signs of chronic laminitis in varying degrees, apparent as elongated claws with ridges and concave dorsal claw wall, as well as discoloration of the sole horn. The comparison of this result with other studies is difficult due to different or missing information on the feed and its ingredients (see also Table 3). The HEP bulls in this trial received a diet containing 11.4 MJ ME (metabolizable energy) /kg DM, 328 g non-structural carbohydrates ((NSC) sugar and starch) /kg DM, and 155 g CP/kg DM. Trials that used pure oligofructose to induce acute laminitis (THOEFNER et al., 2004; DANSCHER et al., 2009; DANSCHER et al., 2010; SOUSA et al., 2020) are not comparable to the present study.

A comparable diet was used in the study of PILACHAI et al. (2019). In this trial, one group received a TMR with 328 g NSC/kg DM and 151 g CP/kg DM. However, the trial lasted only 13 days, and the objective was to induce acute laminitis. None of the animals of the current study showed acute laminitis. Observation of chronic laminitis in 92 % of 65 animals fed 50 % concentrate of DM with 13.5-15.5 % protein (of DM) over an extended period of time confirm the results found in the present trial (GREENOUGH & GACEK, 1987). Also a subsequent feeding trial of GREENOUGH et al. (1990) had similarities to the present study. Fattening bulls from 13.5 months of life to about 17 months of life were investigated and 72 % of 74 animals showed ridges of the dorsal claw wall at the end of experimental time.

Thus, in the present study the occurrence of laminitis has been expected, but it was surprising that all HEP bulls were affected. Possible reasons for the clear and homogeneous pattern of the results could be the following. The present study focused on the long-term effects of intensive feeding on the metabolism and health of the animals. Experimental feeding was conducted over a long period of time (seven months) and the animals were already quite old with an average age of 20 months, compared to slaughter recommended in practice, which are for Holstein bulls at about 15 months of life (GEUDER et al., 2012). Retrospectively, a more frequent

documentation of claw health would have been very interesting, to document the onset and progression of laminitis. The diets were conducted to promote high differences between the feeding groups. The rations were therefore oriented toward the upper (HEP) and lower (LEP) limits of the guidelines of the Bavarian State Research Center for Agriculture (Bayerische Landesanstalt für Landwirtschaft (LfL), 2014). However, these recommendations were for fattening of Simmental bulls, but there were no corresponding recommendations for Holstein bulls. The protein supply was above, energy intake of HEP bulls was within the recommendations. The energy and protein intake of the LEP bulls was below the recommendations mentioned. The obtained effects of the experimental feeding were only considered as a whole, no differentiation of protein- or starch-rich feeding effects was possible. It should be mentioned that all animals of the study originated from the same farm and had a similar genetic background and experienced the same rearing management. This standardization, even before the feeding trial, may also have contributed to the mainly homogeneous results of the study.

Conformation of the claws changed during the feeding trial. Only observed in HEP, length of the dorsal claw wall at slaughter was longer in comparison to baseline and at the hind legs, the outer digit was more prominent than the inner digit. This was also observed in dairy cows fed a high protein diet and was associated with increased lameness of the animals (MANSON & LEAVER, 1988b). However, a feeding trial in a feedlot did not reveal an influence of protein content on claw health. Unfortunately, length of the dorsal claw wall was not measured in that trial (GREENOUGH et al., 1990).

The thickness of the sole was reduced over time in both groups, which were contrary results to GREENOUGH et al. (1990), who observed an increase of sole horn thickness with time and a reduction in sole horn thickness by increasing protein in the diet. But animals used in that study were younger (calves and yearlings), from a different breed (Charolais) and the experimental period was shorter.

Occurrence of dermatitis interdigitalis and heel horn erosion increased over time in both groups. Probably due to environmental conditions seen on slatted floor with rubber mats, like suboptimal cleanliness and humidity, the occurrence of infectious claw diseases was promoted (HOBLET & WEISS, 2001; MAGRIN et al., 2020a). The signs of laminitis were only mild, with maximal documented score of 32 (of possible 72 score points in worst case) and were not associated with a significant increased presence of sole ulcers in HEP, as it was observed in dairy cows affected by chronic laminitis (BRADLEY et al., 1989; BERGSTEN, 1994). The

mildness of the laminitis itself, as well as the low incidence of other laminitis-associated claw lesions, were probably due to the relatively soft ground conditions provided by the rubber mats protecting claws of further traumatic effects (BERGSTEN, 2003).

5.2 General response to feeding with potential metabolic consequences

The feed intake in HEP was significantly higher than in LEP, which on the one hand could be due to the higher concentrate content and thus better palatability and lower volume of TMR, and on the other hand, feed intake of LEP could also have been reduced by the increased fiber content in LEP diet. Digestion of long fibers result in a longer retention time in the rumen, in comparison to easily digestible carbohydrates like starch (NOZIÈRE et al., 2010). Feed intake was increased during the first months of the trial in both groups, possibly caused by adaption of the gastrointestinal tract to the new diet. In dairy cows, adaption to a new TMR takes about four to six weeks, including adaption of ruminal mucosa and microbiome (KLEEN et al., 2003). Feed intake of LEP remained on a similar level until the end of the feeding trial. In HEP, feed intake decreased at the age of 18 months until the end of the trial. This could be an indication that physiological mechanisms to control energy intake and weight gain were still effective. Similar results were observed in a trial with dairy cows, whose feed intake were also reduced at the end of the 15-week positive energy balance trial (DÄNICKE et al., 2014).

The HEP feeding resulted in higher gains and final weights, as well as higher muscle and fat classes. This is in line with other studies which compared different fattening intensities (NOGALSKI et al., 2014; LIU et al., 2020). However, mean ADG was equal at the last two months of the feeding trial, although feed intake, energy and nutrient intake was higher in HEP. Within HEP, fat class was negatively correlated with some long-chain ACs. This indicates that animals were in an anabolic state and that animals with a higher fat class degraded fewer fatty acids and therefore had less long-chain AC in the plasma. The association of increased energy and protein intake with increased gains was also reflected in the correlation of blood urea with mean daily weight gains during the experimental period. Assuming that the urea concentration was mainly influenced by protein intake in otherwise healthy animals (PRESTON et al., 1965; PREWITT et al., 1971), this correlation indirectly showed that the animals within the group had different feed intakes and thus influenced urea concentrations and gains. It would have been interesting to document individual feed intake, in order to compare it with these results.

Differences in the metabolic status were also demonstrated by correlations of concentration of NEFA in blood with expression of mTOR in the liver. The concentrations of NEFA were low

in both feeding groups with concentrations between 0.09 - 0.22 mmol/L. Interestingly; the correlation was negative in HEP, but positive in LEP. The mTOR complex connects nutrient signals and metabolic processes for cell growth (BEN-SAHRA & MANNING, 2017), therefore its expression could be associated with fine tuning of energy metabolism and potential for cell growth. In humans with DM2, turnover of NEFA is impaired and concentrations are increased (PEREIRA et al., 2016). Thus, a negative correlation of NEFA and mTOR in the liver in HEP animals could indicate that less insulin sensitive animals had higher NEFA concentrations and lower expressions of mTOR, due to a relative lack of energy and therefore higher need to generate energy substrates through lipolysis. In contrast, a positive correlation of NEFA and mTOR in the liver in LEP could indicate that bulls, which had a higher metabolic flexibility, used predominantly NEFA instead of glucose to generate energy and thereby were able to express more mTOR in the liver. Furthermore, these correlations could implicate that metabolism of LEP prioritized protein synthesis and not lipogenesis at slaughter, in contrast to HEP animals.

In total, the chronic increased energy and protein intake of HEP led to greater muscle fullness, but even more clearly to an adiposity of the animals. This could indicate that the 20 months old animals reached nearly the limit of their protein accretion capacity and excess energy has been used mainly to increase fat depots. Energy intake influences the time to achieve the genetically determined potential of muscle fullness, but not the ratio of body composition (WALDMAN et al., 1971; HONIG et al., 2020). As a dairy breed, Holstein bulls have a lower ability of muscle accretion and a higher preference for fat deposition in comparison to beef breeds (PFUHL et al., 2007). The bulls in this trial had almost reached the end of their growth potential and further body mass gain occurred through the accumulation of fat tissue (GEUDER et al., 2012). A shortened fattening period to compensate for this breed-related feature was not implemented, which possibly increased the negative effects of the HEP diet on the animals.

A change in body mass composition towards higher fat content is often associated with negative metabolic consequences. Summarized in various reviews, excess nutrient supply and obesity trigger mitochondrial dysfunction, oxidative stress and also membrane and ER stress, thereby promoting impaired insulin signaling and increased inflammatory cytokine production (ODEGAARD & CHAWLA, 2013). Obesity in humans is associated with increased inflammatory mediators in plasma (MONTEIRO & AZEVEDO, 2010), insulin resistance and DM2 (CARAYOL et al., 2017; DESPRÉS & LEMIEUX, 2006). Similar observations have been made in horses, where increased body condition score is a predisposition factor for insulin dysregulation and laminitis (TREIBER et al., 2006). In the case of cattle, studies mainly focused

on dairy cows. It was observed, that overweight cows in dry period had more metabolic disturbances to the onset of lactation than lean ones (RUKKWAMSUK et al., 1999; AMETAJ et al., 2005).

These associations are consistent with the results observed in HEP bulls. Blood biochemistry of HEP bulls showed first indications of a potentially tensed metabolic situation, indicated by increased concentrations of phosphate, urea, BHBA and activity of the enzyme GLDH. These alterations could indicate higher dietary intake of phosphate, protein and starch. High protein intake was associated with increased ammonia concentrations in the rumen (BUNTING et al., 1989) and a higher burden for the liver to bound excessive nitrogen during the urea cycle (MARINI & VAN AMBURGH, 2003). In Holstein cows fed a concentrate rich diet, with increasing intake of concentrate (including high protein components), ammonia concentration in rumen and urea concentration in blood increased (DÄNICKE et al., 2014). This assumption is further supported by increased concentrations of arginine and ornithine in plasma of HEP. These metabolites are also part of the urea cycle.

Concentrations of the most measured AA were higher in HEP (Figure 9), which could reflect the increased supply of these AA by feed and ruminal microbes. Starch is degraded by ruminal microbes, producing inter alia butyrate in the rumen, which could be metabolized to BHBA by the rumen epithelium (QUIGLEY et al., 1991). Especially, BHBA concentrations and activities of GLDH had high inter-individual variations within HEP. Increased activities of GLDH were associated with liver cell damage (WEST, 1977). Liver abscesses were often observed in feedlot cattle (NAGARAJA & CHENGAPPA, 1998), but were not observed during the routine meat inspection of these bulls. However, this macroscopic examination does not exclude liver cell damage. Increased concentrations of various potentially toxic metabolites, like methylamines and N-nitrosodimethylamines were observed in the rumen of dairy cows fed grain-rich diets (AMETAJ et al., 2010), which could have led to liver cell damage after intestinal absorption.

5.3 Metabolites reflecting a pro-inflammatory phenotype

A suspected inflammatory phenotype was further characterized with the AbsoluteIDQ p180 Kit (Biocrates, Innsbruck, Austria). Glycerophospholipids were found to be the metabolites with the greatest differences between the dietary groups (Figure 9). The concentrations of nearly all measured PCs (59 of 76 PC) were lower in HEP. This distinct result underlined the strong differences in metabolism of the feeding groups. Metabolomic studies often revealed alterations

in the class of PCs. However, results were commonly difficult to explain from a biological point of view, because knowledge about the specific functions of individual PCs is incomplete.

These lipids are main components of mammalian cell membranes, influencing fluidity of membranes and cell signaling (TREEDE et al., 2007; VAN DER VEEN et al., 2017). Various signaling molecules are generated from different PCs, like diacylglycerol, lyso-PCs, phosphatidic acid and arachidonic acid (CUI & HOUWELING, 2002). As an example, from PC aa/ae 40:4 and chain length above, arachidonic acid (C20:4) and palmitate (C16:0) are generated. Alterations of PCs were observed in diseases like DM2 (SUHRE et al., 2010) and obesity (CARAYOL et al., 2017). Cell culture studies revealed anti-inflammatory (TREEDE et al., 2007) and protective effects of PCs on ethanol-injured hepatocytes by inhibition of lipid peroxidation (ZHANG et al., 2014). In dairy cows around parturition, PC concentrations in blood dramatically decreased and slowly increased again over weeks of early lactation (IMHASLY et al., 2015; KENÉZ et al., 2016). Therefore, decreased blood concentrations of PCs could reflect consumption of PCs, due to increased cell damage and oxidative stress, increasing the need of cell and tissue remodelling. The other way around, increased concentrations observed in LEP could reflect a less tensed metabolic situation and a higher ability to resist metabolic stress.

Phospholipase A2 hydrolyses PCs to arachidonic acids and lyso-PCs. This pathway is activated by inflammatory signals (KABAROWSKIA et al., 2002). Three lyso-PCs showed higher concentrations in HEP (lyso-PC acyl C14:0, C16:0, C18:2). Decreased concentrations of PCs and increased concentrations of lyso-PCs appeared to be associated with a proinflammatory status. Increased concentrations of lyso-PC acyl C18:2 and lyso-PC acyl 14:0 were also observed as predictive biomarker for the occurrence of disease after calving, in comparison to healthy cows. Cows which had higher concentrations of these lyso-PCs four weeks before parturition were afterwards affected by mastitis, metritis, retained placenta, laminitis or even combinations of these diseases (HAILEMARIAM et al., 2014).

The class of SMs also refer to the phospholipids and are components of cell membranes. The enzyme sphingomyelinase degrades SMs to ceramides and then to other bioactive lipids, like sphingosine or ceramide-1-phosphate. This pathway is activated by inflammatory stimuli, like oxidative stress, LPS or TNF α. It further activates cytokine production and increases permeability of endothelium (MACEYKA & SPIEGEL, 2014; HANNUN & OBEID, 2008). Lower concentrations of SMs observed in HEP bulls were also found in dairy cows around parturition (KENÉZ et al., 2016; RICO et al., 2017), indicating high metabolic stress and the

need to repair tissue damage. The SMs play a role in research because of their connection to ceramides. Ceramides are known to have pathophysiological functions in inflammation and inhibit PKB, resulting in insulin resistance (MACEYKA & SPIEGEL, 2014; LARSEN & TENNAGELS, 2014; HOLLAND & SUMMERS, 2008). This was also shown in bovine adipocytes (RICO et al., 2018). These observations are a possible link between decreased SMs and impaired insulin sensitivity and inflammation. This is also supported by the observation of lower plasma concentrations of some SMs in insulin resistant veal calves (PANTOPHLET et al., 2017). Results were not as clear as in the present study, but the feeding trial only lasted for 13 weeks and the animals were much younger than the animals in this study.

5.4 Insulin insensitivity as special inflammatory feature in Holstein fattening bulls

The further results link the suspected pro-inflammatory phenotype with impaired insulin sensitivity in HEP bulls. However, the determination of whole-body insulin sensitivity in vivo, via hyperinsulinemic-euglycemic clamp test was not possible in this study. In this test, it is measured how much glucose is absorbed into the cell, induced by insulin, to evaluate changes in sensitivity and responsiveness of the tissue (ACHMADI et al., 1993; ANDERSEN et al., 2002; STERNBAUER & LUTHMAN, 2002). Instead, parameters associated with insulin sensitivity were analyzed. The concentration of insulin, certain metabolites and expressions and extents of phosphorylation of components of the insulin signaling cascade were used as indicators for insulin sensitivity. The physiological relationship of insulin to stimulate phosphorylation of these components has been shown in various studies; higher extents of phosphorylation of InsR, PKB, mTOR and AMPK were observed in tissues of healthy horses, by intravenously injected insulin (WARNKEN et al., 2017) and also in an ex vivo stimulation of tissues with insulin in dairy cows (KENÉZ et al., 2019).

The HEP bulls had twofold higher insulin concentrations than LEP bulls. ADAMIAK et al. (2005) defined hyperinsulinemia as >37.20 µIU/ml in dairy heifers. Mean concentration of insulin of HEP bulls was 63.59 µIU/ml, and of LEP bulls was 30.12 µIU/ml, indicating hyperinsulinemia in HEP. It should be noted, that different assays were used to determine insulin and comparability was not verified. Increased concentrations of insulin were also observed in other studies feeding a high level of nutrition in cattle (RÖPKE et al., 1994; LOCHER et al., 2015; PILACHAI et al., 2019) and in sheep (ACHMADI et al., 1993).

However, the glucose concentration in blood did not differ between the feeding groups. This could therefore be termed as a compensated metabolic status, indicating either peripheral

insulin insensitivity or dysregulation of pancreatic insulin secretion. The glucose-to-insulin ratio, as a well-known marker for disturbances in insulin-glucose homeostasis in humans (LEGRO et al., 1998; SILFEN et al., 2001), consequently indicate a decreased insulin sensitivity in HEP bulls. Reduced insulin sensitivity was also found in a model of positive energy balance in nonlactating, nonpregnant dairy cows (LOCHER et al., 2015) and in intensively fed veal calves (PANTOPHLET et al., 2017). Impaired insulin metabolism in horses was termed as insulin dysregulation and included hyperinsulinemia and insulin resistance of tissues (FRANK & TADROS, 2014).

The excessive energy intake of HEP resulted in a higher expression and phosphorylation of proteins of the insulin signaling cascade in the liver. It is assumed that the extent of phosphorylation reflects the activation of these proteins (SASAKI, 2002). Increased extent of phosphorylation of mTOR in the liver implied an increased activation of the cascade there. This was expected according to the function of insulin signaling pathway which should be more activated by higher energy and nutrient intake and consequently higher secretion of insulin and activation of the cascade (TRENKLE, 1970). This physiologically described relationship is also reflected in positive correlations in liver of p-mTOR and PKB, InsR, and insulin x InsR, respectively (see also Figure 1). The positive correlation of p-mTOR and Leu found in this study also points to an enhanced activation of mTOR by Leu described in the literature (WOLFSON et al., 2016). The expression of the proteins within the insulin signaling cascade were positively correlated with each other; including AMPK, which acts as a stress and energy deficit sensor in the cell (HARDIE, 2004). Thus the regulation of the expression of proteins within the cascade could be synchronized and co-regulated in these tissues.

In the liver, higher availability of AAs in the blood seems to be associated with higher expression and phosphorylation of the insulin signaling cascade. The expression of PKB in the liver correlated positively with the concentrations of BCAAs, proline, lysine and urea; indicating that high energy and nutrient supply allowed to express larger amounts of proteins. However, increased expression of PKB was also positively correlated with p-AMPK in the liver. This could indicate that an excess of AAs was associated with a (relative) energy deficiency or metabolic stress in liver cells of HEP bulls. Positive correlations of AAs with the expression of insulin signaling proteins were only observed in the liver, as central distribution organ. In the peripheral tissues muscle and fat, other correlations were found. In fat tissue arginine, Val and urea correlated negatively with the InsR, in the muscle no respective correlations were found.

In all three organs and both groups, p-InsR and thus assumed activation of InsR was not detectable, as well as mTOR in the muscle. This could be due to the advanced age and BW of the animals. HEP already reached 77% of maturity BW, based on average mature weight of 1044 kg (CALO et al., 1973) and the growth curve appeared to have reached an almost horizontal course, as the ADG did not differ anymore between the groups and decreased at the end of the experimental period. Other authors observed reduced daily gains in Holstein bulls from 14 months of age on (GEUDER et al., 2012). Insulin sensitivity decreased with age (PAGANO et al., 1981) and in addition, anabolic reactions shifted away from muscle growth to synthesis of adipose tissue (HONIG et al., 2020). This could result in lower activation of the cascade, thereby diminished especially protein synthesis in the muscle. Another possibility would be that the last feed intake before transport and subsequent slaughter was too long ago and therefore the cascade was not activated anymore. However, there were only a few hours in between and when the animals were slaughtered there was still plenty of rumen filling. The rumen is an intermediate feed store for several hours, so this argument has probably less relevance.

The low activation of the cascade in muscle, reflected by low extent of phosphorylation of the proteins, is particularly interesting in the HEP animals, as they did not achieve increased activation even with the higher insulin concentrations. Similar results were seen in adipose tissue, where the LEP bulls expressed more InsRs and achieved the same activation of the cascade as HEP, but with lower insulin concentrations in the blood. These results indicated that HEP had lower insulin sensitivity in the peripheral tissues than LEP. Furthermore, the expression of InsR in muscle was negative correlated with insulin concentration and this relationship was even stronger in adipose tissue. This could indicate that higher concentrations of insulin in plasma reduced the expression of InsR in peripheral tissue, as it was discussed as pathophysiological mechanism in human insulin resistance and DM2 (PAGANO et al., 1981; ZHOU et al., 2009). This hypothesis is supported by the observed negative correlation of InsR in the muscle and InsR in the fat with BHBA. The BHBA is formed during ketogenesis at starvation, and could indicate a relative lack of cellular energy due to reduced glucose uptake, as observed in DM2 (DEPCZYNSKI et al., 2019). Although there would be sufficient glucose available in the blood, the reduced insulin sensitivity of the tissues is responsible for insufficient glucose uptake into the cells. Similar effects were observed in nonpregnant, nonlactating Holstein cows fed a concentrate-rich ration for 15 weeks. The BHBA concentrations increased at the end (week 12) of the trial period and at the same point of time indicators of insulin resistance were the highest (LOCHER et al., 2015).

5.5 Correlative findings implicating associations with insulin insensitivity

Interestingly, the concentration of insulin correlated negatively with birth weight over all bulls of HEP and LEP. Although the measured insulin concentrations of HEP were higher than those of LEP, bulls with the highest insulin concentrations were in each case the lightest at birth. This phenomenon has also been widely observed in human medicine and it has been suspected that low birth weight follows reduced fetal growth due to deficiency or stress (NEWSOME et al., 2003). This could lead to an imprinting of the metabolism focused on energy deficiency, to save excess energy in stores. In the case of energy and nutrient oversupply in later life, metabolic disorders such as obesity, insulin resistance and DM2 are more likely to occur. This concept was summarized as “thrifty phenotype hypothesis” (HALES & BARKER, 2001). The inverse correlation of birth weight and insulin concentration was identified in both groups, indicating a relative nutrient over-supply in both groups in relation to their requirements at slaughter. This was also reflected in the documented fat classes at slaughter. As observational evidence, the musculature of LEP bulls was also covered almost everywhere with fat and showed some slight fat depositions in the thoracic cavity. Furthermore, within HEP documented fat class was negatively correlated with InsR F and p-PKB L, indicating that animals with higher insulin sensitivity had lower fat classes. The fat class also correlated negatively with the concentration of methionine (Met). However, reduced Met concentrations were associated with increased insulin sensitivity and weight loss in humans (OLSEN et al., 2020). In cattle, relationship appears to be different, possibly due to the purely plant-based diet. Cows fed a diet enriched with fat and ruminal protected Met had increased plasma Met concentrations and improved insulin sensitivity compared to animals fed the same diet without Met supplementation (FUKUMORI et al., 2015).

5.6 Metabolites indicating disturbances in insulin-glucose homeostasis

In humans, there are also other indicators of insulin resistance. Elevated concentrations of BCAAs and AAAs were found years before DM2 was diagnosed (AHOLA-OLLI et al., 2019; NEWGARD et al., 2009; WANG et al., 2011). Such a relationship has not been established in cattle, yet. In the present study, BCAAs and AAA concentrations were increased in HEP, and BCAAs concentrations correlated positively with blood insulin concentrations, confirming these associations also in fattening bulls. Furthermore, increased concentrations of BCAAs correlated positively with p-AMPK in the liver, indicating that increased concentrations of BCAAs in plasma were associated with metabolic stress in liver, for example relative energy deficiency, which was activating AMPK.

Pathophysiological mechanisms between increased concentrations of BCAAs and insulin resistance are still unclear. Discussed were increased dietary intake of BCAAs or obesity-related mechanisms resulting in reduced degradation and consequent accumulation of BCAAs and BCAAs related metabolites in the body (LYNCH & ADAMS, 2014). Furthermore, enhanced proteolysis due to insulin resistance should be conceived. All of these pathways could be possible in HEP. Although no exact amino acid profile was determined in the feed, the protein uptake was strongly increased, and also likely to increase the BCAAs intake. Additionally, bacterial amino acid production was improved by higher intake of easy digestible carbohydrates, in presence of sufficient nitrogen in the rumen. Another discussed possibility was, that transcriptional factors, which were activated to increase lipogenesis, reduced at the same time the expression of enzymes related to BCAAs degradation pathways (WHITE et al., 2018). High fat class scoring at slaughter documented forms of obesity. The HEP bulls had great visceral and subcutaneous adipose tissue depots. However, AC C3 and C5 were formed during the degradation of BCAAs (HARPER et al., 1984) and were positively correlated with body mass index in humans (CARAYOL et al., 2017) and obesity (NEWGARD et al., 2009). A positive correlation between BCAAs and the sum of C3 and C5 concentrations was also observed in HEP bulls. This correlation existed only within the HEP group, but not in LEP. Thus, the degradation of BCAAs was increased in HEP, possibly to counteract the excessive intake. In LEP, other metabolic pathways apparently led to increased levels of these AC independently of the BCAAs. In a study with rats fed a high fat and BCAAs diet, obesity and insulin resistance was observed and was linked to a chronic phosphorylation of mTOR and IRS1, resulting in uncoupling of insulin signaling pathway (NEWGARD et al., 2009). This could be a link between increased intake of BCAAs and development of insulin resistance.

The present data set revealed further connections between metabolite profile in blood and insulin signaling cascade in insulin sensitive tissues. It was noticed that some lysoPCs were associated with reduced insulin sensitivity. Especially InsR in the fat was negatively correlated with lysoPC a C14:0, C16:0, C18:0, C18:2 (Figure 13); as well as InsR in the muscle with lysoPC a C16:0 and InsR in the liver with lyso PC a C18:1. Currently, evidence about the relationship of the class of lysoPC and insulin resistance is rare. But results are in line with a study in veal calves diagnosed as insulin resistant; they showed increased concentrations of lysoPC a C18:2 in comparison to moderately insulin sensitive calves, based on results from an hyperinsulinemic euglycemic clamp test (PANTOPHLET et al., 2017). Results of human studies were inconsistent. In a study with adults, concentrations of lysoPC a C17:0, C18:1, C18:2, and C28:0 were negatively correlated with the body mass index (CARAYOL et al.,

2017). However, increased concentrations of lysoPC 14:0 have been suggested as a predictive biomarker for obesity in childhood in babies (RZEHAK et al., 2014). MOTLEY et al. (2002) discovered that lysoPCs inhibited the insulin-induced activation of PKB and IRS1 (in vascular smooth muscle cells), but did not have an influence on phosphorylation of InsR or binding of insulin. In rats with diet-induced insulin resistance, experimental feeding with polyphenolic compounds improved their insulin sensitivity and reduced the concentrations of various lysoPCs (RUBIO-RODRÍGUEZ et al., 2021). Furthermore, a receptor was discovered which enhanced glucose-dependent insulin secretion by lysoPCs in β-pancreatic mouse cells. In conclusion, there is evidence that lysoPCs do have an influence on insulin sensitivity, but mechanisms and interactions are still unclear in cattle.

In humans, a reduced insulin sensitivity was considered to be the initiating factor to develop DM2 (DEFRONZO & TRIPATHY, 2009). Physical activity or exercise reduced insulin resistance in humans (JELLEYMAN et al., 2015). Exercise could hypothetically also prevent or decrease insulin resistance in fattening bulls. On the one hand, this would result in reduced feed efficiency, due to the fact that energy would also be required for exercise. On the other hand, muscle activity could lead to an increase in muscle fullness through hypertrophic growth independent of the genetically determined number of muscle fibers. It remains questionable what type and intensity of physical activity would be an improvement. A trial with veal calves could not detect any positive effects on insulin sensitivity by physical activity. The exercise group in that study had to do light work on a treadmill, 5 to 15 min at 10 to 12 occasions during four to five weeks (STERNBAUER & ESSÉN-GUSTAVSSON, 2002). It was suspected that higher or more frequent physical activity could have beneficial effects. Further research on this topic would be appreciated, for example it would be interesting if pasturage or increased space offer would have a positive influence on insulin sensitivity and therefore increasing metabolic health of fattening bulls.

5.7 Insulin insensitivity and pro-inflammation led to laminitis in bulls

All alterations in the metabolite profiles of HEP bulls had in common that they were reflecting a tensed or even pathophysiological metabolic situations, which were associated with the occurrence of laminitis. But altered metabolites did not correlate with severity of laminitis. As bulls had chronic laminitis and changes of the claws only became visible after weeks (UWIERA et al., 2017), it is plausible that alterations of the current metabolite profiles did not correlate with the observed changes of the claws.

Correlations of laminitis score and parameters measured in the tissues or constitution of the animals, potentially better reflecting long term effects, revealed connections between liver metabolism and insulin insensitivity. HEP bulls with high laminitis score had also increased expressions of mTOR in the liver and concentrations of albumin in the blood. Increased concentrations of albumin were also observed in nonpregnant, nonlactating dairy cows in positive energy balance (DÄNICKE et al., 2014). High energy and nutrient supply could have activated metabolism of the liver, increasing the expression of mTOR and the production of albumin. However, this activation might also have led to higher production of certain proteins, which were not analyzed in this study, for example complement and coagulation factors. This possibly led to an imbalance, promoting a pro-inflammatory situation and resulted to a more severe degree of laminitis.

The enzyme aspartate-aminotransferase was negatively correlated with laminitis score. This correlation was not expected, because this enzyme is located in cytoplasm and mitochondria of cells of various organs and increased concentrations in the blood are caused by cell damage. However, this could indicate that animals of the HEP group had different focuses in their inflammatory events and animals with low laminitis scoring had probably more cell damage in other organs.

Additionally, laminitis score was negatively correlated with phosphorylation of mTOR in the fat and documented conformation class. This could indicate that increasing laminitis score is associated with an increasing pro-inflammatory status and insulin resistance. A possible explanation for reduced muscle mass is that bulls with high laminitis scores had a reduced activation of insulin signaling cascade in muscle for an extended period of time. Eventually, insulin resistance had even led to increased degradation of muscle tissue and consequently reduced muscle mass. Furthermore, these animals achieved lower activation of mTOR in the fat tissue, which supports the assumption of increased insulin resistance.

The chronic excessive nutrient intake likely disturbed metabolic pathways in different ways to such an extent that biological balances could no longer be maintained. Figure 15 summarizes how the described results might be related to laminitis. The excessive intake of easily digestible carbohydrates and protein could repeatedly cause inflammatory insults by an increase of LPS and toxic metabolites in the digestive tract (AMETAJ et al., 2010; LIU et al., 2020), promoting local inflammation in metabolic organs, summarized as diet-induced inflammation (KHIAOSA-ARD & ZEBELI, 2018). The anabolic effect of chronic high energy and nutrient intake over seven months caused excessive fat deposition, potentially causing obesity related

inflammation. Resulting in chronic low-grade inflammation and insulin resistance (ODEGAARD & CHAWLA, 2013). Furthermore, like in insulin dysregulated horses (FRANK & TADROS, 2014) it could be assumed, that bulls had an excessive insulin response to nutrient intake, resulting in hyperinsulinemia and insulin resistance in peripheral tissues. Hyperinsulinemia was identified to trigger laminitis in horses (ASPLIN et al., 2010; LAAT et al., 2010a; KARIKOSKI et al., 2015) and was also associated with laminitis in cattle (PILACHAI et al., 2019). It is hypothesized that the proinflammatory metabolic status and hyperinsulinemia caused impaired vascularization of the blood vessels of the claws and consequently, chronic laminitis in HEP bulls.

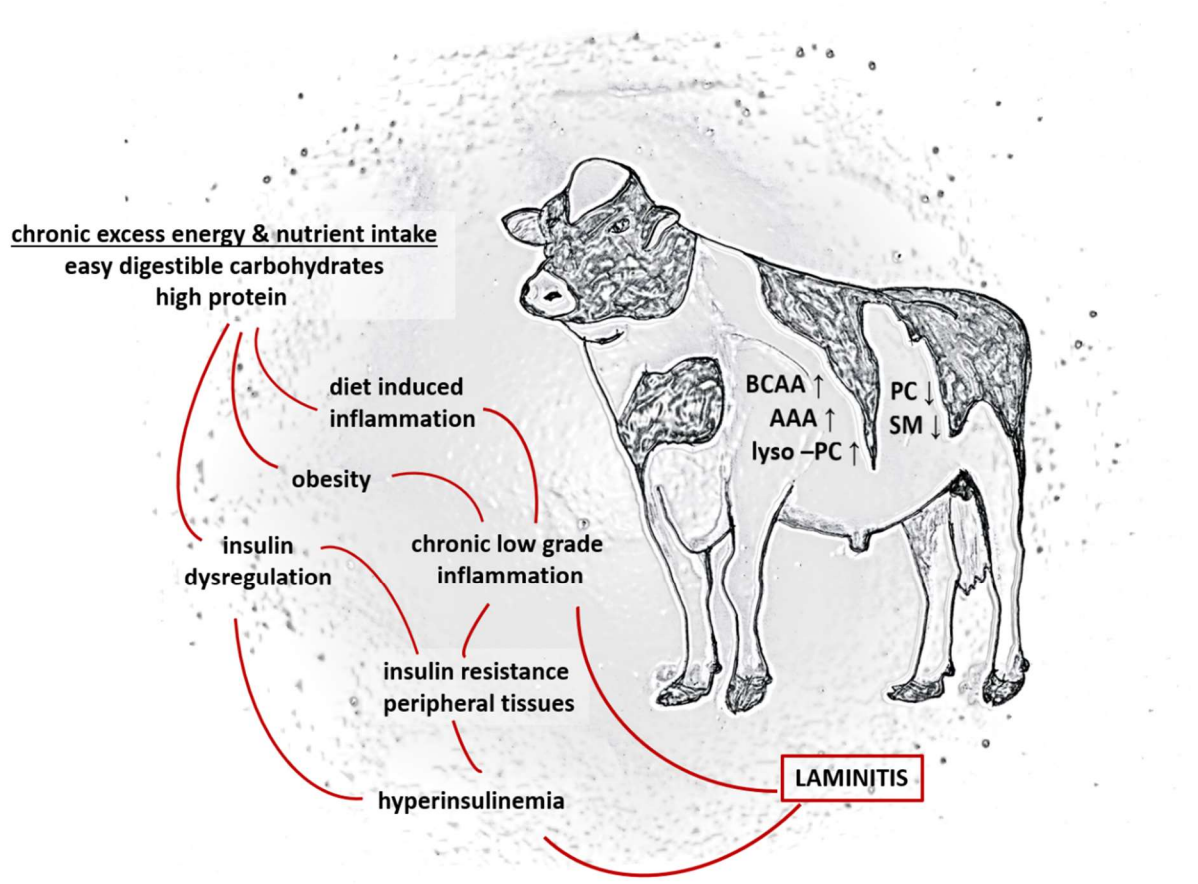

Figure 15. Proposed pathway of chronic excessive energy and nutrient intake towards laminitis in fattening bulls. Associated alterations of metabolite profiles in Holstein bulls fed a high energy and protein diet were increased concentrations of branched chain amino acids (BCAA), aromatic amino acids (AAA), lyso-phosphatidylcholins (lyso-PC), and decreased concentrations of phosphatidylcholines (PC) and sphingomyelins (SM).

6 CONCLUSION

This study provided new insights in metabolic consequences and long-term effects of chronic excessive energy and nutrient supply in Holstein bulls. Feeding an experimental diet with increased content of easily digestible carbohydrates and protein promoted wide ranging alterations of metabolism, indicated by metabolite profiling in blood and insulin signaling cascade in muscle, retroperitoneal adipose tissue and liver. Bulls developed an inflammatory phenotype, characterized by increased concentrations of AAs, especially AAA and BCAAs; as well as reduced concentrations of PCs, SMs and increased concentrations of lyso-PCs. Furthermore, bulls were less insulin sensitive, with reduced activation of signaling cascades in peripheral tissues and compensatory hyperinsulinemia. Metabolic disturbances were further associated with chronic laminitis. However, this study cannot provide information about causative relationships between observed alterations, as they can always be cause or consequence of each other.

Parallels to diabetic alterations in humans as well as to insulin dysregulated horses with laminitis have been repeatedly observed, indicating conserved pathophysiological mechanisms across different species. Further research is needed to confirm these results and hypotheses, in order to understand pathogenesis of laminitis and interactions of metabolic pathways and individual metabolites. These considerations would be of great importance not only for optimizing feeding of cattle, but also for nutritional physiology of other species affected by excessive nutrient intake.

Based on the results of this study, long and intensive fattening is not recommended for Holstein bulls, although high gains and final weights were achieved. Fattening based only on forage resulted in less negative metabolic alterations and allowed healthier growth of the bulls. Further studies are warranted to investigate whether a shortened fattening period attenuates the negative metabolic consequences of intensive fattening and how other breeds cope with this metabolic burden. In addition to that, it is also important to investigate if adapted husbandry and management of the animals, for example higher space offer to improve physical activity, would improve metabolic health of intensively fed Holstein bulls.

7 SUMMARY

Consequences of intensive fattening: Influence on claw health, blood metabolite profile and the insulin signaling cascade in tissues of Holstein fattening bulls

Sonja Christiane Bäßler

Intensive fattening based on high amounts of easy digestible and starch-rich concentrate is a common practice to fatten bulls in order to achieve productivity targets. However, excessive intake of carbohydrate-rich diet is also known to cause rumen acidosis, laminitis or tail tip necrosis, commonly referred to as production diseases. Affected metabolic pathways and underlying mechanisms are not fully understood, yet. Therefore, the major objective of this study was to investigate metabolic consequences of an experimental diet in 20 months old Holstein bulls, comprising a chronically increased nutrient (energy, starch and protein) intake, delivered by additional dietary concentrate feed, in comparison to a feeding regimen only based on forage. Aims of this study were to characterize alterations of metabolite profiles with a targeted metabolomics approach, to elucidate changes in insulin signaling pathway and finally to combine data of claw health and fattening performance with metabolic data, in order to generate new hypotheses of (patho)physiological relationships between chronic excessive energy intake and laminitis.

Holstein bulls intended for beef production were randomly assigned to a high energy and protein diet (HEP; metabolizable energy [ME] intake 169.0 ± 1.4 MJ/day; crude protein [CP] intake 2.3 ± 0.02 kg/day; calculated means ± SEM; n = 15) or a low energy and protein diet (LEP; ME intake 92.9 ± 1.3 MJ/day; CP intake 1.0 ± 0.01 kg/day; n = 15) for the last seven months of the fattening period. Claw health was documented and blood samples were collected at the beginning and at the end of the experimental period (slaughter). The bulls were slaughtered at the age of 20 months. Insulin concentrations (ELISA; Mercodia AB, Uppsala, Sweden), the metabolite profiles (AbsoluteIDQ® p180 kit, Biocrates, Innsbruck, Austria) and the blood biochemistry (Cobas c311 Analyzer, ROCHE, Mannheim, Germany) were analyzed in blood. Protein expression and extent of phosphorylation of insulin receptor (InsR; at Tyr1150), protein kinase B (PKB; at Ser473), AMP-activated kinase (AMPK; at Thr172) and mechanistic target of rapamycin (mTOR; at Ser2448) were semi-quantified in liver, muscle and retroperitoneal adipose tissue, by Western blot.

The final body weight of the bulls was 807 ± 9 kg and 712 ± 12 kg (mean ± SEM) in the HEP and LEP groups, respectively. All HEP bulls showed signs of laminitis at slaughter, but none

of LEP. Multivariate statistics revealed complex alterations in metabolite profiles of HEP bulls, 91 of 182 analyzed metabolites were significantly different between feeding groups. Mainly lower concentrations in HEP bulls were found in the substance classes of phosphatidylcholines (PCs) and sphingomyelins. In contrast, the concentrations of lyso-PC and amino acids were higher in intensively fed bulls. Especially the concentrations of branched-chain amino acids (BCAAs), with isoleucine, leucine and valine were higher in HEP bulls. The concentration of insulin was also increased, but not of glucose, in blood. The extent of phosphorylation of p-InsR in all three tissues and p-mTOR in muscle were under the limit of detection. In the liver expression of PKB, mTOR and AMPK were higher in HEP, as well as the extent of phosphorylation of mTOR. In adipose tissue the expression of InsR was decreased in HEP bulls, but in muscle there were no significant differences between the feeding groups.

Strong differences in plasma metabolite profiles, signs of reduced insulin sensitivity in muscle and retroperitoneal adipose tissue were observed. These metabolic alterations were associated with an inflammatory state of diet-related chronic laminitis in Holstein bulls fed a HEP diet. Hyperinsulinemia and enhanced BCAAs are also characteristic for a prediabetic state in humans, indicating a conserved pathophysiological role of these metabolites. Triggers of laminitis in cattle and horses could be similar, proposing a relationship of diet induced and obesity related inflammation, further promoting insulin resistance and culminating in chronic laminitis. Future research is warranted to understand (patho-)physiological pathways of laminitis in cattle and how adapted husbandry and management is able to improve health of Holstein fattening bulls.

8 ZUSAMMENFASSUNG

Konsequenzen intensiver Mast: Einfluss auf die Klauengesundheit, das metabolische Profil im Blut und die Insulinsignalkaskade in Geweben von Holstein Mastbullen

Sonja Christiane Bäßler

Die intensive Fütterung mit hohen Mengen an stärke- und proteinreichem Kraftfutter ist eine gängige Praxis in der Bullenmast, um Leistungsziele zu erreichen. Eine übermäßige Aufnahme von leichtverdaubaren Kohlenhydraten kann bei Wiederkäuern aber zu Pansenazidose, Klauenrehe oder Schwanzspitzennekrose führen. Die betroffenen Stoffwechselwege und die zugrundeliegenden Mechanismen sind allerdings noch nicht vollständig geklärt. Daher war der Zweck dieser Studie, die metabolischen Folgen einer experimentellen Fütterung, die durch eine erhöhte Energie- und Nährstoffzufuhr (Stärke und Eiweiß) über sieben Monate bei Holstein-Bullen gekennzeichnet war, zu untersuchen. Eine Fütterung die nur auf Grundfuttermitteln basierte, diente als Vergleich. Ziele dieser Studie waren, erstens Veränderungen der metabolischen Profile mit einem gezielten Metabolomics-Ansatz zu charakterisieren. Zweitens Veränderungen im Insulin-Signalweg in Leber, Muskel und retroperitonealem Fettgewebe zu untersuchen. Um schließlich drittens, Verbindungen zwischen den Daten der Klauengesundheit und Mastleistung, sowie der Stoffwechseldaten zu erkennen, um neue Hypothesen über (patho)physiologische Zusammenhänge zwischen chronisch übermäßiger Energiezufuhr und Klauenrehe zu generieren.

Holstein-Bullen, die für die Rindfleischproduktion bestimmt waren, wurden nach dem Zufallsprinzip entweder mit einem Futter mit hohem Energie- und Proteingehalt (HEP; Zufuhr von umsetzbarer Energie [ME] 169,0 ± 1,4 MJ/Tag; Zufuhr von Rohprotein [CP] 2,3 ± 0,02 kg/Tag; berechnete Mittelwerte ± SEM; n = 15) oder mit einem Futter mit niedrigem Energie- und Proteingehalt (LEP; Zufuhr von umsetzbarer Energie 92,9 ± 1,3 MJ/Tag; Zufuhr von Rohprotein 1,0 ± 0,01 kg/Tag; n = 15) gefüttert. Die Klauengesundheit wurde dokumentiert und Blutproben wurden zu Beginn und am Ende des Versuchszeitraums (Schlachtung) entnommen. Die Bullen wurden im Alter von 20 Monaten geschlachtet. Im Blut wurden die Insulinkonzentration (ELISA; Mercodia AB, Uppsala, Schweden), das metabolische Profil (AbsoluteIDQ® p180 Kit, Biocrates, Innsbruck, Österreich) und die Blutbiochemie (Cobas c311 Analyzer, ROCHE, Mannheim, Deutschland) analysiert. Außerdem wurde die Proteinexpression und das Ausmaß der Phosphorylierung des Insulinrezeptors (INSR; an Tyr1150), der Proteinkinase B (PKB; an Ser473), der AMP-aktivierten Kinase (AMPK; an

Thr172) und des mechanistischen Ziels von Rapamycin (mTOR; an Ser2448) in der Leber, dem Muskel und dem retroperitonealen Fettgewebe mittels Western Blot semiquantifiziert.

Das Endgewicht der Bullen betrug 807 ± 9 kg und 712 ± 12 kg (Mittelwert ± SEM) in der HEP- bzw. LEP-Gruppe. Alle HEP-Bullen zeigten bei der Schlachtung Anzeichen von Klauenrehe, aber keiner der LEP Bullen. Multivariate Statistiken zeigten komplexe Veränderungen in den metabolischen Profilen der HEP-Bullen, 91 von 182 analysierten Metaboliten unterschieden sich signifikant zwischen den Fütterungsgruppen. Vor allem in den Substanzklassen der Phosphatidylcholine (PC) und Sphingomyeline wurden bei HEP-Bullen geringere Konzentrationen gefunden. Im Gegensatz dazu waren die Konzentrationen der Lyso-PCs und Aminosäuren bei den intensiv gefütterten Bullen höher. Insbesondere die Konzentrationen der verzweigtkettigen Aminosäuren (BCAAs), Isoleucin, Leucin und Valin waren bei HEP-Bullen höher. Die Insulinkonzentration im Blut der HEP Bullen war ebenfalls erhöht, nicht jedoch die Glukosekonzentration. Das Ausmaß der Phosphorylierung von p-InsR in allen drei Geweben und von p-mTOR im Muskel lag unter der Nachweisgrenze. In der Leber war die Expression von PKB, mTOR und AMPK bei HEP höher, ebenso das Ausmaß der Phosphorylierung von mTOR. Im Fettgewebe war die Expression von InsR bei HEP-Bullen verringert, im Muskel gab es keine signifikanten Unterschiede zwischen den Fütterungsgruppen.

Es wurden große Unterschiede in den Plasmastoffwechselprofilen und Anzeichen für eine verringerte Insulinsensitivität im Muskel- und retroperitonealen Fettgewebe beobachtet. Diese Stoffwechselveränderungen wurden mit einem entzündlichen Zustand von ernährungsbedingter chronischer Klauenrehe, bei Holstein-Bullen die eine HEP-Fütterung erhielten, assoziiert. Hyperinsulinämie und erhöhte Konzentrationen von BCAAs wurden auch als Charakteristika für einen prädiabetischen Zustand beim Menschen identifiziert. Dies könnte auf eine konservierte pathophysiologische Rolle dieser Metaboliten, über verschiedene Spezies hinweg, hinweisen. Auch die Auslöser der Klauen- bzw. Hufrehe bei Rindern und Pferden könnten ähnlich sein. Ein möglicher Zusammenhang zwischen chronisch exzessiver Energie- und Nährstoffaufnahme und Klauenrehe könnte sein, dass ernährungsbedingte und durch Fettleibigkeit bedingte Entzündungen eine Insulinresistenz weiter fördern und sich in chronischer Klauenrehe manifestieren. Weitere Untersuchungen sind erforderlich, um die (patho-)physiologischen Wege der Klauenrehe bei Rindern zu verstehen und um herauszufinden, ob eine andere Haltung und ein angepasstes Management die Gesundheit von intensiv gefütterten Holstein Bullen verbessern könnte.

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10 APPENDIX

10.1 List of figures

10.2 List of tables

10.3 List metabolites measured by p180 Absolute IDQ

Metabolite	HEP	LEP	Unit	P value
Alanine	214.9 ± 8.9	233.1 ± 10.4	µmol/L	0.1947
Arginine	195.0 ± 6.7	175.3 ± 6.3	µmol/L	< 0.05
Asparagine	38.9 ± 2.2	32.2 ± 1.4	µmol/L	< 0.05
Aspartate	9.9 ± 0.8	8.9 ± 0.4	µmol/L	0.2390
Citrulline	60.6 ± 1.9	58.4 ± 2.2	µmol/L	0.4529
Glutamine	389.5 ± 18.4	419.8 ± 18.9	µmol/L	0.2604
Glutamate	84.1 ± 5.3	89.4 ± 4.0	µmol/L	0.4363
Glycine	281.5 ± 15.7	307.6 ± 12.3	µmol/L	0.2008
Histidine	85.7 ± 5.0	76.6 ± 3.0	µmol/L	0.1308
Isoleucine	136.8 ± 5.8	103.0 ± 5.1	µmol/L	< 0.001
Leucine	164.8 ± 9.7	113.0 ± 6.1	µmol/L	< 0.001
Lysine	142.5 ± 8.3	107.4 ± 4.8	µmol/L	< 0.01
Methionine	31.2 ± 2.2	28.0 ± 0.9	µmol/L	0.1819
Ornithine	60.3 ± 2.5	47.6 ± 1.7	µmol/L	< 0.001
Phenylalanine	55.8 ± 2.9	45.1 ± 1.7	µmol/L	< 0.01
Proline	74.6 ± 3.9	66.7 ± 2.4	µmol/L	0.0988
Serine	69.2 ± 3.2	72.8 ± 2.6	µmol/L	0.3904
Threonine	75.0 ± 5.4	52.6 ± 1.9	µmol/L	< 0.001
Tryptophan	54.0 ± 3.1	46.1 ± 1.9	µmol/L	< 0.05
Tyrosine	68.2 ± 3.2	58.2 ± 1.9	µmol/L	< 0.05
Valine	259.3 ± 11.5	199.3 ± 7.7	µmol/L	< 0.001

Metabolite	HEP	LEP	Unit	P value
Acetylornithine	2.3 ± 0.2	3.8 ± 0.3	µmol/L	< 0.001
Asymmetric dimethylarginine	1.0 ± 0.1	1.0 ± 0.0	µmol/L	0.4656
α-Aminoadipic	2.9 ± 0.3	2.5 ± 0.2	µmol/L	0.2555
Carnosine	16.2 ± 1.5	17.6 ± 0.9	µmol/L	0.4152
Creatinine	67.4 ± 3.8	83.0 ± 2.2	µmol/L	< 0.01
Histamine	0.3 ± 0.3	0.0 ± 0.0	µmol/L	0.2979
Kynurenine	5.9 ± 0.4	6.7 ± 0.4	µmol/L	0.1860
Methionine sulfoxide	1.6 ± 0.2	1.4 ± 0.0	µmol/L	0.2479
Nitrotyrosine	1.0 ± 0.3	0.7 ± 0.2	µmol/L	0.5085
Putrescine	0.07 ± 0.03	0.06 ± 0.01	µmol/L	0.6926
Sarcosine	1.8 ± 0.3	1.6 ± 0.1	µmol/L	0.4727
Symmetric dimethylarginine	0.4 ± 0.0	0.0 ± 0.4	µmol/L	0.2093
Serotonin	6.4 ± 1.2	6.8 ± 0.9	µmol/L	0.7610
Spermidine	1.2 ± 0.4	0.4 ± 0.2	µmol/L	0.0782
Taurine	43.4 ± 4.7	32.5 ± 2.6	µmol/L	0.0512
Trans-4-Hydroxyproline	28.1 ± 1.0	32.3 ± 1.4	µmol/L	< 0.05
lyso PC a C14:0	4.4 ± 0.1	4.1 ± 0.1	µmol/L	< 0.05
lyso PC a C16:0	17.9 ± 0.9	14.2 ± 0.6	µmol/L	< 0.01
lyso PC a C16:1	1.2 ± 0.1	1.1 ± 0.0	µmol/L	0.3744
lyso PC a C17:0	1.5 ± 0.1	1.6 ± 0.1	µmol/L	0.3499
lyso PC a C18:0	18.0 ± 1.0	15.7 ± 1.0	µmol/L	0.1043
lyso PC a C18:1	11.2 ± 0.6	11.6 ± 0.5	µmol/L	0.6238

Metabolite	HEP	LEP	Unit	P value
lyso PC a C18:2	14.0 ± 0.7	10.5 ± 0.4	µmol/L	< 0.001
lyso PC a C20:3	1.1 ± 0.1	0.9 ± 0.0	µmol/L	0.1106
lyso PC a C20:4	1.4 ± 0.1	1.6 ± 0.1	µmol/L	0.0941
lyso PC a C24:0	0.06 ± 0.00	0.06 ± 0.00	µmol/L	0.8828
lyso PC a C26:0	0.09 ± 0.01	0.11 ± 0.01	µmol/L	0.2796
lyso PC a C26:1	0.05 ± 0.00	0.04 ± 0.00	µmol/L	0.4043
lyso PC a C28:0	0.2 ± 0.0	0.2 ± 0.0	µmol/L	0.3761
lyso PC a C28:1	0.2 ± 0.0	0.2 ± 0.0	µmol/L	0.8212
SM (OH) C14:1	7.4 ± 0.3	10.6 ± 0.6	µmol/L	< 0.001
SM (OH) C16:1	6.2 ± 0.3	8.2 ± 0.5	µmol/L	< 0.001
SM (OH) C22:1	9.6 ± 0.7	8.9 ± 0.5	µmol/L	0.4504
SM (OH) C22:2	4.2 ± 0.2	5.4 ± 0.3	µmol/L	< 0.01
SM (OH) C24:1	1.1 ± 0.1	1.2 ± 0.1	µmol/L	0.2393
SM C16:0	70.3 ± 3.1	74.5 ± 3.4	µmol/L	0.3640
SM C16:1	7.6 ± 0.5	8.5 ± 0.4	µmol/L	0.2067
SM C18:0	9.6 ± 0.5	11.7 ± 0.6	µmol/L	< 0.05
SM C18:1	4.0 ± 0.2	4.8 ± 0.2	µmol/L	< 0.05
SM C20:2	0.2 ± 0.0	0.2 ± 0.0	µmol/L	0.5251
SM C24:0	18.2 ± 1.2	31.7 ± 1.6	µmol/L	< 0.001
SM C24:1	6.6 ± 0.4	7.0 ± 0.3	µmol/L	0.4589
SM C26:0	0.28 ± 0.02	0.36 ± 0.03	µmol/L	< 0.05
SM C26:1	0.2 ± 0.0	0.2 ± 0.0	µmol/L	0.1866

Metabolite	HEP	LEP	Unit	P value
Hexoses	4182.4 ± 148.8	4181.6 ± 134.6	µmol/L	0.9968
C0	8.6 ± 0.4	9.0 ± 0.4	µmol/L	0.4498
C2	1.6 ± 0.1	1.7 ± 0.1	µmol/L	0.2634
C3	0.36 ± 0.02	0.46 ± 0.04	µmol/L	< 0.05
C3-DC (C4-OH)	0.03 ± 0.00	0.03 ± 0.00	µmol/L	0.9641
C3-OH	0.02 ± 0.00	0.02 ± 0.00	µmol/L	< 0.05
C3:1	0.02 ± 0.00	0.02 ± 0.00	µmol/L	1.000
C4	0.15 ± 0.00	0.14 ± 0.00	µmol/L	0.4233
C4:1	0.03 ± 0.00	0.03 ± 0.00	µmol/L	0.2466
C5	0.12 ± 0.00	0.13 ± 0.00	µmol/L	0.1001
C5-DC (C6-OH)	0.02 ± 0.00	0.02 ± 0.00	µmol/L	0.2770
C5-M-DC	0.03 ± 0.00	0.03 ± 0.00	µmol/L	< 0.01
C5-OH (C3-DC-M)	0.04 ± 0.00	0.04 ± 0.00	µmol/L	0.2977
C5:1	0.06 ± 0.00	0.07 ± 0.00	µmol/L	0.1103
C5:1-DC	0.02 ± 0.00	0.02 ± 0.00	µmol/L	0.8179
C6:1	0.03 ± 0.00	0.04 ± 0.00	µmol/L	< 0.05
C6 (C4:1 DC)	0.06 ± 0.00	0.08 ± 0.01	µmol/L	< 0.05
C7-DC	0.02 ± 0.00	0.02 ± 0.00	µmol/L	0.6923
C8	0.09 ± 0.00	0.09 ± 0.00	µmol/L	0.2576
C9	0.04 ± 0.00	0.04 ± 0.00	µmol/L	0.3090
C10	0.16 ± 0.00	0.16 ± 0.00	µmol/L	0.4270
C10:1	0.06 ± 0.00	0.06 ± 0.00	µmol/L	0.0624

Metabolite	HEP	LEP	Unit	P value
C10:2	0.05 ± 0.00	0.06 ± 0.00	µmol/L	0.6587
C12	0.08 ± 0.00	0.08 ± 0.00	µmol/L	0.7895
C12-DC	0.19 ± 0.00	0.19 ± 0.00	µmol/L	0.7852
C12:1	0.11 ± 0.00	0.09 ± 0.01	µmol/L	< 0.01
C14	0.05 ± 0.00	0.06 ± 0.00	µmol/L	0.0566
C14:1	0.02 ± 0.00	0.03 ± 0.00	µmol/L	0.1745
C14:1-OH	0.03 ± 0.00	0.03 ± 0.00	µmol/L	0.1979
C14:2	0.01 ± 0.00	0.01 ± 0.00	µmol/L	0.1105
C14:2-OH	0.03 ± 0.00	0.03 ± 0.00	µmol/L	0.2553
C16	0.04 ± 0.00	0.04 ± 0.00	µmol/L	0.0858
C16-OH	0.04 ± 0.00	0.04 ± 0.00	µmol/L	0.5364
C16:1	0.03 ± 0.00	0.04 ± 0.00	µmol/L	0.1959
C16:1-OH	0.02 ± 0.00	0.03 ± 0.00	µmol/L	0.0591
C16:2	0.02 ± 0.00	0.02 ± 0.00	µmol/L	0.3973
C16:2-OH	0.02 ± 0.00	0.03 ± 0.00	µmol/L	0.0949
C18	0.03 ± 0.00	0.04 ± 0.00	µmol/L	0.1000
C18:1	0.05 ± 0.00	0.06 ± 0.00	µmol/L	0.4111
C18:1-OH	0.03 ± 0.00	0.04 ± 0.00	µmol/L	0.1431
C18:2	0.03 ± 0.00	0.04 ± 0.00	µmol/L	0.0756
PC aa C24:0	0.03 ± 0.00	0.03 ± 0.00	µmol/L	0.7736
PC aa C26:0	0.4 ± 0.0	0.4 ± 0.0	µmol/L	0.4570
PC aa C28:1	1.0 ± 0.1	0.9 ± 0.0	µmol/L	0.3320

Metabolite	HEP	LEP	Unit	P value
PC aa C30:0	1.7 ± 0.1	1.7 ± 0.1	µmol/L	0.7553
PC aa C30:2	0.1 ± 0.0	0.2 ± 0.0	µmol/L	< 0.01
PC aa C32:0	4.4 ± 0.2	5.1 ± 0.2	µmol/L	< 0.01
PC aa C32:1	4.6 ± 0.2	5.3 ± 0.2	µmol/L	< 0.05
PC aa C32:2	4.2 ± 0.3	6.0 ± 0.4	µmol/L	< 0.001
PC aa C32:3	10.1 ± 1.0	10.0 ± 0.7	µmol/L	0.9164
PC aa C34:1	63.2 ± 3.6	71.4 ± 3.5	µmol/L	0.1117
PC aa C34:2	109.7 ± 6.0	85.3 ± 3.7	µmol/L	< 0.01
PC aa C34:3	16.2 ± 1.0	23.7 ± 1.3	µmol/L	< 0.001
PC aa C34:4	2.7 ± 0.3	3.2 ± 0.2	µmol/L	0.1854
PC aa C36:0	4.1 ± 0.3	10.0 ± 1.0	µmol/L	< 0.001
PC aa C36:1	94.7 ± 4.2	133.4 ± 5.5	µmol/L	< 0.05
PC aa C36:2	183.4 ± 8.2	166.7 ± 6.7	µmol/L	0.1286
PC aa C36:3	57.2 ± 2.3	71.2 ± 11.7	µmol/L	< 0.01
PC aa C36:4	19.9 ± 1.1	23.9 ± 1.2	µmol/L	< 0.05
PC aa C36:5	4.2 ± 0.2	6.4 ± 0.4	µmol/L	< 0.001
PC aa C36:6	1.3 ± 0.1	1.9 ± 0.1	µmol/L	< 0.01
PC aa C38:0	1.5 ± 0.1	4.2 ± 0.3	µmol/L	< 0.001
PC aa C38:1	2.8 ± 0.2	9.7 ± 0.6	µmol/L	< 0.001
PC aa C38:3	28.2 ± 2.0	38.4 ± 2.0	µmol/L	< 0.01
PC aa C38:4	34.9 ± 30.6	44.4 ± 2.1	µmol/L	< 0.01
PC aa C38:5	11.5 ± 0.7	18.6 ± 1.3	µmol/L	< 0.001

Metabolite	HEP	LEP	Unit	P value
PC aa C38:6	2.2 ± 0.1	3.4 ± 0.2	µmol/L	< 0.001
PC aa C40:1	0.2 ± 0.0	0.3 ± 0.0	µmol/L	< 0.001
PC aa C40:2	0.2 ± 0.0	0.5 ± 0.0	µmol/L	< 0.001
PC aa C40:3	2.4 ± 0.2	5.8 ± 0.5	µmol/L	< 0.001
PC aa C40:4	9.1 ± 0.8	18.9 ± 1.3	µmol/L	< 0.001
PC aa C40:5	13.3 ± 0.8	20.9 ± 1.2	µmol/L	< 0.001
PC aa C40:6	3.6 ± 0.3	6.3 ± 0.5	µmol/L	< 0.001
PC aa C42:0	0.06 ± 0.00	0.07 ± 0.00	µmol/L	< 0.001
PC aa C42:1	0.06 ± 0.00	0.09 ± 0.00	µmol/L	< 0.001
PC aa C42:2	0.09 ± 0.00	0.11 ± 0.00	µmol/L	< 0.001
PC aa C42:4	0.2 ± 0.0	0.3 ± 0.0	µmol/L	< 0.001
PC aa C42:5	0.6 ± 0.0	2.3 ± 0.2	µmol/L	< 0.001
PC aa C42:6	0.3 ± 0.0	1.1 ± 0.1	µmol/L	< 0.001
PC ae C30:0	0.37 ± 0.02	0.44 ± 0.01	µmol/L	< 0.05
PC ae C30:1	0.7 ± 0.1	0.8 ± 0.1	µmol/L	0.2394
PC ae C30:2	0.2 ± 0.0	0.2 ± 0.0	µmol/L	0.1746
PC ae C32:1	1.7 ± 0.1	2.1 ± 0.1	µmol/L	< 0.01
PC ae C32:2	3.7 ± 0.2	4.2 ± 0.2	µmol/L	0.0877
PC ae C34:0	1.9 ± 0.1	2.8 ± 0.1	µmol/L	< 0.001
PC ae C34:1	9.1 ± 0.4	12.8 ± 0.6	µmol/L	< 0.001
PC ae C34:2	12.2 ± 0.7	16.4 ± 0.8	µmol/L	< 0.001
PC ae C34:3	13.3 ± 1.2	12.0 ± 0.7	µmol/L	0.3433

Metabolite	HEP	LEP	Unit	P value
PC ae C36:0	1.5 ± 0.1	2.8 ± 0.2	µmol/L	< 0.001
PC ae C36:1	11.9 ± 0.5	18.2 ± 0.9	µmol/L	< 0.001
PC ae C36:2	17.8 ± 0.8	20.8 ± 0.9	µmol/L	< 0.05
PC ae C36:3	6.8 ± 0.3	9.3 ± 0.5	µmol/L	< 0.001
PC ae C36:4	4.7 ± 0.4	4.7 ± 0.2	µmol/L	0.9329
PC ae C36:5	3.5 ± 0.3	4.0 ± 0.2	µmol/L	0.1878
PC ae C38:0	1.0 ± 0.1	2.1 ± 0.1	µmol/L	< 0.001
PC ae C38:1	1.6 ± 0.1	3.2 ± 0.2	µmol/L	< 0.001
PC ae C38:2	2.4 ± 0.1	3.6 ± 0.2	µmol/L	< 0.001
PC ae C38:3	2.7 ± 0.1	3.6 ± 0.2	µmol/L	< 0.001
PC ae C38:4	3.6 ± 0.2	5.1 ± 0.2	µmol/L	< 0.001
PC ae C38:5	2.7 ± 0.2	3.3 ± 0.2	µmol/L	< 0.05
PC ae C38:6	2.2 ± 0.2	2.5 ± 0.1	µmol/L	0.2427
PC ae C40:1	0.2 ± 0.0	0.4 ± 0.0	µmol/L	< 0.001
PC ae C40:2	0.7 ± 0.0	1.0 ± 0.0	µmol/L	< 0.001
PC ae C40:3	0.7 ± 0.0	0.9 ± 0.0	µmol/L	< 0.001
PC ae C40:4	0.9 ± 0.0	1.2 ± 0.1	µmol/L	< 0.001
PC ae C40:5	1.3 ± 0.1	2.0 ± 0.1	µmol/L	< 0.001
PC ae C40:6	0.7 ± 0.0	1.0 ± 0.0	µmol/L	< 0.01
PC ae C42:0	0.4 ± 0.0	0.4 ± 0.0	µmol/L	0.1548
PC ae C42:1	0.1 ± 0.0	0.2 ± 0.0	µmol/L	< 0.001
PC ae C42:2	0.15 ± 0.01	0.22 ± 0.01	µmol/L	< 0.001

Metabolite	HEP	LEP	Unit	P value
PC ae C42:3	0.1 ± 0.0	0.2 ± 0.0	µmol/L	< 0.001
PC ae C42:4	0.1 ± 0.0	0.2 ± 0.0	µmol/L	< 0.001
PC ae C42:5	0.5 ± 0.0	0.7 ± 0.0	µmol/L	< 0.001
PC ae C44:3	0.07 ± 0.00	0.08 ± 0.00	µmol/L	< 0.001
PC ae C44:4	0.08 ± 0.00	0.1 ± 0.00	µmol/L	< 0.01
PC ae C44:5	0.07 ± 0.00	0.09 ± 0.00	µmol/L	< 0.001
PC ae C44:6	0.06 ± 0.00	0.07 ± 0.00	µmol/L	< 0.001

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