Untersuchungen zum Einsatz von konjugierten Linolsäuren (CLA) und Vitamin E, sowie deren Wechselwirkungen, als Interventionsmaßnahme zur Verminderung einer ketotischen Stoffwechselbelastung und des oxidativen Stresses bei peripartalen Milchkühen

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I Abbreviations

(used in Introduction, Background, General Discussion, Conclusion and Summary)

Δ9-desaturase	stearoyl-CoA desaturase
AA	amino acids
a.p.	antepartum
apo B	apolipoprotein B
BCS	body condition score
BHB	β-hydroxybutyrate
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation
CLA	conjugated linoleic acid
CoA	coenzyme A
ConA	concavalin A
CPT-I	carnitine palmitoyltransferase I
DMI	dry matter intake
EB	energy balance
Epi	epinephrine
HMG-CoA-synthase	3-hydroxy-3-methylglutaryl-CoA-synthase
IFNγ	interferon γ
Ig	immunoglobulin
IL	interleukine
LFI	liver functionality index
LIR	lactational incidence rate
ME	metabolizable energy
MFD	milk fat depression
NEFA	non-esterified fatty acids
NE_L	net energy of lactation
NOX	NADPH oxidase
PC	pyruvate carboxylase
PBMC	peripheral blood mononuclear cells
PEPCK	phosphoenolpyruvate kinase

PMN	polymorphonuclear leukocytes
p.p.	postpartum
PPARγ	peroxisome proliferator activated receptor gamma
ROS	reactive oxygen species
RSEI	residual energy intake
SCK	subclinical ketosis
SREBP	sterol response element-binding protein
ST	somatotropin
TAG	triacylglycerol
TCA	tricarboxylic acid cycle
TNFα	tumor necrosis factor α
Vit. E	Vitamin E
VLDL	very low density lipoproteins

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General Discussion

1. Introduction

During the transition period, lasting from three weeks before to three weeks after parturition dairy cows are challenged by a variety of physiological, endocrinological and metabolic changes (Mallard et al., 1998). The increased energy and nutrient demand due to fetal growth and onset of lactogenesis is accompanied by a reduced dry matter intake (**DMI**), which results in a negative energy balance (**EB**) (Bell, 1995). Genetic advancements as well as management improvements resulting in an average annual milk yield of 8,453 kg/cow in 2016 in Germany (Deutscher Verband für Leistungs- und Qualitätsprüfungen e.V., 2017) aggravate this imbalance. In order to counterbalance the negative EB fatty acids from the adipose tissue are mobilized and transported to the liver for β-oxidation. However, due to a relative lack of oxaloacetate, which is mainly needed for gluconeogenesis, fatty acids can only partly be metabolized. As a consequence, triglycerides accumulate in the liver and ketone bodies are formed. Increased levels of non-esterified fatty acids (**NEFA**) and β-hydroxybutyrate (**BHB**) might play an important role in the impairment of the functionality of the immune system during the transition period (Contreras and Sordillo, 2011).

Another challenge for the immune system especially in high-conditioned dairy cows (Bernabucci et al., 2005) might be oxidative stress caused by an imbalance between antioxidative capacity and reactive oxygen species (**ROS**) (Betteridge, 2000). According to Ingvartsen (2006) most cases of production diseases are observed in the phase of early lactation, which might lead to impairment of productivity and consequently economic losses for dairy farmers. In consequence, focuses in research for nutrition of dairy cows are on strategies to reduce the negative EB and following lipomobilisation as wells as on improving the functionality of the immune system during late dry-period and early lactation.

Conjugated linoleic acid (**CLA**) has been successfully proven to reduce milk fat content and milk fat yield and thereby leading to a more efficient use of the metabolizable energy (**ME**) (Baumgard et al., 2000). However, supplementation with CLA might also result in increased daily milk yield and consequently repartition of energy (Bauman et al., 2008). However, the observed effects of CLA on lipomobilisation are not consistent. Both a protection against lipomobilisation (von Soosten et al., 2011) and lipolytic or antilipogenic influences (Akter et al., 2011) were described.

Vitamin E (**Vit. E**) has been proven to have antioxidant properties (Burton and Traber, 1990;

Nakamura and Omaye, 2010). Therefore, supplementation with Vit. E might be helpful to improve the antioxidative capacity and thereby the health situation of high-conditioned dairy cows during the transition period. However, it has been observed that CLA increases the excretion of Vit. E with the milk in dairy cows (Gessner et al., 2015) as well as the storage in tissues in mice (Chen et al., 2012). This might counteract the positive influences of Vit. E supplementation on the health status of dairy cows.

In this investigation, an animal model established by Schulz et al. (2014), which allows to create animal groups sensitive for high lipomobilisation and ketosis by an excessive energy intake in the dry-period, a high body condition score (**BCS**) at the beginning of the transition period and a decreased energy supply due to a decelerated increase in concentrate proportion postpartum (**p.p.**) was used to assess the effects of supplementation with CLA and Vit. E and their interaction on performance and health of dairy cows in the transition period and early lactation.

2. Background

2.1 Metabolic processes and adaptation mechanisms around parturition

According to Grummer (1995) the transition period of the dairy cow comprises late dry-period and early lactation and is defined as the time period from 3 weeks before parturition until 3 weeks p.p. During this time the high-yielding dairy cow is challenged by a diversity of massive metabolic, endocrine and immunological changes (Mallard et al., 1998). Hormonal regulation is responsible for changes in metabolism and repartition of nutrients and energy (Bauman and Currie, 1980; Ingvartsen, 2006). According to Bauman and Currie (1980) the endocrine system acts via two different regulatory methods, homeostasis and homeorhesis. Homeostasis is used to regulate the physiological equilibrium with the environment in the short term (Ingvartsen, 2006). Conversely, homeorhesis is defined as those metabolic changes, which take place to adapt to a new physiological state like gestation in the long term. While energy and nutrient demand increase due to growth of the fetus and mammary tissue (Bauman and Currie, 1980; Bell, 1995) as well as for the onset of lactation, the feed intake decreases and reaches a minimum in the first week of lactation (Janovick and Drackley, 2010). This imbalance between energy expenditure and energy intake results in a negative EB (Grummer, 1995; Bell, 1995). Dairy cows partly compensate this by mobilizing energy reserves, particularly fat from adipose tissue. This mobilization results in a decreased BCS (Garnsworthy, 2006). The substrates used for oxidation by the fetus consist of 50 – 60% glucose and lactate (Hay et al., 1983), 30- 40% amino acids (Faichney and White, 1987) and 10 – 15% acetate (Comline and Silver, 1976). The fetus itself cannot utilize the mobilized fatty acids due to low placental transport of fatty acids in ruminants (Bell, 1993). However, the maternal use of these fatty acids might spare glucose and amino acids for the fetus (Bell et al., 1995). Drackley et al. (2001) have observed that the glucose demand of the cow increases from ca. 1,000 up to 2,500 g daily within 3 weeks after calving. As there is a daily supply of 2,000g, a shortcoming of ca. 500g glucose per day remains. In order to ensure sufficient glucose supply for the fetus during late dry-period and adequate glucose for lactogenesis during early lactation despite this lack of adequate glucose the rate of hepatic gluconeogenesis is increased. Additionally, the glucose uptake and utilization by adipose and muscle tissue is decreased. According to (Bell, 1995; Hayirli, 2006) this can be attributed to a decreased insulin sensitivity of these peripheral tissues. Bell (1995) reported that the protein hormone soma-

totropin is an important regulatory hormone for the tissue responses to insulin.

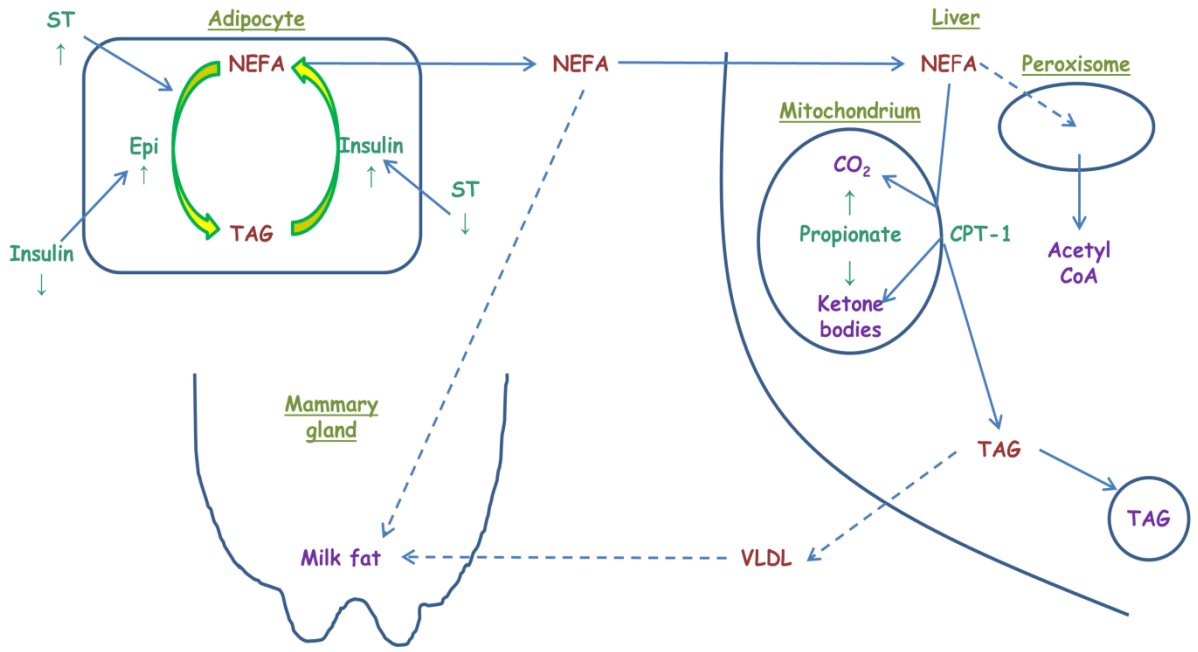

Figure 1: Schematic representation of lipid metabolism in dairy cows during the transition period, adapted from Drackley (1999). ↑ indicate stimulation, while ↓ indicate inhibition. Processes illustrated with dashed arrows occur at low rates or only during certain physiological states. Abbreviations: CPT-I = carnitine palmitoyltransferase I, Epi = epinephrine, ST = somatotropin, TAG = triacylglycerol, VLDL = very low density lipoproteins.

Sechen et al. (1990) observed that somatotropin reduced the glucose response to insulin and thereby decreased the ability of insulin to initiate lipogenesis (Figure 1). Bauman et al. (1989) ascribe the reduced glucose uptake of muscle tissue also to somatotropin, which modulates the effect of insulin. Ingvartsen and Andersen (2000) reported that the ratio of somatotropin to insulin is increased during early lactation. This high ratio results in increased stimulation of the adipose tissue to release fatty acids, which circulate in the blood as NEFA and form an important energy source for the cow (Ingvartsen, 2006). The magnitude and duration of increased lipolysis and decreased lipogenesis depend on energy intake, milk production and stage of lactation (McNamara, 1991; Theilgaard et al., 2002) and might partly be attributed to an altered reaction to the catecholamines epinephrine and norepinephrine. It has been observed that cows in early lactation release significantly more NEFA when subjected to an epinephrine challenge compared to cows in later stages of lactation (Theilgaard et al., 2002; Underwood et al., 2003). Whereas Ingvartsen (2006) attribute the increased lipolysis to an increased sensitivity to epinephrine and norepinephrine, McNamara (1991) reports that not the sensitivity but the maximal response to these catecholamines is higher in cows with higher

milk production. Other mechanisms involved in an increase of lipolysis in early lactation are the upregulation of β-adrenergic receptors, hormone-sensitive lipase and perilipin (Sumner and McNamara, 2007). When released into the blood NEFA are bound to albumin for transportation (Roche et al., 2009). Three pathways are known for further metabolization of circulating NEFA (Figure 1). They can be used for milk fat synthesis in the mammary gland, re-esterified to triacylglycerides (**TAG**) in the liver, partly oxidized to ketone bodies in the liver or completely oxidized in the liver or skeletal muscle to act as energy source (Drackley, 1999; Roche et al., 2009). There are 3 key factors determining the further metabolism of NEFA in the liver (Drackley et al., 2001). Firstly, the of hepatic NEFA flood, secondly, the activity of carnitine palmitoyltransferase I (**CPT-1**), which regulates the entry of acyl-coenzyme A (**CoA**) into the mitochondria where acyl-CoA is subjected to either β-oxidation to CO_2 or formation of ketone bodies, and thirdly, the activity of the 3-hydroxy-3-methylglutaryl-CoA-synthase (**HMG-CoA-synthase**) in the mitochondria, which catalyzes the reaction of acetoacetyl-CoA to HMG-CoA and thereby regulates the formation of ketone bodies (Hegardt, 1999). The activity of CPT-1 has been demonstrated to be inhibited by malonyl-CoA as well as by methylmalonyl-CoA in sheep liver (Brindle et al., 1985). The concentration of malonyl-CoA itself depends on the activity of the acetyl-CoA carboxylase, which can be activated by insulin (Zammit, 1996). Furthermore, insulin reduces the CPT-1 activity, probably by antagonizing cyclic AMP and thereby decreasing the transcription rate of the CPT-1 gene (Zammit, 1996). Additionally, the sensitivity of CPT-1 to inhibition by malonyl-CoA is reduced in states of low blood insulin concentrations (Zammit, 1996). Dann and Drackley (2005) reported that feed intake antepartum (**a.p.**) does not alter the CPT-1 sensitivity to malonyl-CoA. The activity of CPT-1 is increased in cows, which are in an energy deficiency a.p. But even if fed ad libitum a.p. no differences were detected p.p. According to Dann et al. (2000) the CPT-1 activity is upregulated on d 1 and d 21 p.p. compared to d 21 a.p. with enzyme activity being reduced to a.p. levels on d 65 p.p. Methylmalonyl-CoA is an intermediate in the metabolism of propionate to succinyl-CoA. Succinyl-CoA itself has been demonstrated to elicit antiketogenic effects due to inhibiting the HMG-Co-synthase and thereby reducing the formation of ketone bodies (Drackley et al., 2001). Whereas in all mammals β-oxidation in the liver can occur in mitochondria and in peroxisomes (Poirier et al., 2006), the peroxisomal proportion of total hepatic β-oxidation depends on the individual fatty acids and for females also on species (Grum et al., 1994). Piot et al. (1998) detected for male animals no species differences between rats and cattle for the peroxisomal contribution of total hepatic β-oxidation. Drackley et al. (2001) suggest that the proportion of β-oxidation in the peroxisomes

is increased in situations of energy restriction and consequently higher NEFA flood to the liver, which might assist in NEFA oxidation during the transition period by being independent of CPT-1. In case of incomplete β-oxidation ketone bodies are formed. This occurs in states of negative EB due to a relative lack of oxaloacetate in the citrate cycle, when oxaloacetate is mainly needed for gluconeogenesis (Baird et al., 1968). Whereas these ketone bodies can be utilized by the mammary gland as a substrate for milk fat synthesis and can be oxidized by skeletal and heart muscle, the kidney, the mammary gland as well as the gastrointestinal tract, it has been demonstrated that the percentage of ketone bodies, which are extracted by these organs from the blood remains constant (Heitmann et al., 1987). As a consequence, elevated ketone body formation leads to elevated blood concentrations, which might result in subclinical ketosis (BHB-levels > 1.2 mmol/L (Nielen et al., 1994)), whose prevalence has been reported to amount up to 36.6% (Suthar et al., 2013). Though subclinical ketotic cows show no clinical signs of ketosis, their health and productivity might still be impaired (Drackley, 1999). This is reflected by the highest incidence of production diseases, which occurs during early lactation (Ingvartsen, 2006). Massive mobilization of adipose tissue during the transition period might lead to levels of hepatic NEFA uptake, which exceed the oxidation and secretion capacity by the liver. Whereas Kleppe et al. (1988) report rates of TAG-formation of goats being comparable to that of rats, the secretion rate of TAG with very-low density lipoproteins (VLDL) is reduced. Though a low synthesis of apolipoprotein B (**apo B**) (Hocquette and Bauchart, 1999) or a deficiency of microsomal TAG transfer protein (Greffat-Mouty et al., 1999) were suggested to be responsible for low VLDL secretion, the cause has not been clarified (Drackley, 2001). As a consequence of insufficient VLDL secretion, TAG might accumulate in the liver and cows might be subjected to a fatty liver syndrome, which is observed in up to 50% of dairy cows during early lactation (Jorritsma et al., 2001) and is accompanied by a decrease in health status, reproductive performance and productivity (Wensing et al., 1997).

As described above, dairy cows in early lactation experience a daily glucose deficiency from digestible energy intake of up to 500 g, which is mainly compensated by increased rates of gluconeogenesis in the liver. According to Huntington (1997) gluconeogenesis is responsible for up to 50% of glucose supply in cows fed with medium or high concentrate diets. Different substrates might be used as glucogenic precursors in the liver. The proportion of propionate as a precursor of the net glucose release has been reported to be from 46% (Lomax and Baird, 1983) to 55% (Reynolds et al., 1988) in lactating cows fed ad libitum, whereas amino acids

(**AA**) have been demonstrated to contribute 17% (Reynolds et al., 1988), with alanine and glutamine being accountable for 40% to 60% of glucogenesis from AA (Bergman and Heitmann, 1978). The proportion of lactate has been observed to range from 16% (Lomax and Baird, 1983) to 23% (Baird et al., 1980). Another precursor is glycerol, whose maximum proportion has been reported to be between 15% and 20% (Bell, 1995). The rate of hepatic gluconeogenesis from lactate and glucogenic AA is regulated mainly by the enzymes pyruvate carboxylase (**PC**) and phosphoenolpyruvate kinase (**PEPCK**). The carbon derived from propionate is metabolized via the tricarboxylic acid cycle (**TCA**), whereas carbons derived from lactate and glucogenic AA are metabolized to pyruvate first. PC catalyzes the reaction of pyruvate to oxaloacetate, whereas PEPCK is responsible for the conversion of oxaloacetate to phosphoenolpyruvate. In rat hepatocytes it has been shown that insulin decreases the abundance of cytosolic PEPCK mRNA, whereas glucagon, which acts via cyclic adenosine monophosphate (**cAMP**), increases it (Granner et al., 1983; Sasaki et al., 1984). She et al. (1999) observed the same effects of glucagon and insulin on PEPCK mRNA in lactating cows. However, according to Aiello and Armentano (1987) the mitochondrial PEPCK, which is insensitive for hormonal influences, is responsible for over 60% of gluconeogenesis in hepatocytes of ruminants. Therefore, PEPCK might not be as important for limiting gluconeogenesis in ruminants as in rats (Drackley et al., 2001). Whereas propionate is the principal contributor of hepatic gluconeogenesis, the capacity of the liver to convert propionate depends on its supply originating from ruminal fermentation (Drackley et al., 2001). Consequently, the contribution of propionate as glucogenic precursor might be impaired during reduced DMI in the transition period (Drackley et al., 2001). This assumption is emphasized by a significant correlation of gluconeogenesis to fat-free net energy of lactation ($\mathbf{NE_L}$)-intake on d 1 and d 21 p.p. (Drackley et al., 2001). However, Overton et al. (1998) reported an increase of isolated liver slices in propionate conversion from d 1 p.p. to d 21 p.p. of 19% and 29% compared to d 21 a.p. which might be attributed to an increased efficiency of hepatocytes to utilize propionate (Drackley et al., 2001). Another factor affecting gluconeogenesis might be fatty liver. Cadórniga-Valiño et al. (1997) reported a reduction of conversion of 24% in bovine hepatocytes which accumulated TAG from exposure with oleate in vitro. Overton et al. (1999) reported an impairment of gluconeogenesis from propionate by increased ammonia concentrations. This might be explained by an observed decreased ability of fatty livers to synthesize urea in order to detoxify ammonia in vitro (Strang et al., 1998) as well as in vivo (Zhu et al., 1998).

The proportion by which AA contribute to gluconeogenesis might be depending on their supply from the gastrointestinal tract as well as the skeletal muscle to the liver (Danfær et al., 1995). According to Bell (1995) skeletal muscle is mobilized during the transition period in order to supply AA. In agreement, Overton et al. (1999) suggested that the potential to utilize AA is increased in situations of a sudden glucose demand such as parturition and onset of lactation. This is emphasized by an observed increase of gluconeogenesis from alanine from d 1 to d 21 p.p. of 98% and 50% compared to d 21 a.p. respectively (Overton et al., 1998). Furthermore, rates of 3-methyl histidine to creatinine in urine, which is an indication of protein degradation from skeletal muscle, were increased 3 times on d 3 p.p. (Overton et al., 1998). The cause for an increased capacity of alanine conversion compared to propionate conversion might be an increased activity of PC. Greenfield et al. (2000) observed an increase of mRNA for PC by 750% in liver slices from d 28 a.p. to d 1 p.p.

Whereas theoretically an increased activity of PC should result in an increased gluconeogenesis rate from lactate, lactate utilization for gluconeogenesis in fact results from a recycling of carbon (Drackley et al., 2001). According to Nocek (1997) lactate is not produced in considerable quantities in the rumen when cows are fed typical diets during the transition period, but rather results from glucose catabolism in peripheral tissues or propionate catabolism in visceral epithelial tissues (Drackley et al., 2001). Investigations on the influence of the production cycle on lactate to glucose conversion are divergent. Whereas Baird et al. (1983) and Bell (1995) suggested that the proportion of lactate for gluconeogenesis was lower during early lactation than during late pregnancy due to the gravid uterus releasing lactate (Bell, 1995), Mills et al. (1986) did not observe differences.

The utilization of glycerol for gluconeogenesis might also be understood as recycling (Drackley et al., 2001). However, in contrast to lactate conversion this recycling depends on mobilization of adipose tissue and does not occur on a minute to minute base but over a whole production cycle (Drackley et al., 2001). While Bell (1995) reported a proportion of glycerol for gluconeogenesis of 15% to 20% on d 4 p.p. the proportion decreases to between 2% and 5% during lactation when mobilization of adipose tissue slows down (Drackley et al., 2001).

2.2 Health situation and immune system during the transition period

It has been demonstrated that the highest incidence of production diseases in dairy cows occurs during early lactation (Ingvartsen, 2006) with lameness showing the highest lactational

incidence rate (**LIR**) ranging from 1.8% to 60% (Ingvartsen et al., 2003). Following were early metritis (1.7% to 43.8%) and mastitis (2.8% to 39%), whereas the LIR of retained placenta range from 3.1% to 13% (Ingvartsen et al., 2003). For ovarian cysts LIR of 3.1% to 12.4% were reported, followed by clinical ketosis with a LIR of 0.2% to 10% (Ingvartsen et al., 2003). The reported LIR of periparturient paresis ranged from 0.2% to 8.9% and was followed by a left displaced abomasum showing LIR of 0.6% to 6.3% (Ingvartsen et al., 2003). Dystocia played a minor role with a LIR of 1% to 2.1% (Ingvartsen et al., 2003). The incidence rate for subclinical ketosis (**SCK**; BHB-levels from 1.2 to 2.9 mmol/L (McArt et al., 2012)) has been reported to be 43.2%, showing a peak prevalence of 28.9% at d 5 p.p. (McArt et al., 2012). Although cows being subclinical ketotic do not show clinical signs of ketosis, they suffer from a greater risk to develop production diseases like a displaced abomasum or metritis (Ospina et al., 2010) as well as from a decreased milk production and a decreased conception rate at first service (McArt et al., 2012). Important risk factors for the development of ketosis are a higher parity (Gröhn et al., 1984) and a BCS of 3.5 and higher (Gillund et al., 2001). Additionally, silages high in butyric acid were identified as a risk factor for ketosis (Andersson and Lundström, 1985). According to Holtenius and Holtenius (1996) two types of ketosis can be differentiated. Type I occurs in high-producing cows 3 to 6 weeks p.p. due to the high glucose demand for lactation and is associated with low plasma concentrations of glucose and insulin and a low risk to develop a fatty liver. Type II is observed in early lactation and is caused mainly by overfeeding during the dry period. Plasma concentrations of glucose and insulin as well as lipolysis and lipid accumulation in the liver are increased. This finding is emphasized by Jorritsma et al. (2001), who reported prevalence of moderate or severe fatty liver (more than 50mg TAG/ 1g wet liver tissue (Bobe et al., 2004) for 54% of dairy cows in early lactation. Overfeeding during the dry period resulting in a high BCS (Fronk et al., 1980) accompanied by a low DMI around calving (Bertics et al., 1992) which leads to considerable lipolysis were identified as major risk factors. However, Jorritsma et al. (2001) observed that fatty liver is associated with low blood glucose concentrations, which contradicts the characterization of type II ketosis by Holtenius and Holtenius (1996). Fatty liver itself is associated with a diversity of diseases and decreased reproductive performance with the strongest connection with a displaced abomasum and ketosis, whereas the association with an impaired immune system, metritis and mastitis were moderate (Bobe et al., 2004).

Another challenge faced by dairy cows during the transition period is oxidative stress (Bernabucci et al., 2005), which is defined as an imbalance between the production of differ-

ent ROS as well as other oxidative radicals like reactive chlorine species and the antioxidant mechanisms of the organism (Betteridge, 2000). Cows with a high BCS a.p. accompanied by a high BCS loss p.p. and elevated concentrations of NEFA and BHB are particularly sensitive to oxidative stress (Bernabucci et al., 2005). ROS are formed under physiological conditions and are necessary e.g. as a co-factor for enzymes (Halliwell, 1996) and for retention of endothelial function in the vascular system during an inflammation (Kvietys and Granger, 2012) as well as a part of the defence against infections (Miller et al., 1993). Dahlgren and Karlsson (1999) described that ROS production is mediated by the NADPH oxidase (**NOX**) during phagocytosis processes by human polymorphonuclear leukocytes (**PMN**). However, an excess of ROS might result in damage to DNA, lipids, proteins and polysaccharides (Miller et al., 1993) and might especially affect immune cells, because their cell membranes incorporate high concentrations of polyunsaturated fatty acids which are very sensitive to peroxidative processes (Spears and Weiss, 2008). The oxygen metabolism of dairy cows during the transition period is elevated up to a level where the antioxidative mechanisms which are either scavenging ROS or catalysing them to less reactive molecules (Cadenas, 1997) are depleted (Bernabucci et al., 2002, Sordillo and Aitken, 2009).

During the transition period cows are often in inflammatory conditions (Cappa et al., 1989), probably caused by a release of proinflammatory cytokines such as tumor necrosis factor α (**TNFα**) and interleukines (**IL**) (Grimble, 1990; Van Miert, 1995). Reasons for this release might be an injury occurring during parturition or gastrointestinal disorders (Bertoni et al., 2008) or environmental challenges and oxidative stress (Drackley et al., 2005). Inflammatory processes are accompanied by a change of liver metabolism (Gruys et al., 2005), which results in an acute phase reaction leading to a change in acute-phase proteins. Synthesis of positive acute-phase proteins such as haptoglobin is increased, whereas negative acute-phase proteins such as albumin are produced to a lesser extent (Fleck, 1989). It has been demonstrated that haptoglobin and serum amyloid A are important acute-phase proteins in cattle and might be used to distinguish acute from chronic inflammations (Alsemgeest et al., 1994). Uchida et al. (1993) demonstrated that high haptoglobin levels are associated with high NEFA concentrations and fatty liver p.p.

According to literature (Tienken et al., 2015; Drong et al., 2016b) different changes in populations of immune cells and their functions during the transition period are associated with over conditioning and resulting high lipomobilisation. They are accompanied by elevated NEFA and BHB concentrations p.p. which are altering certain immunoreactions. Whereas neutro-

phils in bloodstream are reported to increase towards calving (Orrù et al., 2012), cows after calving experience a state of leukopenia (Gilbert et al., 1993; Detilleux et al., 1995), which is probably caused by neutrophils migrating to the reproductive tract (Gunnink, 1984). The ability of lymphocytes and neutrophils to phagocyte is impaired (Ishikawa, 1987; Detilleux et al., 1995). According to Lacetera et al. (2005) peripheral blood mononuclear cells (**PBMC**) synthesized less DNA and secreted less immunoglobulin (**Ig**) M 1 week a.p. compared to 2 and 5 weeks p.p., whereas interferon γ (**IFNγ)** was not affected by parturition but by over conditioning a.p. This finding is emphasized by Ster et al. (2012), who reported an impairment of IFNγ production due to high NEFA concentrations. Additionally, the decreased IgM secretion p.p. was more pronounced in overconditioned cows (Lacetera et al., 2005). Lacetera et al. (2007) also reported that the proliferation of PBMC was impaired in vitro when concentrations of oleic, palmitic, stearic or linoleic acid, which simulate the situation of moderate to intense lipomobilisation, were applied. Renner et al. (2013) observed a dose-dependent decreased proliferation of PBMC in vitro upon exposure to different fatty acids (both CLA-isomers, phytanic acid, linoleic acid and a mixture of fatty acids), whereas no alteration of the spectrum of cytokines produced by the PBMC was found. A similar in vitro study showed that NEFA concentrations associated with high lipomobilisation increased oxidative burst activity of PMN while simultaneously decreasing the viability and increasing necrosis of these cells (Scalia et al., 2006). NEFA were also shown to increase ROS synthesis by neutrophils (Schönfeld and Wojtczak, 2008). Hoeben et al. (1997) reported that high BHB concentrations are responsible for an impairment of the oxidative burst activity of PMN as well. Furthermore, high BHB concentrations decreased the chemotaxis ability of leukocytes (Suriyasathaporn et al., 1999). Populations of T-lymphocytes are affected by parturition, too. However, reported changes are contradicting each other. Shafer-Weaver et al. (1996) observed a decrease in population of T-cells accompanied by a shift from cytotoxic to suppressor cluster of differentiation (**CD**) 8+-cells shortly p.p. compared to mid-lactation, which might contribute to an impaired immunity following parturition (Shafer-Weaver and Sordillo, 1997). In a study by Harp et al. (1991) an increase of the CD4+-population p.p. was observed while the CD8+-population stayed constant. Contradicting results were observed by Kimura et al. (1999), who reported a decrease of CD4+-cells towards calving, which was not regained 2 weeks p.p., whereas CD8+-cells remained unaffected. This finding is contradicted by Van Kampen and Mallard (1997), who found that both T-cell-populations decreased between 3 weeks a.p. and calving and returned to the initial populations by week 16 p.p. Tienken et al. (2015) reported a decrease of both populations around calving and a following increase there-

after. Drong et al. (2016b) did not detect any influence of parturition on the proportions of CD4+- and CD8+-lymphocytes.

2.3 Conjugated linoleic acids (CLA)

The term conjugated linoleic acids (**CLA**) relates to a group of di-unsaturated fatty acids, which are derived from linoleic acid (Figure 2) and whose double bonds are separated by only one single bond in between. As the double bonds can have a *cis* or a *trans* configuration, 28 different isomers can be formed (Banni, 2002). However, the highest biological activity has been observed in *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers (Figure 2) (Banni, 2002).

CLA has been reported to elicit influences on milk composition and lactation performance, body composition, energy balance as well as on the immune system of the dairy cow. According to Baumgard et al. (2000) the *trans*-10, *cis*-12 isomer leads to a reduction of milk fat content by inhibiting the *de novo* synthesis of milk fat. The mechanisms behind milk fat content reduction might be the decreased mRNA expression of different enzymes necessary for uptake and transport of circulating fatty acids as well as for lipogenesis. Baumgard et al. (2002) observed a reduction of mRNA expression for the enzymes acetyl CoA carboxylase, fatty acid synthetase, Δ9-desaturase, lipoprotein lipase, fatty acid binding proteins, glycerol phosphate acyltransferase and acylglycerol phosphate acyltransferase upon 5 d abomasal infusion of 13.6 g/d *trans*-10, *cis*-12 CLA.

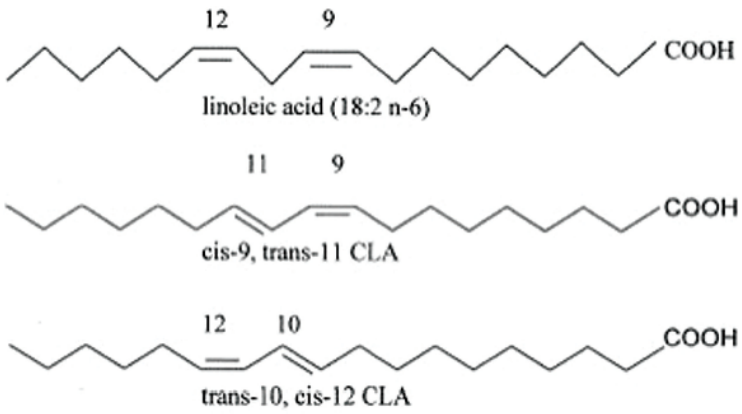

Figure 2: Structural formulas of linoleic acid, *cis*-9, *trans*-11 conjugated linoleic acid (CLA) and *trans*-10, *cis*-12 CLA, adapted from Evans et al. (2002).

CLA isomers are abundant in ruminant products such as milk and meat with the *cis*-9, *trans*-11 constituting 80% to 90% of CLA in milk fat (Bauman et al., 2000). Two ways to synthesize CLA isomers in the cow are known. They can either be produced by microbial biohydro-

genation of linoleic acid to stearic acid in the rumen (Harfoot and Hazlewood, 1997) or produced in adipose and mammary gland tissue from *trans*-vaccenic acid, which is an intermediate in the metabolism of linoleic and linolenic acid to stearic acid in the rumen (Griinari et al., 2000).

The reaction of intestinally absorbed *trans*-vaccenic acid to *cis*-9, *trans*-11 CLA is catalysed by the enzyme stearoyl-CoA desaturase (Δ9-desaturase) (Griinari et al., 2000).According to Peterson et al. (2004) the reduction in mRNA of lipogenic enzymes is due to an inhibition of the sterol response element-binding protein (**SREBP**)-1, which is responsible for activation of lipogenic genes, by *trans*-10, *cis*-12 CLA, whereas the *cis*-9, *trans*-11 isomer did not elicit any effect. According to Chilliard et al. (2000) 40% of milk fatty acids derive from de novo synthesis producing fatty acids with 16 or less carbon-chain-lengths, whereas 60% are originated from fatty acids, which are taken up from the blood stream. As the majority of fatty acids from de novo synthesis are saturated, because the activity of Δ9-desaturase is low on fatty acids with a chain length shorter than 18 carbons, supplementation of CLA might change the fatty acid profile of milk towards longer-chain fatty acids. Hussein et al. (2013) reported a decrease of *de novo* synthesised fatty acids with chain-lengths <16 of 15% (proportion) and 27% (daily yield) in ewes supplemented with 1.5 g/d *trans*-10, *cis*-12 CLA. According to Chouinard et al. (1999) abomasal infusion of 4.7 g/d *trans*-10, *cis*-12 CLA and 7.3 g/d *cis*-9, *trans*-11 resulted in a decrease of short and medium chain-length fatty acids from C4:0 to C16:0, whereas the proportions of C18:0, C18:2 and C18:3 were increased. Chouinard et al. (1999) reported transfer rates of 22.5% for *cis*-9, *trans*-11 and of 10.2% for *trans*-10, *cis*-12 CLA from abomasal infusion supplement to milk. The transfer rates from lipid-encapsulated supplements ranged from 4.6% (Moallem et al., 2010) to 7.9% (Perfield et al., 2004a). Pappritz et al. (2011a) reported a transfer rate of 6% for *trans*-10, *cis*-12 CLA from feed to milk when supplementing 3.1g/d. Thereby, supplementation of CLA might enhance CLA excretion with the milk. The described effects of CLA supplementation on milk protein content are divergent. Whereas Piamphon et al. (2009), Moallem et al. (2010) and von Soosten et al. (2011) observed a reduction of milk protein content, Medeiros et al. (2010) reported an increase. However, in those studies no effect on daily protein synthesis was observed. Therefore, the decrease of protein content might be attributed to an increase of milk yield. However, most studies show no effect of CLA supplementation on milk protein content (Bernal-Santos et al., 2003; Castaneda-Gutierrez et al.; 2005, Castañeda-Gutiérrez et al., 2007; Sigl et al., 2010; Hutchinson et al., 2011). Whereas a decreased milk fat content upon supplementation of

trans-10, *cis*-12 CLA is reported in a variety of studies (Bernal-Santos et al., 2003; Castaneda-Gutierrez et al., 2005; Piamphon et al., 2009; Pappritz et al., 2011b; von Soosten et al., 2011), effects on milk fat yield differ. Whereas some studies observed a lower milk fat yield (Medeiros et al., 2010; Moallem et al., 2010; von Soosten et al., 2011) other studies reported no effect (Castañeda-Gutiérrez et al., 2007; Piamphon et al., 2009; Pappritz et al., 2011b). The decreased milk fat content might be accompanied by a higher milk yield leading to a repartition of energy to increased milk yields. Consequently, no milk fat depression (**MFD;** reduction of milk fat content and milk fat yield) was observed. Because milk fat is the major energy source in milk, a MFD might be favourable in order to counteract the negative EB of dairy cows during the transition period. Nevertheless, an improvement of EB was observed in several studies. Kay et al. (2006) reported an increased EB of 24.4 MJ NE_L/d compared to the control group in cows receiving 21 g/d *trans*-10, *cis*-12 CLA. Sippel et al. (2009) observed a positive EB in daily doses of *trans*-10, *cis*-12 CLA from 13 g on. Studies, which did not observe a positive effect of CLA on the EB, attribute this to a reduced DMI in cows treated with CLA (Selberg et al., 2004; Castaneda-Gutierrez et al.; 2005, Harvatine et al., 2009; Pappritz et al., 2011b). As described above, mobilization of adipose tissue results in increased NEFA concentrations in the blood. According to Odens et al. (2007) NEFA concentrations in plasma were decreased by CLA supplementation. This finding is emphasized by Hutchinson et al. (2011) who reported a decrease of the peak NEFA concentration as well. However, Moore et al. (2004) did not observe any effect of CLA on NEFA concentrations. Whereas increased NEFA concentrations reflect lipolysis, CLA might also have a direct influence on body composition. Studies in rodents report a decrease of adipose tissue for mice (Park et al., 1997; DeLany et al., 1999; Tsuboyama-Kasaoka et al., 2000; Clément et al., 2002; Warren et al., 2003; Poirier et al., 2005) and rats (Stangl, 2000; Yamasaki et al., 2003b) after supplementation with CLA. A reduction of adipose tissue after CLA supplementation was also reported for humans with metabolic syndrome (Risérus et al., 2001) or diabetes type II (Belury et al., 2003) as well as for growing pigs (Ostrowska et al., 1999). Whereas Park et al. (1997) suggested that a decreased lipogenesis is responsible for the observed effects, Tsuboyama-Kasaoka et al. (2000) observed that supplementation with CLA resulted in an induction of apoptosis of adipocytes. According to Yamasaki et al. (1999) CLA leads to decreased triglyceride concentrations in adipose tissue cells. These findings indicate that CLA might not only decrease lipogenesis but also increase lipolysis. Several mechanisms behind these influences on adipose tissue were discussed. Park et al. (1997) holds the inhibition of lipoprotein lipase accountable for the reduced adipose storage. Tsuboyama-Kasaoka et al.

(2000) observed that concentration of the TNFα was increased 12 times after treatment with CLA. TNFα is responsible for induction of apoptosis in adipocytes and increases the expression of uncoupling protein 2 mRNA, which might result in an increased expression of energy expenditure. Kang et al. (2003) reported that supplementation of *trans*-10, *cis*-12 CLA decreased the mRNA of the fatty acid synthase, the adipocyte lipid binding protein, the enhancer binding protein alpha and the peroxisome proliferator activated receptor gamma (**PPARγ**) in mice preadipocytes. These mRNA reductions might inhibit the differentiation of preadipocytes. Conversely, *trans*-10, *cis*-12 CLA reduced adipocyte volume, but not the total number of adipocytes in rats (Azain et al., 2000). Whereas Park et al. (1997) reported that CLA increased the activity of CPT-1 and thereby might lead to an increased fatty acid oxidation, these results were obtained only for activity in adipose tissue and skeletal muscle in mice. However, according to Martin et al. (2000) CLA did not increase activity of skeletal muscle, heart or hepatic CPT-1, whereas activity in adipose tissue was increased by the *trans*-10, *cis*-12 isomer in rats. Investigations on the effects of CLA on exact body composition of cows are spare and results vary. Harvatine et al. (2009) reported an increase in enzymes responsible for lipogenesis accompanied by MFD upon abomasal supplementation of 7.5 g/d *trans*-10, *cis*-12 CLA for 4 days. Consequently, it was suggested that the energy spared due to MFD is repartitioned to the adipose tissues. Von Soosten et al. (2011) supplemented primiparous dairy cows with 6 g/d *trans*-10, *cis*-12 CLA and observed a decrease in mobilization of the retroperitoneal adipose tissue depot compared to the control group, whereas omental, mesenterial and subcutaneous adipose tissue depots were not affected by treatment. Consequently, it was concluded that the retroperitoneal adipose tissue depot might be the most sensitive one in dairy cows. Akter et al., 2011 investigated the adipocyte sizes of these cows and observed that they were smaller after 42 days of treatment with 6 g/d *trans*-10, *cis*-12 CLA in subcutaneous sternum fat as well as in the mesenterial adipose tissue. After 105 days of supplementation adipocyte size in subcutaneous fat of the tail head, and also the omental, mesenterial and retroperitoneal adipose tissue was decreased. These findings suggest a lipolytic or antilipogenic effect of CLA, which contradicts the findings of Harvatine et al. (2009). This contradiction might possibly be attributed to a decreased duodenal availability of the used rumen-protected supplement compared to the abomasal infusion.

As explained above, the immune function of dairy cows is impaired during the transition period and animals are subjected to oxidative stress. Several studies suggested that CLA might improve immune function. Hayek et al. (1999) observed an increased production of spleno-

cyte IL-2 in mice regardless of age. According to Kelley et al. (2002) both CLA isomers resulted in increased IL-4, IL-6 and TNFα concentrations in mice as well. Yamasaki et al. (2003a) reported that *trans*-10, *cis*-12 CLA increased the proportion of B lymphocytes in mice, whereas the *cis*-9, *trans*-11 isomer decreased it. Additionally, the proportion of CD4+-cells was increased by *trans*-10, *cis*-12 CLA, whereas the proportion of CD8+-cells was increased by *cis*-9, *trans*-11 CLA and a 1:1 mixture of both isomers. Regarding immunoglobulins an increase of IgA and IgM production was observed for animals supplemented with *trans*-10, *cis*-12 CLA upon stimulation with concavalin A (**ConA**), whereas *cis*-9, *trans*-11 CLA did not elicit any effect on Ig production. However, Ramírez-Santana et al. (2009) investigated the influence of a 80:20 mixture of *cis*-9, *trans*-11: *trans*-10, *cis*-12 on Ig concentrations of rats and reported an increase of IgA, IgG and IgM concentrations in those animals, who already received this supplementation through placental uptake. These findings indicate that the effects of CLA on the immune system of non-ruminants might be depending on the isomers in the supplement, the species as well as the time of supplementation. IgG concentrations in serum of goats were increased after supplementation of 12 g/kg DM feed/d (50:50 mixture of *cis*-9, *trans*-11: *trans*-10, *cis*-12) from the 3rd month of lactation on. IgG concentrations in colostrum remained unaffected (Castro et al., 2006). This finding is confirmed by Odens et al. (2007), who detected no differences of IgG in colostrum of dairy cows on d 9 p.p. after supplementation of 3.3 or 9.7 g/d *trans*-10, *cis*-12 CLA. Renner et al. (2012) reported that the basal expression of several cytokines (IL-4, IL-10, IL-12, TNF-α, IFNγ) was not altered by supplementation with 6.0 g/d *trans*-10, *cis*-12 and 5.7 g/d *cis*-9, *trans*-11 CLA of unstimulated PBMC and splenocytes *ex vivo*. The mRNA of the acute phase protein haptoglobin was reduced in omental and subcutaneous whither fat, whereas serum and liver mRNA were not affected by treatment with CLA (Saremi et al., 2012). Basiricò et al. (2015) and Basiricò et al. (2017) investigated the effects of CLA on bovine mammary cells in vitro and observed an improved reaction to oxidative stress, particularly in cells treated with the *trans*-10, *cis*-12 isomer, due to increased glutathione concentrations which were accompanied by an NADPH increase, as well as increased activity of γ-glutamyl-cysteine ligase, superoxide dismutase, glutathione peroxidase and glutathione S-transferase activity and a decreased activity of glutathione reductase.

2.4 Vitamin E (Vit. E)

The term "Vitamin E" is used for a group of different tocochromanols, consisting of alpha, beta-, gamma- and delta-tocopherol as well as corresponding tocotrienols, which differ in side chain and substitutes (Figure 3).

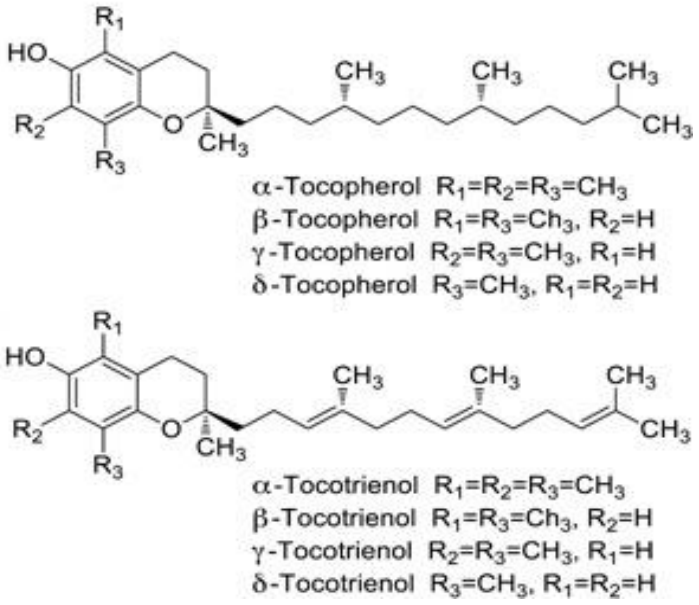

Figure 3: Structural formulas of Vit. E, adapted from Xu et al. (2015).

Each isomer has 3 stereocenters in the side chain, which leads to 32 possible tocopherol isomers. α-tocopherol is the predominant form in mammalian tissues (Jiang et al., 2001) and its natural form has the RRR-conformation of the side chain's chiral centers. Due to its higher stability to oxygen the synthetic DL- α-tocopheryl acetate is used in supplements (Burton and Traber, 1990), which must be activated in the body and consist of a mixture of isomers.

Natural sources for Vit. E are plants, in particular germs (McDowell et al., 1996), whereby the dominant form is γ-tocopherol (Jiang et al., 2001). According to McDowell et al. (1996) suitable feeds for ruminants containing high amounts of Vit. E are green forages with the highest amounts being found in alfalfa. However, natural Vit. E has been reported to be unstable, which results in losses of activity during processing and storage (Coelho, 1991). This instability might be attributed to conditions often occurring in processing (heat, moisture, oxygen) and trace elements as iron salts interacting with Vit. E (McDowell et al., 1996). The Vit. E content of forages is reduced remarkably by storage as well as dehydration which might lead to losses of up to 73% (McDowell et al., 1996). King et al. (1967) reported losses of 80% due to haymaking, whereas rapid dehydration and ensiling resulted in conservation of most Vit. E.

However, according to Kivmae and Carpena (1973) Vit. E content in silages is decreased up to 1/6 compared to freshly cut forages. For corn a reduction of α-tocopherol from 5.7 mg/kg DM to 1 mg/kg DM due to ensiling is reported (Young et al., 1978). Consequently, Vit. E concentrations in plasma of cows fed with high proportions of silage were observed to decrease (Goff and Stabel, 1990; Politis et al., 1995). Investigations on ruminal degradation of Vit. E are contradicting. Alderson et al. (1971) reported that ruminal Vit. E disappearance increased with increasing proportion of corn in the diet, whereas no preintestinal absorption of Vit. E was observed in ewes. Ruminal disappearance of up to 52% depending on application form were registered for steers (Shin and Owens, 1990). Chikunya et al. (2004) investigated the disappearance of Vit. E in sheep as well and observed that proportional losses were lower in animals supplemented with higher doses. This finding might indicate that there is an absolute rather than a relative amount, which can disappear in the rumen. However, the mechanism behind disappearance is unclear. According to Alderson et al. (1971) transport through the rumen epithelium does not account for Vit. E losses. Leedle et al. (1993) suggest that Vit. E is not degraded in the rumen by microorganisms but rather incomplete extraction of Vit. E in samples for analysis might be responsible.

Vit. E exerts a diversity of effects in the body. The biohydrogenation shift from *trans*-11 to *trans*-10 fatty acids in the rumen is decreased. This decrease results in higher milk fat content and yield as well as a reduction of the proportion of *trans*-10 C18:1 fatty acid (Pottier et al., 2006). Additionally, the RRR form of α-tocopherol serves as a precursor for α-tocopherolquinone, which is necessary in the mitochondria as a co-factor for fatty acid desaturases. High concentrations of Vit. E in the feed might thereby increase the activity of the Δ9-desaturase, which in turn results in an increased conversion of *trans*-11 octadecenoic acid to CLA (Infante, 1999). However, on the other hand α-tocopherolquinone has been shown to be a precursor for ROS. This mechanism might be responsible, that high doses of Vit. E do not elicit positive effects in humans (Crisostomo et al., 2007). Vit. E has strong antioxidant properties and acts by scavenging radicals with the free hydroxyl group (Burton and Traber, 1990; Nakamura and Omaye, 2010). Vit. E is particularly important for the integrity of cell membranes due to its lipophilicity (Cheeseman et al., 1986). When scavenging radicals α-tocopherol is converted to the inactive α-tocopheryl and can be reactivated by Vitamin C, ubiquinol-10 or bilirubin (Burton and Ingold, 1986). Furthermore, Vit. E binds to the PPARγ (Rimbach et al., 2002) and thereby increases the expression of a diversity of antioxidative enzymes such as superoxide dismutase and catalase (Nakamura and Omaye, 2010). Next to its

antioxidant properties, Vit. E influences different cell signaling pathways and thereby inhibits proliferation of smooth muscle cells and inflammatory reactions (Boscoboinik et al., 1991; Rimbach et al., 2002).

The described actions of Vit. E might lead to beneficial effects on the immune system of cows and therefore an improvement of the health situation during the transition period. Studies showed that Vit. E improved the ability of neutrophils to kill bacteria, whereas the phagocytic activity remained unaffected (Gyang et al., 1984; Hogan et al., 1990; Hogan et al., 1992). Politis et al. (1995) observed that supplementation of 3,000 IU/d Vit. E was sufficient to prevent a decrease of IL-1 and superoxide anion production by neutrophils during the transition period, thereby preventing a decreased reaction against pathogens. Additionally, this dose also prevented the decreased chemotaxis of neutrophils around parturition (Politis et al., 1996). It was demonstrated that 3,000 IU/d Vit. E improved IL-1 as well as major histocompability class II antigen expression of blood monocytes, whereas the function of macrophages in the mammary gland and the ability of lymphocytes to react to stimulation with ConA were not improved (Politis et al., 1995; Politis et al., 1996). Tengerdy et al. (1973) reported an increase of Ig production in mice supplemented with Vit. E. Smith et al. (1984) observed a reduction of clinical mastitis by 37% in cows, which were supplemented with 740 IU/d Vit. E during the dry period. Weiss et al. (1997) supplemented cows with either 1,000 IU/d or 4,000 IU/d and found a reduction of mastitis incidence by 30% and 89% respectively. These findings suggest, that the effect of Vit. E supplementation on mastitis incidence might be highly dose-dependent. Conversely, Batra et al. (1992) reported no effect of 1,000 IU/d Vit. E supplementation on mastitis incidence. However, cows in that trial were also selenium-deficient, which might have also had influences on the functionality of the immune system (Batra et al., 1992). According to Miller et al. (1993) and Erskine et al. (1997) Vit. E supplementation did also decrease the incidence of placenta retention. This effect, however, depends on the serum α-tocopherol concentrations a.p., as LeBlanc et al. (2002) reported. Vit. E tended to reduce the incidence of retained placenta in cows having serum α-tocopherol/cholesterol mass ratios lower than 2.5×10^{-3} a.p., whereas the incidence in cows with ratios higher than 2.5×10^{-3} a.p. was not reduced.

3. Scope of the thesis

As presented in the background, dairy cows in the transition period are challenged by various endocrinological and metabolic changes, which might compromise immune-reactivity. Whereas individual effects of CLA and Vit. E are described in literature, it has also been indicated that they might interact with each other and thereby might elicit interactive effects. The milk-fat depression caused by CLA-supplementation on dairy cows is well investigated. However, the influences of CLA on lipomobilisation are inconsistently described in literature. CLA has been reported to increase excretion of Vit. E with milk. Vit. E in turn might increase the activity of desaturases and thereby increase the CLA excretion with milk. While Vit. E acts as an antioxidant and has been demonstrated to elicit positive effects on the health of dairy cows during the transition period, the influence of CLA on the bovine immune system is less well investigated. Therefore, this thesis aimed to test the following hypotheses:

1. Supplementation with CLA results in MFD, which results in spared energy. As a consequence lipolysis and lipid accumulation in the liver is decreased, while supplementation with Vit. E does not affect milk fat production or mass of adipose tissues (**Paper I**)
2. Supplementation with CLA and Vit. E shows a synergistic effect above supplementation with solely CLA on excretion of CLA and the vitamins retinol, β-carotene and Vit. E with milk (**Paper II**)
3. Supplementation with CLA leads to a higher reduction of Vit. E in the serum around parturition due to higher Vit. E excretion with milk (**Paper II**)
4. Supplementation with CLA and Vit. E increases the ability of dairy cows to cope with the immune suppression caused by parturition (**Paper III**)

To investigate these topics an animal model which had been shown to be suitable to create cow groups sensitive to excessive lipomobilisation p.p. and ketosis type II was applied. This model consists of a combination of a high concentrate proportion during the dry period (60% of total DMI), a high BCS before parturition and a decelerated increase of the concentrate proportion p.p. (from 30 to 50% of total DMI in three weeks). Sixty-four cows were allocated to the experimental groups. The aim was a mean BCS higher than 3.5 with a SD of 0.5 in each group. Therefore, only cows with a BCS of 3 and higher were enrolled in the study. The treatment groups received either 8.4 g of *trans*-10, *cis*-12 and 8.4 g of *cis*-9, *trans*-11 CLA

(BASF Lutrell, Lampertheim, Germany) per day per animal (CLA, n = 16), 2,327 IU of vitamin E/d per animal (BASF Lutavit E 50; Vit. E, n = 15), or both supplements (CLA + Vit. E, n = 12). Animals in the control group (n = 16) and the Vit. E group received a rumen-protected control fat supplement (BASF Silafat consisting of hydrogenated vegetable fat, 11% of which is C16:0, 89% is C18:0) to ensure caloric balance of the diets. All diets were isocaloric and were fed from day 42 a.p. until day 70 p.p. This experimental design and setup was used to test the above formulated hypotheses.

4. Paper I

Influence of conjugated linoleic acid and vitamin E on performance, energy metabolism, and change of fat depot mass in transitional dairy cows

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Influence of conjugated linoleic acid and vitamin E on performance, energy metabolism, and change of fat depot mass in transitional dairy cows

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ABSTRACT

The objective of this experiment was to determine the effects of conjugated linoleic acid (CLA) and vitamin E as well as their interaction on performance variables and lipomobilization during late pregnancy and early lactation (wk 6 antepartum until wk 10 postpartum). For this purpose, 59 pluriparous German Holstein cows were assigned to 4 dietary groups in a 2 × 2 design with the factors CLA and vitamin E at 2 levels. For this trial, we selected cows with a high body condition score because they are more likely to mobilize fat and consequently are at a higher risk of developing ketosis. Furthermore, concentrate proportions were adjusted to provoke ketosis. Lactation performance variables were analyzed in 3 periods (d 42 antepartum until calving, 1 to 21 d in milk, 22 to 70 d in milk). Dry matter intake and net energy intake were reduced in animals receiving CLA. Milk fat content was reduced in the CLA group compared with the control group (4.83 vs. 5.46% in period 2; 3.36 vs. 4.57% in period 3). In the vitamin E and the CLA + vitamin E groups, reduction of milk fat content was observed in period 3 (3.76 vs. 4.57% compared with the control group). Milk yield was not affected by treatment. β-Hydroxybutyrate concentrations and liver lipid contents were not influenced by CLA or vitamin E. Moreover, longitudinal changes of adipose tissue depot mass were not affected by dietary treatments. Results suggest that the effects CLA had on milk composition were compensated by an increased milk yield and a decreased dry matter intake. Reduced milk energy output in CLA-treated animals was compensated by a reduced dry matter intake. Therefore, the net energy balance was not affected by either treatment. Consequently, we found no group effect on the mobilization of adipose tissue.

Key words: dairy cow, conjugated linoleic acid, vitamin E, fat depot

INTRODUCTION

During the transition period, dairy cows are confronted with profound changes in their metabolic status. The increased nutrient demand caused by the growth of the fetus and the onset of lactogenesis cannot be compensated due to a reduced feed intake (Grummer et al., 1995). This results in a negative energy balance, which is partly compensated by the mobilization of fatty acids from adipose tissue and β-oxidation with the help of oxaloacetate in the liver. In a state of energy deficit, oxaloacetate is mainly needed for gluconeogenesis; therefore, a relative lack of oxaloacetate exists in the citrate cycle. As a consequence, the fatty acids can only partly be metabolized by the liver and ketone bodies (acetate, acetoacetate, and BHB) are produced from acetyl-CoA. Although ketone bodies can serve as an energy source for skeletal muscle, heart, kidney, and mammary glands in ruminants, Heitmann et al. (1987) showed that the extraction percentages of ketone bodies from blood by these organs are constant. Consequently, a higher ketone body production in the liver results in higher blood levels. Levels of BHB higher than 1.2 mmol/L are defined as the threshold for subclinical ketosis (Nielen et al., 1994). According to Schulz et al. (2014), a high concentrate proportion before calving, a high BCS at calving, and a delayed increase of concentrate proportion after parturition stimulate lipolysis; as a consequence, cows are more sensitive to develop a subclinical ketosis. Subclinical ketotic cows show no clinical signs of ketosis, but their reproductive health and economic productivity might be impaired (Drackley, 1999; Janovick et al., 2011). Therefore, the main objective of feeding in the transitional phase is the reduction of

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the negative energy balance. As milk fat is the major source of energy in milk, a reduction of milk fat percentage leads to a reduced milk energy output and, consequently, might partly counterbalance the negative energy balance. Baumgard et al. (2000) showed that the *trans*-10,*cis*-12 isomer of CLA is specifically responsible for the reduction of milk fat content and total milk fat yield (milk fat depression) by reducing the de novo milk fat synthesis in the udder, which results in a more efficient use of the ME. However, according to Bauman et al. (2008), supplementation of CLA in the state of early lactation or underfeeding also leads to a higher total milk yield and, therefore, repartitioning of energy. Consequently, CLA might have no significant effect on the total energy balance of the animal. Energy-dense diets antepartum (**a.p.**) lead to an increased DMI and increased accretion of internal fat depots without influencing carcass weight or BCS (Drackley et al., 2014). Pronounced lipomobilization caused by high fat depot mass is a risk factor for fatty liver and ketosis (Grummer, 1993; Bobe et al., 2004) and enhances the sensitivity to oxidative stress (Bernabucci et al., 2005), whereas vitamin E acts as an antioxidant (Rimbach et al., 2002; Nakamura and Omaye, 2010). Conjugated linoleic acid has been shown to have a decelerating influence on the mobilization of the retroperitoneal adipose tissue depot (von Soosten et al., 2011). To the contrary, Akter et al. (2011) reported a lipolytic or antilipogenic effect of CLA on the adipose tissue. This makes it necessary to evaluate the influence of CLA on lipomobilization. Literature indicates possible interactions between CLA and vitamin E, as CLA enhances α-tocopherol concentration in muscle tissue (Schlegel et al., 2012), probably by preventing the degradation of vitamin E in the liver (Chao et al., 2010). Furthermore, Pottier et al. (2006) observed an influence of vitamin E on biohydrogenation pathways in the rumen, where vitamin E counteracted the *trans*-11 to *trans*-10 shift.

Therefore, the current experiment aimed to investigate the effect that CLA has on lactation performance, energy metabolism, and lipomobilization, as well as the interactions between CLA and vitamin E in dairy cows during the transition period and the first 10 wk of lactation. Our objective was to clarify the influence that CLA has on lipomobilization of fat depots and resulting subclinical ketosis. Furthermore, we wanted to evaluate whether treatment with vitamin E reduces the milk fat-decreasing effect of CLA.

MATERIALS AND METHODS

The study was conducted at the experimental station of the Friedrich Loeffler Institute in Brunswick, Germany. The experiment was carried out in accordance with the German Animal Welfare Act approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany).

Experimental Design and Diets

The experimental design was a 2 × 2 factorial design with CLA and vitamin E as main factors, resulting in 3 intervention groups (CLA, vitamin E, CLA + vitamin E) and 1 control group. We used the experimental strategies as proposed by Schulz et al. (2014) to generate cow groups suited for testing of possible protective effects of CLA and vitamin E. Sixty-four pluriparous German Holstein cows were allocated to these groups 8 wk prior (−42 d a.p.) to the calculated calving date based on their BCS (Edmonson et al., 1989), which was targeted higher than 3.5 with a standard deviation of 0.5 in each group. Further criteria were milk yield and milk composition of the previous lactation, BW, and number of lactation. In the control group, cows were in the second (n = 10), third (n = 4), fourth (n = 1), and fifth (n = 1) lactation. For the CLA group, cows in the second (n = 9), third lactation (n = 3), fourth (n = 1), sixth (n = 1), eighth (n = 1) and ninth (n = 1) lactation were included. In the vitamin E group, 10 cows were in their second (n = 10), third (n = 3), and fourth (n = 2) lactation. Cows in the CLA + vitamin E group were in their second (n = 5), third (n = 4), fifth (n = 1), and eighth (n = 2) lactation. The study was divided into 3 periods: period 1 from 42 d a.p. until calving, period 2 from calving until d 21 postpartum (**p.p.**), and period 3 from d 21 until 70 p.p.

The animals were fed a standardized partial mixed ration (**PMR**) during the whole experiment, which was provided ad libitum by self-feeding stations (type RIC, Insentec B.V., Marknesse, the Netherlands). Concentrate was administered at 3 kg/d per animal via computerized self-feeding stations (Insentec B.V.).

The components and the chemical composition of the feedstuffs are presented in Table 1. During the first period the ration was composed of 60% concentrate and 40% silage (50% maize, 50% grass silage on a DM basis). After parturition the concentrate proportion increased from 30 to 50% until d 21 p.p, where it remained until d 70 p.p. The treatment groups received either conjugated linoleic acid (BASF Lutrell, Lampertheim, Germany) containing 8.4 g of *trans*-10,*cis*-12 and 8.4 g of *cis*-9,*trans*-11 per day per animal (**CLA**, n = 16), 2,327 IU of vitamin E/d per animal (BASF Lutavit E 50; **Vit. E**, n = 15), or both supplements (**CLA + Vit. E**, n = 12) from d 42 a.p. to 70 p.p. The control group (n = 16) as well as the Vit. E group received a

rumen-protected control fat supplement (BASF Silafat) for caloric balance. Silafat consists of hydrogenated vegetable fat, 11% of which is C16:0 (palmitic acid), 89% is C18:0 (stearic acid). All diets were isocaloric.

Sample Collection

Samples of roughage feed were taken twice weekly and pooled to a collective sample for periods of 4 wk. Concentrate samples were taken once weekly for every group and also pooled to a collective sample for periods of 4 wk.

Dry matter intake was recorded daily for each individual cow by computerized feeding stations for PMR and concentrate. The BCS was assessed by the same person weekly on a 5-point scale according to Edmonson et al. (1989) during the whole experiment. Live weight was recorded weekly in period 1. In period 2 and 3, cows were weighed twice daily after each milking.

Milk yield was assessed twice daily during milking at 0530 and 1530 h via automatic milk counters (Lemmer Fullwood GmbH, Lohmar, Germany). Milk samples were taken twice weekly and stored at 4°C until further analysis.

Blood samples were taken after morning milking on d 42, 14, 7, and 3 a.p. and d 1, 3, 7, 10, 14, 21, 28, 36, 42, 56, and 70 p.p. from a jugular vein using serum and EDTA-plasma tubes. Serum and plasma samples were

Table 1. Ingredients and chemical composition of concentrate and roughage during the experimental period from d 42 antepartum until d 70 postpartum[1]

	Concentrate					Roughage[2]	
Item	LAC[3]	CLA[4]	SF[5]	VE[6]	DRY[7]	Maize silage	Grass silage
Ingredient, %							
Wheat	41	41	41	41	41		
Dried sugarbeet pulp	30.3	24.95	26.05	29.55	30.5		
Rapeseed meal	20	20	20	20	20		
Soybean meal	6.5	6.5	6.5	6.5	6.5		
CLA supplement	—	5.5	—	—	—		
Silafat	—	—	4.4	—	—		
Vitamin E	—	0.05	0.05	0.75	—		
Vitamin/mineral premix[8]	—	2.0	2.0	—	2.0		
Vitamin/mineral premix[9]	2.0	—	—	2.0	—		
Calcium carbonate	0.2	—	—	0.2	—		
Chemical analysis							
DM, g/kg	879	884	883	886	875	340	302
Nutrient, g/kg of DM							
Crude ash	64	67	57	66	59	36	112
CP	188	183	187	187	191	59	124
Ether extract	25	64	67	28	25	31	34
Crude fiber	107	93	95	102	98	199	283
NDF	267	243	262	258	272	399	496
Energy,[10] MJ/kg of DM							
ME	12.9	13.4	13.6	12.9	13.0	10.9	9.8
NE_L	8.2	8.6	8.6	8.2	8.2	7.0	5.4
DL-α-tocopheryl acetate, IU/kg of DM	—	217	188	2,453	—	—	—
trans-10,*cis*-12 CLA, g/kg of DM	<0.01	4.7	<0.01	<0.01	—	—	—

[1]Concentrate allocation (original substance) per group before parturition: control group: 2 kg of SF, 1 kg of LAC; CLA group: 2 kg of CLA, 1 kg of LAC; vitamin E group: 2 kg of SF, 1 kg of vitamin E; CLA + vitamin E group: 2 kg of CLA, 1 kg of vitamin E. Concentrate allocation (original substance) per group after parturition: control group: 2 kg of SF, 1 kg of LAC; CLA group: 2 kg of CLA, 1 kg of LAC; vitamin E group: 2 kg of SF, 1 kg of vitamin E; CLA + vitamin E group: 2 kg of CLA, 1 kg of vitamin E.

[2]50% maize silage, 50% grass silage on a DM basis.

[3]Concentrate for lactation (LAC).

[4]CLA concentrate.

[5]Silafat (SF) concentrate, BASF, Lampertheim, Germany.

[6]Vitamin E concentrate.

[7]Concentrate for dry cows.

[8]Ingredients per kilogram of mineral feed: 50 g of Ca; 120 g of Na; 70 g of P; 50 g of Mg; 7 g of Zn; 4.8 g of Mn; 1.3 g of Cu; 100 mg of I; 50 mg of Se; 35 mg of Co; 800,000 IU of vitamin A; 100,000 IU of vitamin D_3.

[9]Ingredients per kilogram of mineral feed: 140 g of Ca; 120 g of Na; 70 g of P; 40 g of Mg; 6 g of Zn; 5.4 g of Mn; 1 g of Cu; 100 mg of I; 40 mg of Se; 25 mg of Co; 1,000,000 IU of vitamin A; 100,000 IU of vitamin D_3.

[10]Calculation based on equations for calculation of energy content in feedstuffs published by the GfE (2001, 2008, 2009).

centrifuged (Heraeus Varifuge 3.0R Heraeus, Osterode, Germany; 2,123 × *g*, 15°C, 15 min) and stored at −80°C until analysis.

Liver biopsies of approximately 400 mg/animal were taken from 31 cows at d 42 a.p. and d 7, 28, and 70 p.p. Of the 31 cows, 8 were in the control, CLA and Vit. E groups, respectively. In the CLA + Vit. E group, biopsies were taken from 7 cows. All biopsies were collected under local anesthesia and sonographic control using the Bard Magnum biopsy system with corresponding sterile needles (C.R. Bard Inc., Murray Hill, NJ). Samples were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Ultrasonic measurements for the assessment of adipose tissues (subcutaneous, retroperitoneal, mesenteric, and omental) were performed at different sites on the animal according to Raschka et al. (2016). A Mindray M5 Vet (Mindray, Shenzhen, China) diagnostic ultrasound system equipped with a linear (6 MHz, Mindray 6LE5Vs) and a convex probe (3 MHz, Mindray 3C5s) were used. To avoid bias, measurements were done without applying any external pressure and each site done in duplicate. The thickness of the fat layers was measured in millimeters and the following ultrasound sites were used in the subsequent calculation of adipose depots: on 12th rib at the same height as the last point (**R12**), at the point of interception of the line from the last lumbar vertebrae in the ventral direction and the line between the last rib and the greater trochanter parallel to the topline of the animal [skin to end of musculature (**AW1b**)], in the middle of the paralumbar fossa [skin to end of musculature (**AW3b**) and skin to peritioneum (**AW3c**)], backfat thickness (**BFT**), between the 2 next transverse processes cranial from the caudal pole of the kidney from dorsal between the transverse processes of the lumbar vertebrae measuring from skin to peritoneum [skin to peritoneum (**KD2c**)], and between the 2 next transverse processes cranial from KD2 [skin to end of kidney (**KD3b**)].

Analyses

Samples of PMR and concentrate were analyzed according to the standard methods defined by the VDLUFA (1993; method numbers in brackets) for DM (3.1), crude ash (8.1), CP (4.1.2), ether extract (5.1.1), crude fiber (6.1.1), as well as NDF. Concentrate samples were analyzed for tocopherol acetate according to a modified version of the tocopherol acetate JP ed16 method (JP Committee, Pharmaceutical Affairs and Food Sanitation Council, Japanese Minister of Health, Labour and Welfare, 2011). Conjugated linoleic acid isomers were analyzed by HPLC. Samples of maximal 20 g were filled in Erlenmeyer flasks and mixed with 25 mg of butylhydroxytoluol, 300 mg of sodium ascorbate, 45 mL of H_2O, 40 mL of ethanol, and 10 mL of acetic acid. The expected CLA content was 6 to 300 mg. The Erlenmeyer flasks were placed in ultrasonic baths at 60°C for at least 15 min. After cooling to room temperature 100 mL of cyclohexane/ethyl acetate (80:20) were added. The flasks were placed on a magnetic stirrer for 30 min. After adding 70 mL of saturated saline solution, samples were stirred again for at least 10 min. One milliliter of the extract was dried under nitrogen stream in a heating block at 60°C. One milliliter of hydrogen chloride in methanol (1.0 mL of acetyl chloride/100 mL of methanol) was added. After shaking for 20 min, 1 mL of H_2O and 2 mL of cyclohexane were added and samples were shaken. The organic phase was filtered through a 0.45-μm disposable filter into HPLC vials. The HPLC measurement was done using 3 ChromSpher 5 Lipids (5 μm; 4.6 × 250 mm) columns (Varian, Agilent, Santa Clara, CA) and n-hexane/acetonitrile/diethyl ether (994:1:5) as a mobile phase. Ten microliters of sample were injected at a flow rate of 1 mL/min. The oven temperature was kept at 10°C. The wavelength of the detector was 233 nm with a bandwidth of 8 nm. Gamma-tocopherol was analyzed with an in-house method. Milk samples were analyzed by an infrared milk analyzer (Milkoscan FT 6000; Foss Electric, Hillerød, Denmark) for fat, protein, lactose, and urea concentrations. Serum samples were analyzed for BHB, fatty acids, and glucose by a photometric measurement system (Eurolyser VET CCA, Salzburg, Austria). Samples of liver were analyzed for total lipid content using a gravimetrical method by Starke et al. (2010).

Calculations and Statistical Analysis

The performance variables DMI, energy intake, energy balance, milk yield, and milk components were calculated as weekly means before statistical evaluation. The variables of energy metabolism were calculated according to established equations. For calculating the NE_M and NE_L and the milk energy concentration, the equations published by the Society of Nutrition Physiology (GfE 2001) were used, where $BW^{0.75}$ is metabolic BW:

$$NE_M \text{ (MJ of } NE_L/d) = 0.294 \times BW^{0.75},$$

$$NE_L \text{ (MJ of } NE_L/d) = [\text{milk energy concentration (MJ of } NE_L/d) + 0.086] \times \text{milk yield (kg/d)}, \text{ and}$$

$$\text{Milk energy concentration (MJ of NE}_\text{L}\text{/d)} = 0.3 \times \text{milk fat (\%)} + 0.21 \times \text{milk protein (\%)} + 0.95\ \text{NE}_\text{L}\ \text{(MJ of NE}_\text{L}\text{/d)}.$$

The equation by Gaines (1928) was used to calculate the FCM:

$$\text{4\% FCM (kg/d)} = \{[\text{milk fat (\%)} \times 0.15] + 0.4\} \times \text{milk yield (kg/d)}.$$

For calculating the ECM, the equation by Sjaunja et al. (1990) was used:

$$\text{ECM (kg/d)} = \text{milk yield (kg/d)} \times \{[38.3 \times \text{milk fat (g/kg)} + 24.2 \times \text{milk protein (g/kg)} + 16.54 \times \text{milk lactose (g/kg)} + 20.7]/3{,}140\}.$$

The energy intake per day was calculated by multiplying the energy content of the feedstuff by the daily DMI. The following equation was used to calculate the net energy balance:

$$\text{Net energy balance (}\mathbf{NEB}\text{; MJ of NE}_\text{L}\text{/d)} = \text{energy intake (MJ of NE}_\text{L}\text{/d)} - \text{NE}_\text{M}\ \text{(MJ of NE}_\text{L}\text{/d)} - \text{NE}_\text{L}\ \text{(MJ of NE}_\text{L}\text{/d)},$$

Additionally, 13 MJ of NE_L/d were subtracted from wk 6 to 3 a.p., and 18 MJ of NE_L/d for the last 3 wk before calving to account for the additional late gestational requirements. The feed efficiency (kg/kg) was calculated by dividing ECM by DMI. To be able to compare the efficiency of the different feeding groups to the complete herd involved in this experiment, the residual energy intake (**RSEI**; Hurley et al., 2016) was calculated as a second variable of efficiency according to the following equation:

$$\text{RSEI} = \text{NE}_\text{L}\ \text{measured intake} - \text{NE}_\text{L}\ \text{estimated intake}.$$

Variables and coefficients, which were used for calculating the estimated NE_L intake, were evaluated using a nonlinear regression model.

The subcutaneous (SCAT), retroperitoneal (RPAT), omental (OMAT), and mesenteric (MAT) adipose tissue masses were calculated based on ultrasound measurements on different sites using the regressions according to (Raschka et al., 2016):

$$\text{SCAT} = -6.66 + 0.72 \times \text{R12} + 0.31 \times \text{AW3c},$$

$$\text{RPAT} = -9.55 + 0.62 \times \text{R12} + 0.06 \times \text{KD3b},$$

$$\text{OMAT} = -2.32 + 0.55 \times \text{BFT} + 0.37 \times \text{AW3b, and}$$

$$\text{MAT} = -12.8 + 0.38 \times \text{AW1b} + 1.73 \times \text{AW3b} - 1.45 \times \text{AW3c} + 0.07 \times \text{KD2c}.$$

To evaluate accretion or mobilization of each adipose depot, the mean change between d 42 a.p. and 7 p.p. (phase 1), d 8 and 28 p.p. (phase 2), as well as between d 29 and 70 p.p. (phase 3) were calculated and divided by the number of days for the respective phase. To calculate the prevalence of subclinical (1.2 mmol/L < BHB < 3 mmol/L) and clinical ketosis (BHB >3 mmol/L), each cow reaching the subsequent values during the trial was counted as one case.

The statistical analyses were performed using the SAS software package (version 9.4; SAS Institute Inc., Cary, NC). The procedure MIXED for repeated measures was used with a compound symmetry structure (Littell et al., 1998). The fixed effects in the model were CLA, vitamin E, and time and the interaction between them. Each cow within treatment was considered to be a random effect. The day or week of sampling was a repeated measure. For the blood variables and absolute fat depot masses, the value of the d −42 sample was used as a covariate. P-values >0.05 and ≤0.10 were considered to be a trend, whereas P-values ≤0.05 were considered to be statistically significant after Tukey post-hoc test.

RESULTS

Fifty-nine out of the initial 64 cows completed the entire experiment. In the Vit. E group, 1 animal was excluded on d 4 p.p. because of multisystemic health problems. In the CLA + Vit. E group, 4 cows did not complete the trial due to 1 case of prolonged hypocalcemia, 2 cases of abomasal displacement, and 1 case of an impaired locomotion system. We did not observe any influences of treatment on health events.

Conjugated linoleic acid had a reducing influence on the DMI (Table 2; $P = 0.022$); however, differences between groups were not significant and the same holds true for the net energy intake. Dry matter intake as well as net energy intake decreased from period 1 to 2 and increased from period 2 to 3. Both changes were significant in all groups. Neither CLA nor Vit. E affected the calculated NEB (Table 2). We noted a trend for a treatment × period interaction ($P = 0.054$) driven by the Vit. E group, which tended to have a higher positive NEB in period 1 and a lower negative NEB in period 2 than the other groups. However, the dif-

Table 2. Dry matter intake, net energy intake, net energy balance, BCS, and live weight (LSM) of the experimental groups during periods 1 (d 42 antepartum until calving), 2 (d 1 until 21 postpartum), and 3 (d 22 until 70 postpartum)

	Treatment[1]					*P*-value			
Item	Control, n = 16	CLA, n = 16	Vitamin E, n = 15	CLA + vitamin E, n = 12	SEM	CLA	Vitamin E	Period	CLA × vitamin E × period
DMI, kg/d									
Period 1	17.9[b]	16.7[b]	18.1	17.4[b]	0.25	0.022	0.477	<0.001	0.966
Period 2	15.8[c]	14.1[c]	15.8	14.8[c]	0.28				
Period 3	20.9[a]	19.6[a]	20.9	20.2[a]	0.25				
Net energy intake, MJ of NE_L/d									
Period 1	132.7[b]	124.3[b]	134.4[b]	128.9[b]	1.79	0.020	0.448	<0.001	0.986
Period 2	111.8[c]	100.4[c]	112.1[c]	104.5[c]	2.01				
Period 3	150.2[a]	140.7[a]	150.0[a]	145.4[a]	1.75				
Net energy balance, MJ of NE_L/d									
Period 1	77.2[a]	69.3[a]	80.9[a]	77.1[a]	1.78	0.745	0.201	<0.001	0.054
Period 2	−45.1[c]	−45.9[c]	−41.8[c]	−43.1[c]	1.96				
Period 3	−14.8[b]	−11.9[b]	−12.6[b]	−7.8[b]	1.64				
BCS									
Period 1	3.94[a]	3.85[a]	3.68[a]	3.78[a]	0.07	0.933	0.368	<0.001	0.002
Period 2	3.48[b]	3.47[b]	3.35[b]	3.33[b]	0.07				
Period 3	3.13[c]	3.20[c]	3.16[c]	3.05[c]	0.07				
Live weight, kg									
Period 1	758[a]	757[a]	753[a]	771[a]	8.64	0.917	0.829	<0.001	0.482
Period 2	676[b]	676[b]	683[b]	677[b]	8.82				
Period 3	645[c]	651[c]	653[c]	647[c]	8.45				

[a–c]Means with different superscripts within column differ ($P < 0.05$).

[1]Treatment: Before calving cows were fed a concentrate proportion of 60%. Postpartum the concentrate proportion increased from 30 to 50% within 3 wk in all groups. CLA (n = 16) and CLA + vitamin E (n = 12) received 8.4 g of *trans*-10,*cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E (n = 15) and CLA + vitamin E groups received 2,327 IU of vitamin E/d (BASF Lutavit E 50). The control group (n = 16) as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

ferences between groups within each period were not significant. The BCS is presented in Table 2. Neither CLA nor Vit. E had an influence on the course of the BCS; therefore, we did not find differences between the treatment groups. The BCS decreased from period 1 to 2 to 3 in all groups, which was also true for live weight (Table 2). The period influenced all described variables.

Variables of milk performance are presented in Table 3.We found no differences between treatments for milk yield, 4% FCM, ECM, feed efficiency (ECM/DMI and residual energy intake), protein content and yield, as well as lactose content and yield. An influence of CLA ($P < 0.001$) and a treatment × period interaction ($P < 0.001$) were found for the milk fat content (Figure 1). Fat content was lower in the CLA group ($P = 0.042$) than in the control group during the second period. During the third period, milk fat content was lower in the CLA ($P < 0.001$) and the CLA + Vit. E group ($P < 0.001$) compared with the control group. Milk fat content was also reduced in the CLA ($P < 0.001$) and the CLA + Vit. E group ($P = 0.041$) compared with the Vit. E group. Milk fat yield (Figure 2) was not different between the groups in period 2. In period 3, milk fat yield was lower in both groups receiving CLA compared with the control group. Neither milk protein content nor milk protein yield were different between treatments. The same is true for milk lactose content and milk lactose yield. We detected treatment × period interaction for urea, as it was lower in the CLA group in comparison to the control ($P < 0.001$) and the Vit. E group ($P < 0.001$) during period 3. We found a treatment × period interaction ($P < 0.001$) for the milk fat:protein ratio. This ratio was lower in the CLA group in period 2 compared with the control group ($P = 0.048$) and period 3 compared with the control group ($P < 0.001$) and the Vit. E group ($P < 0.001$). Furthermore, we observed differences between the CLA + Vit. E and the control group ($P = 0.006$) in period 3. Conjugated linoleic acid had an influence on the milk energy output ($P = 0.020$). Whereas it tended to be higher in period 3 than in period 2 in all groups, we only found a difference between both periods in the control ($P = 0.030$) and the Vit. E group ($P = 0.016$); however, no differences were found between the 4 treatment groups within period. Period had an influence on all variables described except for 4% FCM. Variables

Table 3. Lactation performance variables (LSM) of the experimental groups during period 2 (d 1 until d 21 postpartum) and period 3 (d 22 until d 70 postpartum)

	Treatment[1]					*P*-value			
Item	Control, n = 16	CLA, n = 16	Vitamin E, n = 15	CLA + vitamin E, n = 12	SEM	CLA	Vitamin E	Period	CLA × vitamin E × period
Milk yield, kg/d									
Period 2	31.5^{B}	30.8^{B}	32.1^{B}	29.5^{B}	0.66	0.723	0.719	<0.001	0.006
Period 3	37.5^{A}	39.0^{A}	37.6^{A}	37.7^{A}	0.62				
Milk fat content, %									
Period 2	5.46^{A,a}	4.83^{A,b}	4.99^{A,ab}	5.18^{A,ab}	0.08	<0.001	0.885	<0.001	<0.001
Period 3	4.57^{B,a}	3.36^{B,b}	4.36^{B,a}	3.76^{B,b}	0.07				
Milk fat yield, kg/d									
Period 2	1.70	1.50^{A}	1.61	1.54	0.03	<0.001	0.834	<0.001	0.002
Period 3	1.71^{a}	1.31^{Bb}	1.62^{ab}	1.40^{b}	0.03				
Milk protein content, %									
Period 2	3.58^{A}	3.62^{A}	3.60^{A}	3.50^{A}	0.04	0.744	0.543	<0.001	0.908
Period 3	3.08^{B}	3.12^{B}	3.10^{B}	3.02^{B}	0.04				
Milk protein yield, kg/d									
Period 2	1.11	1.12^{B}	1.15	1.02^{B}	0.02	0.548	0.506	<0.001	0.007
Period 3	1.15	1.20^{A}	1.16	1.14^{A}	0.02				
Milk lactose content, %									
Period 2	4.70^{B}	4.67^{B}	4.73^{B}	4.65^{B}	0.02	0.039	0.678	<0.001	0.795
Period 3	4.83^{A}	4.80^{A}	4.89^{A}	4.78^{A}	0.02				
Milk lactose yield, kg/d									
Period 2	1.49^{B}	1.47^{B}	1.54^{B}	1.39^{B}	0.03	0.568	0.764	<0.001	0.039
Period 3	1.81^{A}	1.87^{A}	1.83^{A}	1.80^{A}	0.03				
Milk urea, mg/kg									
Period 2	129^{A}	113^{A}	120	129^{A}	3.31	0.006	0.456	<0.001	<0.001
Period 3	106^{B,a}	72^{B,b}	104^{a}	83^{B,ab}	2.77				
Milk fat:protein ratio									
Period 2	1.54^{a}	1.32^{A,b}	1.40^{ab}	1.49^{A,ab}	0.03	<0.001	0.599	<0.001	<0.001
Period 3	1.50^{a}	1.10^{B,b}	1.41^{ac}	1.25^{B,bc}	0.02				
Milk energy concentration, MJ/kg									
Period 2	3.76^{A,a}	3.51^{A,b}	3.54^{A,ab}	3.65^{A,ab}	0.03	<0.001	0.975	<0.001	<0.001
Period 3	3.33^{B,a}	2.86^{B,b}	3.26^{B,a}	3.00^{B,b}	0.03				
Milk energy output, MJ/d									
Period 2	117.6^{B}	108.4	114.3^{B}	108.2	2.08	0.020	0.733	<0.001	0.578
Period 3	124.5^{A}	111.5	121.7^{A}	112.5	1.95				
4% FCM, kg/d									
Period 2	39.1	35.0	38.0	35.8	0.69	0.005	0.811	0.071	0.620
Period 3	40.5	35.3	39.2	35.8	0.64				
ECM, kg/d									
Period 2	37.4	34.7	36.2	34.2	0.65	0.027	0.498	<0.001	0.712
Period 3	39.4	35.5	37.9	35.6	0.61				
Residual energy intake									
Period 2	2.01	−4.44	3.47	0.00	1.69	0.287	0.369	0.772	0.605
Period 3	0.31	−2.98	1.14	1.29	1.54				
ECM/DMI, kg/kg									
Period 2	2.43^{A}	2.58^{A}	2.37^{A}	2.34^{A}	0.04	0.930	0.185	<0.001	0.099
Period 3	1.89^{B}	1.83^{B}	1.87^{B}	1.78^{B}	0.04				

[a–c]Means with different superscripts within row differ ($P < 0.05$).

[A,B]Means with different superscripts within column differ ($P < 0.05$).

[1]Treatment: Before calving cows were fed a concentrate proportion of 60%. Postpartum the concentrate proportion increased from 30 to 50% within 3 wk in all groups. CLA (n = 16) and CLA + vitamin E (n = 12) received 8.4 g of *trans*-10,*cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E (n = 15) and CLA + vitamin E groups received 2,327 IU of vitamin E/d (BASF Lutavit E 50). The control group (n = 16) as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

and coefficients we used to calculate the RSEI were as follows:

$$NE_L \text{ estimated intake} = 16.458 \times \text{lactation week} - 0.932 \times \text{lactation week}^2 + 1.447 \times \text{ECM} + 0.211 \times BW^{0.75}.$$

We found no differences between groups or periods; neither CLA nor vitamin E had an influence on the RSEI (Table 3).

Absolute masses of fat depots per group are presented in Figure 3. Daily change of fat depots is presented in Table 4. We detected no influence of either CLA or

vitamin E on subcutaneous and retroperitoneal adipose tissue. For the omental adipose tissue, on d 28 p.p. we found an influence of vitamin E ($P = 0.031$) as well as an interaction between treatments ($P > 0.001$). The omental adipose tissue of the Vit. E group was higher compared with the control group ($P = 0.046$). For the omental adipose tissue, at d 70 p.p. we observed an interaction between treatments ($P = 0.043$); however, when comparing groups we found no differences. For mesenteric adipose tissue on d 28 p.p., we found an interaction between CLA and vitamin E ($P = 0.028$). When comparing the groups, we detected that the Vit. E group has a higher mesenteric adipose tissue than the control ($P = 0.017$), CLA ($P = 0.030$), and the CLA + Vit. E group ($P = 0.0215$).

The subcutaneous tissue depots increased in all groups during phase 1. During phases 2 and 3, it was mobilized; however, the mobilization in phase 3 was slower than in phase 2. The daily change differed between phase 1 and phase 2 in all groups. We observed no differences between phase 1 and phase 3 as well as phase 2 and phase 3 within groups, except for the Vit. E group, in which the daily change in phase 3 differed from phase 1. Neither CLA nor vitamin E had an influence on the daily change. The retroperitoneal adipose tissue depot increased slightly during the first phase, whereas it was mobilized during the second and the third phase in all groups. In the control and the CLA groups, we found differences between phase 1 and phase 2; no influences of treatment were detected. We detected accretion of the mesenteric adipose tissue depot in phase 1, whereas mobilization was found in phase 2 in all groups. We observed neither differences between groups nor influences of treatment. We found accretion of the omental adipose tissue depot in all groups during phase 1. In phase 2 we detected mobilization, except for the Vit. E group, in which the depot further increased. During phase 3 we did not observe differences between groups. Changes of all described adipose tissue depots were influenced by phase ($P < 0.001$).

The serum concentrations of BHB during the trial are given in Figure 4. Concentrations of serum BHB, fatty acids, and glucose per period are given in Table 5. In the control group, the BHB values tended to be higher in period 2 ($P = 0.066$) and were higher in period 3 ($P = 0.002$) than in period 1. The BHB values in period 2 were enhanced compared with period 1 in CLA ($P = 0.050$) and Vit. E group ($P = 0.005$). Values for BHB in the CLA + Vit. E group did not differ between periods; however, the values tended to be higher in period 2 (P

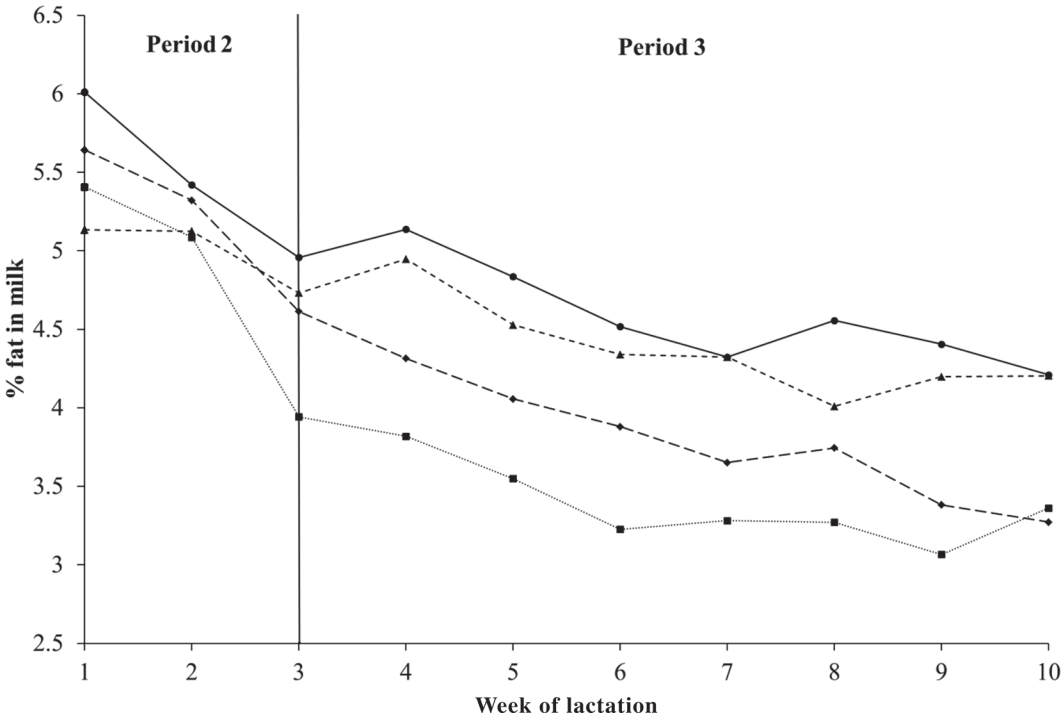

Figure 1. Milk fat percentage (LSM) during the experiment. Cows received a high-concentrate diet with a concentrate:roughage ratio of 60:40 antepartum. After parturition, concentrate:roughage ratio increased stepwise from 30:70 to 50:50 within 21 d in all groups. Groups included a control group (n = 16) (●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, dotted line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dashed line) from d −42 until 70 relative to calving.

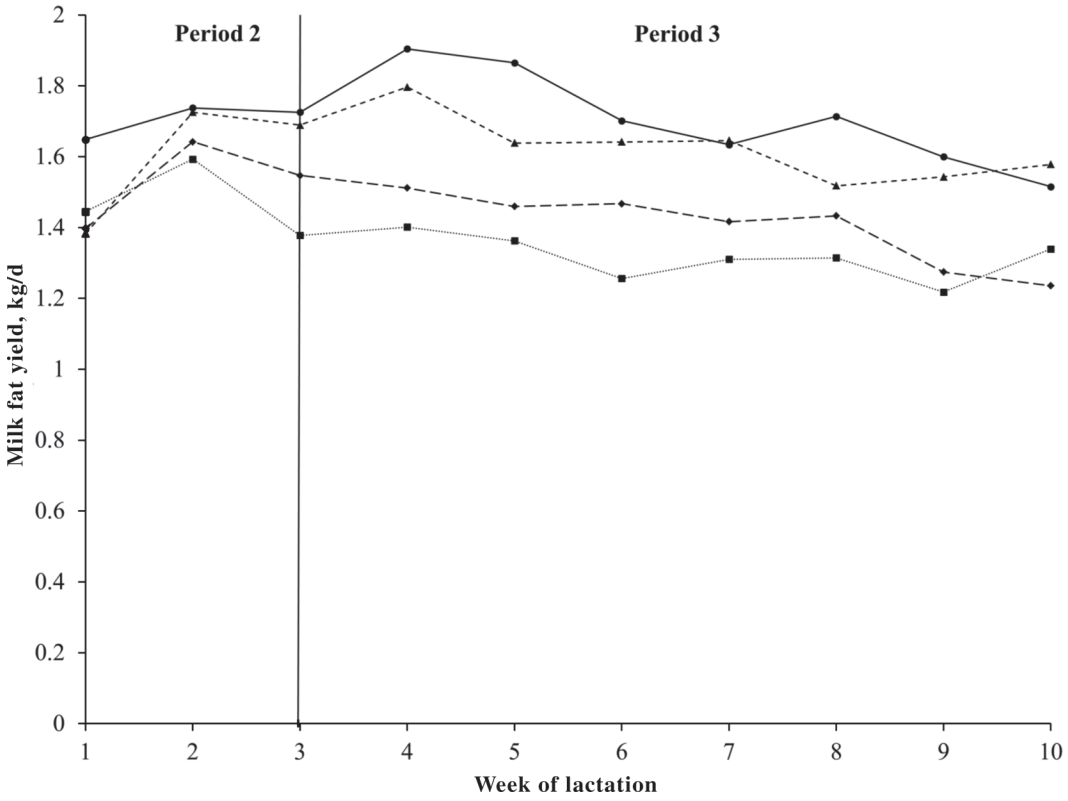

Figure 2. Milk fat yield (LSM) during the experiment. Cows received a high-concentrate diet with a concentrate:roughage ratio of 60:40 antepartum. After parturition concentrate:roughage ratio increased stepwise from 30:70 to 50:50 within 21 d in all groups. Groups included a control group (n = 16) receiving (●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, dotted line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dashed line) from d −42 until 70 relative to calving.

= 0.053) than in period 1. The prevalence of clinical ketosis (BHB >3 mmol/L) during the experiment was 25% in the control group, 27% in the CLA group, 13% in the Vit. E group, and 25% in the CLA + Vit. E group. Subclinical ketosis (1.2 mmol/L < BHB < 3 mmol/L) was detected in 63% of the control cows, 87% of the CLA group, 80% of the Vit. E group, and 67% of the CLA + Vit. E group. We did not find differences in prevalence of subclinical ketosis between groups. The fatty acid levels were higher in the second period compared with the other 2 periods for all groups. In the control group, a trend for higher fatty acid levels in period 3 than in period 1 ($P = 0.073$) was observed. Glucose values for the control group were reduced ($P = 0.003$) in period 2 compared with period 1; we detected no differences for period in the CLA group. In the Vit. E group, we found a reduction of glucose levels in period 2 compared with period 1 ($P = 0.002$) and compared with period 3 ($P = 0.036$). The same holds true for the CLA + Vit. E group, where we detected a P-value <0.001 for the comparison of period 1 and 2 and a P-value of 0.004 for the comparison between period 2 and 3. Neither CLA nor Vit. E had an influence on the BHB, fatty acids, and glucose. The period had a significant influence on all described blood variables.

The results of the total lipid analysis in liver tissue are presented in Table 6. Day of biopsy did elicit an influence ($P < 0.001$); however, only in the CLA group were differences between days of biopsy observed. Lipid levels were higher for biopsy on d 7 ($P = 0.030$) and 28 ($P = 0.003$) compared with d −42.

DISCUSSION

The aim of the current experiment was the evaluation of the possible interaction between CLA and vitamin E and the subsequent effects on ketosis, lipomobilization, and lactation performance variables. It is known from the literature that cows at high body condition before calving are at special risk for postpartum excessive lipomobilization and ketosis (Gillund et al., 2001). Such a metabolic situation was shown to be suited to investigate the efficacy of intervention measures, such as Monensin-release boli (Kexxtone; Elanco, Bad Hom-

burg, Germany) and dietary essential oils (Drong et al., 2016). Thus, to test the possible protective effects of CLA and vitamin E, we used the experimental strategies as proposed by Schulz et al. (2014) to generate suitable cow groups. In this model the combination of a high BCS before calving, a high concentrate proportion during the dry period, and a decelerated increase of the concentrate proportion p.p is responsible for a type II ketogenic metabolic status (Holtenius and Holtenius, 1996). This type of ketosis is characterized by a parallel increase of fatty acids and BHB very early in lactation and remarkable stress for the liver, whereas type 1 comes with a delayed BHB increase at approximately wk 4 of lactation and, typically, cows will not show symptoms of liver stress. The initial BCS at d 42 a.p. was above 3.5 in all 4 groups and increased further until calving, at which time it was at 4 on a 5-point scale. Therefore, it is concluded that the requirements to induce a ketogenic metabolic status in the animals were achieved. This is emphasized by the high incidence of subclinical ketosis in all groups.

The DMI in animals receiving CLA was lower during all periods. Effects of CLA on DMI in literature are controversial. Pappritz et al. (2011) found a decrease of DMI during early lactation starting CLA supplementation 1 d after calving and continuing until wk 26 p.p. To the contrary, a DMI increase has been observed in several studies. The differences in result might also be attributed to a different CLA dose. Pappritz et al. (2011) administered a dose of 9 g/d *trans*-10,*cis*-12 CLA, whereas Odens et al. (2007) provided the animals with 3.25 g of *trans*-10,*cis*-12 CLA daily. Metzger-Petersen (2012) found that 3.29 g/d *trans*-10,*cis*-12 CLA given after parturition for 80 d into lactation increased DMI, whereas 3.22 g given daily for 120 d p.p. decreased DMI. Castañeda-Gutiérrez et al. (2005) reported a DMI increase at a daily dose of 9 g of *trans*-10,*cis*-12 CLA and a decrease at 18 g of *trans*-10,*cis*-12 CLA;

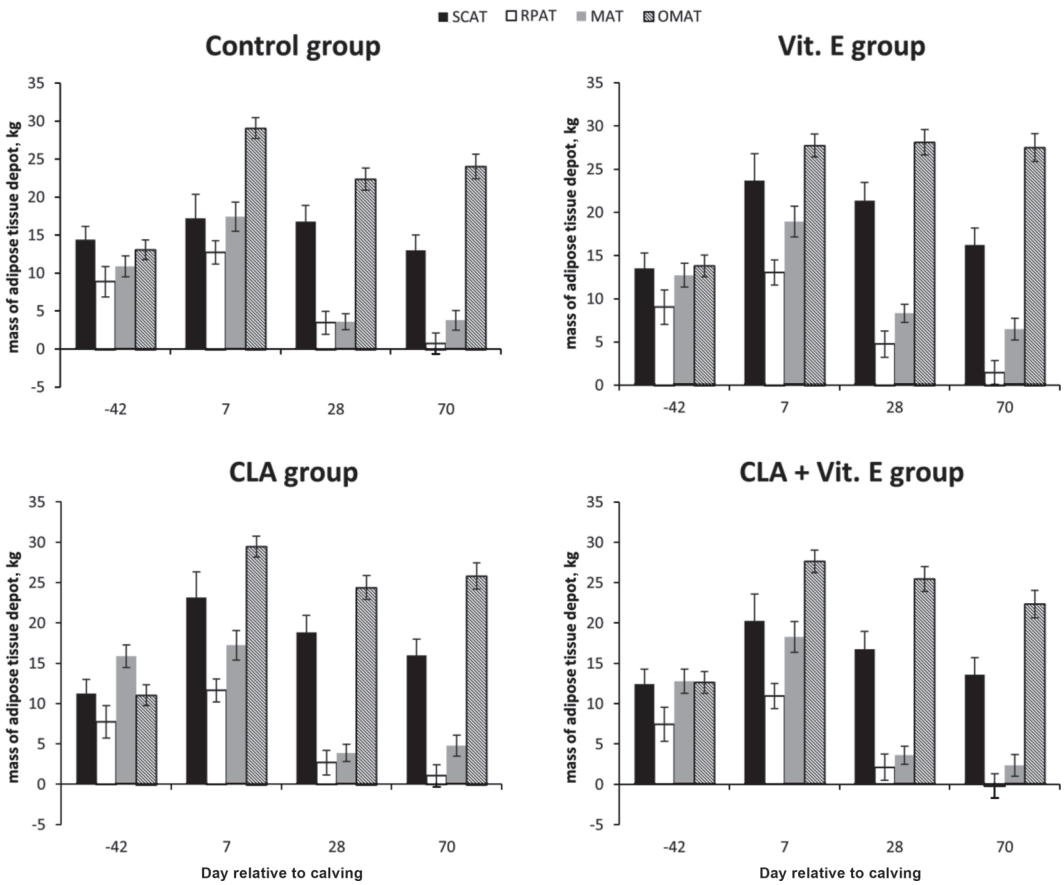

Figure 3. Absolute masses of adipose tissue depots (LSM) during the experiment. Cows received a high-concentrate diet with a concentrate:roughage ratio of 60:40 antepartum. After parturition concentrate:roughage ratio increased stepwise from 30:70 to 50:50 within 21 d in all groups. Groups included a control group (n = 16), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d, and a group (n = 12) receiving both treatments from d −42 until d 70 relative to calving. SCAT, RPAT, MAT, and OMAT = subcutaneous, retroperitoneal, mesenteral, and omental adipose tissue, respectively. Error bars indicate SEM.

Table 4. Change in adipose tissue depot mass (kg/d; LSM) of the experimental groups from d 42 antepartum (a.p.) until d 70 postpartum (p.p.)

	Treatment[1]					*P*-value			
Item	Control, n = 8	CLA, n = 8	Vitamin E, n = 8	CLA + vitamin E, n = 7	SEM	CLA	Vitamin E	Phase	CLA × vitamin E × phase
Subcutaneous adipose tissue depot									
d 42 a.p. until 7 p.p. (phase 1)	0.17^{A}	0.21^{A}	0.19^{A}	0.16^{A}	0.03	0.702	0.956	<0.001	0.945
d 8 until 28 p.p. (phase 2)	-0.20^{B}	-0.19^{B}	-0.12^{B}	-0.18^{B}	0.03				
d 29 until 70 p.p. (phase 3)	-0.10^{AB}	-0.05^{AB}	-0.13^{B}	-0.07^{AB}	0.03				
Retroperitoneal adipose tissue depot									
d 42 a.p. until 7 p.p.	0.09^{A}	0.07^{A}	0.08	0.06	0.02	0.962	0.937	<0.001	0.962
d 8 until 28 p.p.	-0.12^{B}	-0.13^{B}	−0.08	−0.12	0.02				
d 29 until 70 p.p.	-0.07^{AB}	-0.04^{AB}	−0.08	−0.05	0.02				
Mesenteric adipose tissue depot									
d 42 a.p. until 7 p.p.	0.13^{A}	0.03^{A}	0.12^{A}	0.12^{A}	0.03	0.174	0.671	<0.001	0.346
d 8 until 28 p.p.	-0.71^{B}	-0.61^{B}	-0.55^{B}	-0.72^{B}	0.03				
d 29 until 70 p.p.	0.02^{A}	0.01^{A}	-0.04^{A}	-0.03^{A}	0.03				
Omental adipose tissue depot									
d 42 a.p. until 7 p.p.	0.35^{A}	0.36^{A}	0.27^{A}	0.33^{A}	0.03				
d 8 until 28 p.p.	$-0.31^{a,C}$	$-0.24^{a,C}$	$0.02^{AB,b}$	$-0.12^{ab,B}$	0.03	0.683	0.294	<0.001	0.008
d 29 until 70 p.p.	0.04^{B}	0.04^{B}	-0.02^{B}	-0.08^{B}	0.03				

[a,b]Means with different superscripts within row differ ($P < 0.05$).

[A–C]Means with different superscripts within column differ ($P < 0.05$).

[1]Treatment: Before calving cows were fed a concentrate proportion of 60%. Postpartum the concentrate proportion increased from 30 to 50% within 3 wk in all groups. CLA (n = 16) and CLA + vitamin E (n = 12) received 8.4 g of *trans*-10,*cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E (n = 15) and CLA + vitamin E groups received 2,327 IU of vitamin E/d (BASF Lutavit E 50). The control group (n = 16) as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

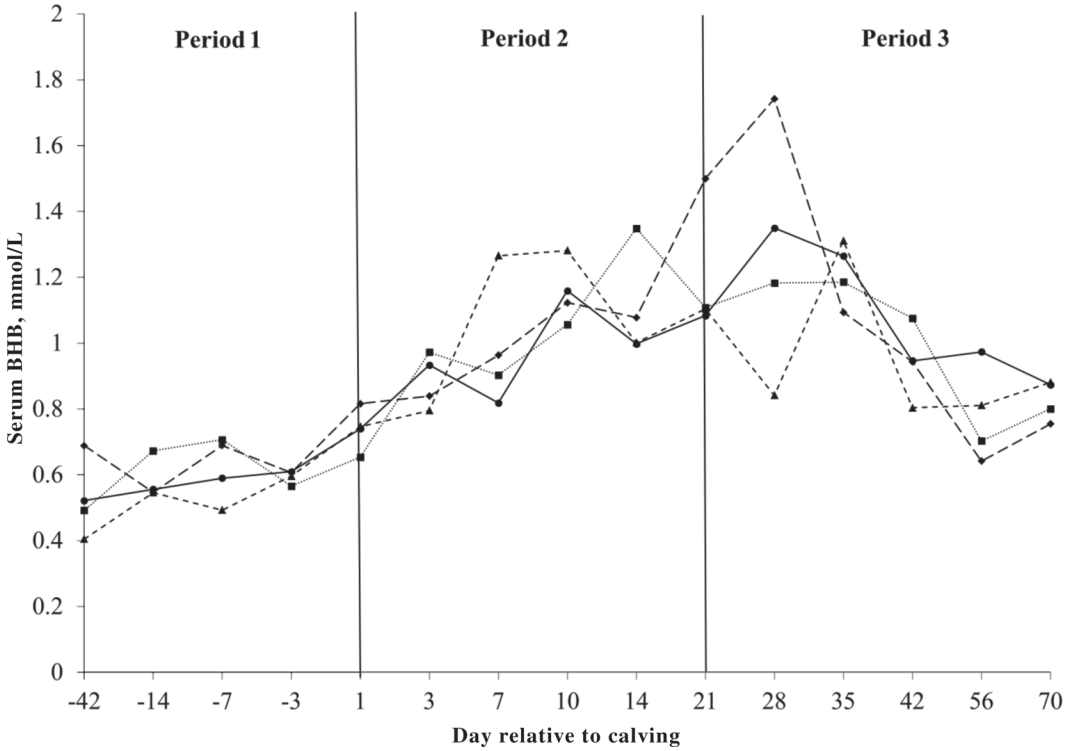

Figure 4. Serum BHB (LSM) during the experiment. Cows received a high-concentrate diet with a concentrate:roughage ratio of 60:40 antepartum. After parturition concentrate:roughage ratio increased stepwise from 30:70 to 50:50 within 21 d in all groups. Groups included a control group (n = 16; ●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, dotted line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dashed line) from d −42 until 70 relative to calving.

Table 5. Concentrations of BHB, fatty acids, and glucose in blood serum (LSM) of the experimental groups during periods 1 (d 42 antepartum until calving), 2 (d 1 until d 21 postpartum), and 3 (d 22 until d 70 postpartum)

	Treatment[1]					*P*-value			
Item	Control, n = 16	CLA, n = 16	Vitamin E, n = 15	CLA + vitamin E, n = 12	SEM	CLA	Vitamin E	Period	CLA × vitamin E × period
BHB, mmol/L									
Period 1	0.57[b]	0.66[b]	0.59[b]	0.59	0.07	0.884	0.906	<0.001	0.734
Period 2	0.98[ab]	1.08[a]	1.10[a]	1.06	0.06				
Period 3	1.09[a]	0.98[ab]	0.95[ab]	1.00	0.06				
Fatty acids, mmol/L									
Period 1	0.20[b]	0.20[b]	0.25[b]	0.33[b]	0.03	0.654	0.603	<0.001	0.404
Period 2	0.64[a]	0.63[a]	0.67[a]	0.60[a]	0.03				
Period 3	0.38[b]	0.31[b]	0.35[b]	0.31[b]	0.02				
Glucose, mmol/L									
Period 1	2.94[a]	2.79	2.92[a]	2.96[a]	0.05	0.693	0.383	<0.001	
Period 2	2.47[b]	2.59	2.42[c]	2.34[b]	0.05				0.218
Period 3	2.80[ab]	3.00	2.77[b]	2.81[a]	0.05				

[a–c]Means with different superscripts within column differ ($P < 0.05$).

[1]Treatment: Before calving cows were fed a concentrate proportion of 60%. Postpartum the concentrate proportion increased from 30 to 50% within 3 wk in all groups. CLA (n = 16) and CLA + vitamin E (n = 12) received 8.4 g of *trans*-10,*cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E (n = 15) and CLA + vitamin E groups received 2,327 IU of vitamin E/d (BASF Lutavit E 50). The control group (n = 16) as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

however, this result might be generated by a different study design, in which cows received CLA not during the complete trial (wk 3 a.p. to 18 p.p) but only from wk 3 a.p. until wk 9 p.p. An increase of DMI was also found by de Veth et al. (2005) and Castañeda-Gutiérrez et al. (2007); however, in both studies, the treatment with CLA began when animals were already in lactation. Therefore, it is suggested that the effect of CLA on the DMI is dose- and time-dependent. As a consequence of the decreased DMI in animals that received CLA, net energy intake was 6 to 11% lower than in control animals in our study. As milk energy output between CLA-treated animals and the other groups did not differ, and CLA-treated animals did not show an enhanced fat depot mobilization, it is assumed that the efficiency was increased. This is reflected by lower, albeit not significant, RSEI in animals receiving CLA. Total liver lipid content did increase p.p. in our experiment; this is in accordance with the findings of Janovick et al. (2011) and Douglas et al. (2006), who observed that high-energy diets did not increase the total liver lipid content a.p. while enhancing it p.p. Milk yield was 2 to 8% higher in period 2 and 3% higher in period 3 in CLA-treated cows. This change in milk yield is confirmed by Von Soosten et al. (2011), who found slight effects of CLA on milk yield. A decrease in milk fat content of 13% for the CLA group and 5% for the CLA + Vit. E group compared with the control group was stated in period 2. The reduction of milk fat content in the Vit. E group was 9%. In period 3 the reduction was 25, 18, and 5% for the CLA, CLA + Vit. E, and Vit. E groups, respectively. The level of milk fat content

Table 6. Total lipid content in liver as mg/g of wet tissue weight of the experimental groups on d 42 antepartum (a.p.), d 7, 28, and 70 postpartum (p.p.) (LSM)

	Treatment[1]					*P*-value			
Lipid content in liver, mg/g of wet tissue weight	Control, n = 8	CLA, n = 8	Vitamin E, n = 8	CLA + vitamin E, n = 7	SEM	CLA	Vitamin E	Day of biopsy	CLA × vitamin E × day of biopsy
d −42	49.2	45.6[b]	58.0	64.3	11.6	0.605	0.503	<0.001	0.589
d 7	146.5	154.6[a]	165.5	103.0	12.0				
d 28	149.5	175.9[a]	123.0	131	11.3				
d 70	122.2	89.6[ab]	103.5	103.5	11.3				

[a,b]Means with different superscripts within column differ ($P < 0.05$).

[1]Treatment: Before calving cows were fed a concentrate proportion of 60%. Postpartum the concentrate proportion increased from 30 to 50% within 3 wk in all groups. CLA (n = 16) and CLA + vitamin E (n = 12) received 8.4 g of *trans*-10,*cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E (n = 15) and CLA + vitamin E groups received 2,327 IU of vitamin E/d (BASF Lutavit E 50). The control group (n = 16) as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

reduction in the CLA group is in accordance with other studies applying similar doses of *trans*-10,*cis*-12 CLA. Perfield et al. (2002) showed that the application of 3 g/d of *trans*-10,*cis*-12 CLA led to a milk fat content decrease of 23% in late lactation, whereas Castañeda-Gutiérrez et al. (2005) demonstrated a reduction of 10% at a dose of 9 g/d of *trans*-10,*cis*-12 CLA during the first 9 wk of lactation.

We found no evidence for a reduction of milk fat content in dairy cows by vitamin E. Pottier et al. (2006) and Liu et al. (2008) showed an increase of milk fat content in cows treated with vitamin E; however, Pottier et al. (2006) used a daily dose of 12,000 IU, whereas Liu et al. (2008) administered doses of 5,000 or 10,000 IU compared with 2,327 IU used in our study. Furthermore, cows were on a high-fat diet in the experiment by Pottier et al. (2006); therefore, it is concluded that higher doses of vitamin E than our 2,327 IU/d are necessary to show a reducing effect on milk fat content. Our results suggest an interaction between vitamin E and CLA, as the reduction of milk fat content was lower in the group receiving both treatments in comparison to the CLA group. Dawson and Herbein (1996) observed that the uptake of CLA by bovine mammary cells is proportional to the level of CLA in the medium. Consequently, it is assumed that the CLA levels in the blood are reduced in the CLA + Vit. E group compared with the group receiving only CLA. According to Pottier et al. (2006), vitamin E in high doses is able to prevent the *trans*-11 to *trans*-10 shift in the rumen; however, vitamin E only partly reverses the shift once it had occurred. Therefore, the *trans*-10,*cis*-12 CLA in the CLA + Vit. E group might partly be transformed to *trans*-11 isomers, resulting in lower blood levels of *trans*-10,*cis*-12 CLA and, consequently, less reduction of milk fat content. As shown by Weiss et al. (1995), vitamin E is not affected in the rumen; therefore, vitamin E might influence CLA at different sites. Kay et al. (2005) reported a change of plasma levels of different *trans* fatty acids after application of α-tocopherol, suggesting that vitamin E might influence biohydrogenation in the rumen. Analysis of CLA levels in duodenal content and in blood is necessary to further clarify the site of interaction between vitamin E and CLA. As a consequence of the reduced milk fat content and a decreased milk yield in period 2, as well as an only slightly increased milk yield in period 3 in the CLA and CLA + Vit. E groups, the total milk fat synthesis was reduced. In our study, a milk yield increase in lactation wk 2 was observed, milk yield then decreased. Different patterns of milk fat yield reduction by CLA are also described in literature. In the study of Perfield et al. (2002), a decrease of milk fat yield occurred from wk 1 on. Metzger-Petersen (2012) reported no decrease of milk fat yield, whereas Moore et al. (2004) observed milk fat yield decrease beginning in wk 2 after parturition, similar to our results.

Von Soosten et al. (2011) found a decelerated decrease in milk fat yield beginning in wk 4 p.p. A reason for the delayed onset of milk fat yield reduction might be that the effect of CLA is masked by enhanced lipomobilization during early lactation. Fatty acids in milk can be derived either from fatty acids circulating in blood or be de novo synthesized in the mammary cells. Conjugated linoleic acid is known to reduce the de novo synthesis of fatty acids from acetate. However, in a state of energy deficiency, fatty acids from body fat mobilization increase proportionally, thereby reducing the proportion of de novo synthesized fatty acids (Bauman and Griinari, 2003). As dairy cows are in a negative energy balance after calving in early lactation, the contribution of fatty acids mobilized from adipose tissues to milk fat synthesis is relatively high.

Nousiainen et al. (2004) conducted a meta-analysis and proved that milk urea depends highly on dietary CP. As dietary CP did not differ between groups in our study, it is suggested that the reduced milk urea content during period 3 is a consequence of the lower DMI, and consequently CP intake, in cows treated with CLA. Milk protein content and yield were not affected by treatment; this is confirmed by other experiments not showing an influence of CLA on milk protein (Bernal-Santos et al., 2003; Moore et al., 2004; Perfield et al., 2004; Metzger-Petersen, 2012). The reduced milk fat:protein ratios and milk energy concentrations in CLA-receiving animals could be explained as a direct consequence of a milk fat reduction while the milk protein stayed constant. The reduced milk energy output in both groups receiving CLA is accompanied by the decreased DM and energy intake. Thus, CLA did not enhance the energy balance in our trial. The residual energy intake of the CLA group was negative in both periods, indicating this group had a more efficient use of energy compared with the other groups.

Changes in adipose tissue of dairy cows during transition period and the effects CLA and vitamin E have on them have not been extensively researched. Animals in our study experienced a daily loss of fat depot mass of 0.10 to 0.66 kg between 8 and 28 DIM, which is comparable to other studies (Andrew et al., 1994; Komaragiri and Erdman, 1997). In our trial, no differences in change of the subcutaneous fat depots between groups were found; as the correlation between back fat thickness and BCS is high (0.96–0.98; Hussein et al., 2013) and BCS between groups did not differ, this finding is in concordance with our other results. In the mouse (DeLany et al., 1999; Tsuboyama-Kasaoka et al., 2000; Clément et al., 2002; Warren et al., 2003; Poirier et

al., 2005) and humans (Risérus et al., 2001; Belury et al., 2003), *trans*-10,*cis*-12 CLA has been shown to be responsible for a reduction of abdominal adipose tissue. DeLany et al. (1999) reported that CLA has a major influence on the retroperitoneal adipose tissue depot in mice. Studies by von Soosten et al. (2011) suggested a decelerated mobilization of the retroperitoneal fat depot for dairy cows. However, the outcomes of our trial indicate that CLA did not influence the change of the retroperitoneal fat depot during the viewed periods. The contradictory results of von Soosten et al. (2011) might be due to the fact that they used only primiparous cows in their study. Berry et al. (2007) and Roche (2007) reported that primiparous cows are more sensitive to lipomobilization after parturition than pluriparous cows, and their ability to compensate this loss is impaired. Furthermore, administration of CLA began after calving compared with 6 wk before parturition in our study.

CONCLUSIONS

It was possible to induce a susceptibility to lipomobilization and subclinical ketosis in cows after parturition using a high concentrate proportion before calving and a decelerated increase in concentrate after calving. Treatment with vitamin E counteracts the milk fat-decreasing effect of CLA and thereby reduces the effect that CLA has on milk energy output. In addition, results suggest that the effects CLA had on milk content, thereby reducing the milk energy output, were compensated by a slightly increased milk yield and a decreased DMI. Therefore, the net energy balance was not affected by either treatment. Consequently, the lipomobilization was not reduced by either treatment. Thus, it was not possible to reduce the prevalence of subclinical ketosis by treatment with CLA. Further studies are needed to clarify the mechanisms behind the interaction effects between CLA and vitamin E on milk fat.

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5. Paper II

Influence of conjugated linoleic acids and vitamin E on milk fatty acid composition and concentrations of vitamin A and α-tocopherol in blood and milk of dairy cows

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Influence of conjugated linoleic acids and vitamin E on milk fatty acid composition and concentrations of vitamin A and α-tocopherol in blood and milk of dairy cows

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Summary

The objective of this trial was to investigate the influences of conjugated linoleic acid (CLA) and vitamin E (Vit. E) and their interactions on fatty acid composition and vitamins in milk (α-tocopherol, retinol and β-carotene) as well as on α-tocopherol in blood of pluriparous cows from week 6 ante partum until week 10 post-partum (p.p.). We assigned 59 pluriparous German Holstein cows to four treatment groups with the treatment factors CLA and Vit. E at two levels in a 2 × 2 factorial design. Milk fatty acid composition and milk vitamins were analysed on lactation days 7 and 28. α-tocopherol in blood serum was analysed on days −42, −7, 1, 7, 14, 28 and 70 relative to parturition. Milk concentration of α-tocopherol was influenced by Vit. E ($p < .001$) and CLA ($p = .034$). Percentage of *cis*-9, *trans*-11 CLA in total milk fat was influenced by treatment with CLA ($p < .001$), while for percentage of *trans*-10, *cis*-12 CLA an interaction between treatment and day ($p = .019$), driven by an increase in both CLA groups from day 7 to day 28, was found. Serum ratios of α-tocopherol to cholesterol were influenced by Vit. E ($p < .001$). Results suggest that treatment with CLA during late pregnancy and early lactation is suitable to enhance the proportion of *trans*-10, *cis*-12 CLA in milk and thereby influencing nutritional properties. As treatment with Vit. E did not have an impact on milk fatty acid composition, it might be possible to increase the antioxidative capacity of the dairy cow without affecting milk properties. Consequently, combined treatment with CLA and Vit. E might elicit synergistic effects on the cow and milk quality by increasing the proportion of CLA in milk fat as well as the excretion of Vit. E and the Vit. E levels in serum.

KEYWORDS

conjugated linoleic acid, dairy cow, milk fatty acids, vitamin A, vitamin E

1 | INTRODUCTION

Different properties of milk are influenced by its fatty acid composition and oxidation of milk fatty acids. It has been observed that unsaturated fatty acids as conjugated linoleic acids (CLA) exert anti-atherogenic and anticarcinogenic effects (Belury, 1995; Parodi, 1999). While it is not clear, which CLA isomer is responsible for these effects, Ha, Storkson, and Pariza (1990) suggest that the anticarcinogenic effect is based on the *cis*-9, *trans*-11 isomer. However Lengler (2011) demonstrated that also the *trans*-10, *cis*-12 isomer has anticarcinogenic activity in caco-2-cells. As milk and other dairy products are a major source for CLA in human nutrition, it is favourable to increase the CLA content in milk. To affect the milk fatty acid composition feeding rumen-protected CLA has been shown to be efficient (Loor & Herbein, 1998).

Infante (1999) suggests that especially the RRR-α-tocopherol is an important precursor for α-tocopherol quinone, which acts as an cofactor for fatty acids desaturases in the mitochondria. Consequently, dietary α-tocopherol might enhance the function of the Δ9-desaturase and thereby increase the conversion from *trans*-11 octadecenoic acid to CLA, which would enhance CLA content in the milk. Moreover, Vit. E has been shown to be an important antioxidant, which might not only influence milk quality by protecting polyunsaturated fatty acids from oxidation but also increase the antioxidant capacity of the cow itself (Burton & Traber, 1990). Zeitz, Most, and Eder (2015) have proven that supplementation with CLA increased the milk concentration of Vit. E as well as retinol in ewes when related to milk fat content. Gessner et al. (2015) have observed the same result in cows. However, Pottier et al. (2006) have shown that Vit. E has an influence on ruminal biohydrogenation. Consequently, supplementation with Vit. E might reduce the effect of CLA on milk excretion of vitamins.

Our objective in this trial was to evaluate the influences of CLA and Vit. E treatments on milk fatty acid composition and excretion of three fat-soluble vitamins (retinol, β-carotene and α-tocopherol) in the milk. We aimed to investigate whether a combined treatment with CLA + Vit. E shows a synergistic effect over supplementation of solely CLA on milk excretion of vitamins and proportion of CLA in milk. In addition we aimed to investigate the effects, which our treatments might have on levels of cholesterol and Vit. E in blood serum during late pregnancy and early lactation.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The study was carried out at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut, Braunschweig, Germany. The whole study design is described detailed in Schäfers et al. (2017). Briefly, 59 pluriparous German Holstein cows were allocated to four treatment groups 8 weeks ante partum (a.p.). The treatment groups received either 8.4 g *trans*-10, *cis*-12 and 8.4 g *cis*-9, *trans*-11 conjugated linoleic acid/d (BASF Lutrell®) (CLA, *n* = 16), or 2,327 IU Vit. E/d (BASF Lutavit® E 50) (*n* = 15) or both supplements (*n* = 12) from day 42 a.p. until day 70 post-partum (p.p.). The control group (*n* = 16) as well as the Vit. E group received a control fat supplement (BASF Silafat®), which consists of hydrogenated vegetable fat, 11% of which is C 16:0 (palmitic acid), 89% is C18:0 (stearic acid) for caloric balance. A standardised partial mixed ration (PMR) was provided during the whole experiment ad libitum by self-feeding stations (type RIC, Insentec B.V., Marknesse, the Netherlands). Additionally, concentrate was administered via computerised self-feeding stations (Insentec B.V.). The ingredients and chemical composition of the feedstuffs are shown in Table 1. The study was divided into three periods: period 1: 42 days a.p. until calving, period 2: calving until day 21 p.p. and period 3: remaining time until day 70 p.p.

2.2 | Measurements and sample collection

Milk samples were taken during evening milking time of day 6 and morning milking time of day 7 as well as evening milking time of day 27 and morning milking time of day 28 of lactation. Due to sampling days being on 4 days a week, sampling days were actual day 7 and day 28 ± 2 days. Milk was taken in 2 × 100 ml samples into wide-necked bottles (Kautex Textron GmbH KG, Bonn, Germany). Milk samples were frozen and stored at −20°C until analysis. Blood samples were taken on days −42, −7, 1, 7, 14, 28 and 70 relative to parturition. Samples were taken from a jugular vein into serum tubes. Blood samples were centrifuged (Heraeus Varifuge® 3.0R, Heraeus, Osterode, Germany, 2,123 *g*, 15°C, 15 min) and stored at −80°C until analysis.

2.3 | Analyses

Samples of PMR and concentrate were analysed according to the standard methods defined by the VDLUFA (1993) (method numbers in brackets) for dry matter (3.1), crude ash (8.1), crude protein (4.1.2), ether extract (5.1.1), crude fibre (6.1.1) as well as neutral detergent fibre (NDFom). Concentrations of tocopherol in blood serum were determined by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). Samples of 250 µl serum were mixed with 750 µl ethanol containing 0.01% butylated hydroxytoluene. After cooling on ice for 10 min samples were mixed with 2 × 1 ml *n*-hexane and centrifuged for 15 min at 4°C and 20,800 *g* (Sorvall® RC-6+, Thermo Fisher Scientific, Waltham, MA, USA). After centrifugation, the upper hexane layers were pipetted into a pear-shaped flask. The hexane phase was evaporated at 40°C under N_2-flow, and the residue was redissolved in 250 µl methanol. Samples were filtered (syringe filter PVDF 13 mm, amchro GmbH, Hattersheim, Germany), and 20 µl of the filtrate were injected into the HPLC-system. High-performance liquid chromatography-measurement was performed using at Inertsil ODS-2 column (150Å; 5 µm; 150 × 3.0 mm) and 100% methanol as mobile phase at a isocratic flow rate of 1.0 ml/min. The oven temperature was kept at 25°C, while the temperature of the autosampler was kept at 4°C. The detection wavelength of the diode array detector was set at 295 nm.

Blood serum samples were analysed for cholesterol by a photometric measurement system (Eurolyser VET CCA, Salzburg, Austria).

Milk fatty acid composition was determined according to Schlegel et al. (2012). In brief, 0.15 g of milk was extracted with 2 ml of hexane and isopropanol (3:2, v/v) respectively, excluding light for 3 hr on a shaker (GFL 3015, GFL, Burgwedel, Germany) at 150 rpm. After addition of 0.5 ml water the tube was vigorously agitated for 30 min. For phase separation, the mixture was centrifuged (10 min at 15°C and 1,000 *g*). A 200 µl aliquot of the upper fat-containing phase was evaporated to dryness under N_2 at 37°C and methylated with 75 µl of trimethylsulfonium hydroxide (Butte, 1983). The fatty acid methyl esters were separated by gas chromatography using a PE Clarus 580 system (Perkin Elmer, Waltham, MA, USA) equipped with an automatic split injector, a polar capillary column (50 m, 0.25 mm i.d., 0.2 µm film thickness; Permabond FFAP, Macherey and Nagel, Düren, Germany),

TABLE 1 Ingredients and analysed chemical composition of concentrate and roughage during the experimental period from day 42 ante partum until day 70 post-partum[a]

	Concentrate					Roughage[b]	
Item	LAC[c]	CLA[d]	SF[e]	VE[f]	DRY[g]	Maize silage	Grass silage
Ingredient, %							
Wheat	41	41	41	41	41		
Dried sugarbeet pulp	30.3	24.95	26.05	29.55	30.5		
Rapeseed meal	20	20	20	20	20		
Soybean meal	6.5	6.5	6.5	6.5	6.5		
CLA supplement	–	5.5	–	–	–		
Silafat	–	–	4.4	–	–		
Vit. E	–	0.05	0.05	0.75	–		
Vit./mineral premix[h]	–	2.0	2.0	–	2.0		
Vit./mineral premix[i]	2.0	–	–	2.0	–		
Calcium carbonate	0.2	–	–	0.2	–		
Chemical analysis							
Dry matter (DM), g/kg	879	884	883	886	875	340	302
Nutrient, g/kg of DM							
Crude ash	64	67	57	66	59	36	112
Crude protein	188	183	187	187	191	59	124
Ether extract	25	64	67	28	25	31	34
Crude fibre	107	93	95	102	98	199	283
NDF_{OM}	267	243	262	258	272	399	496
Energy[j], MJ/kg of DM							
ME	12.9	13.4	13.6	12.9	13.0	10.9	9.8
NE_L	8.2	8.6	8.6	8.2	8.2	7.0	5.4
DL-α-tocopheryl acetate (IU/kg DM)	–	217	188	2,453	–	–	–
Retinol (IU/kg DM)	1,500	1,500	1,100	1,500	1,200	–	–
trans-10, *cis*-12 CLA g/kg DM	<0.01	4.7	<0.01	<0.01	–	–	–

[a]Concentrate allocation (original substance) per group before parturition: control group, 2 kg of SF, 1 kg of LAC; CLA group, 2 kg of CLA, 1 kg of LAC; vitamin E group, 2 kg of SF, 1 kg of vitamin E; CLA + vitamin E group, 2 kg of CLA, 1 kg of vitamin E. Concentrate allocation (original substance) per group after parturition: control group, 2 kg of SF, 1 kg of LAC; CLA group, 2 kg of CLA, 1 kg of LAC; vitamin E group, 2 kg of SF, 1 kg of vitamin E; CLA + vitamin E group, 2 kg of CLA, 1 kg of vitamin E.
[b]50% Maize silage, 50% grass silage on dry matter basis.
[c]Concentrate for lactation (LAC).
[d]CLA concentrate.
[e]Silafat (SF) concentrate.
[f]Vit. E concentrate.
[g]Concentrate for dry cows.
[h]Ingredients per kg mineral feed: 50 g Ca; 120 g Na; 70 g P; 50 g Mg; 7 g Zn; 4.8 g Mn; 1.3 g Cu; 100 mg I; 50 mg Se; 35 mg Co; 800,000 IU Vit. A; 100,000 IU Vit. D_3.
[i]Ingredients per kg mineral feed: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu; 100 mg I; 40 mg Se; 25 mg Co; 1,000,000 IU Vit. A; 100,000 IU Vit. D_3.
[j]Calculation based on equations for calculation of energy content in feedstuffs published by the GfE (2001, 2008, 2009).

and a flame ionisation detector. Helium was used as carrier gas (flow rate 1.2 ml/min). The individual fatty acid methyl esters were identified by comparing their retention times with those of purified standards.

Concentrations of retinol, β-carotene and tocopherols in milk were determined by HPLC (L-7100, LaChrom, Merck-Hitachi, Darmstadt, Germany) according to a modification of the method of Balz, Schulte,

TABLE 2 Concentration of α-tocopherol in blood serum (mmol/L) of the experimental groups at day 42 and 7 ante partum (a.p.), day 1, 7, 14, 28 and 70 post-partum (p.p.) (LSMeans). The concentration on day 42 a.p. was used as a covariable in the statistical analysis

	Treatment†					*p*-Value			
Day	Control *n* = 16	CLA *n* = 16	Vit. E *n* = 15	CLA + Vit. E *n* = 12	*SEM**	CLA	Vit. E	LT	CLA × Vit. E × LT
42 a.p.	3.97^{BC}	3.78^{C}	3.62^{D}	3.87^{CD}	0.18				
7 a.p.	3.95^{BC}	4.11^{C}	5.49^{BC}	5.29^{BCD}	0.18				
1 p.p.	2.92^{C}	3.24^{C}	3.94^{CD}	3.52^{CD}	0.18				
7 p.p.	3.38^{C}	3.69^{C}	4.96^{CD}	4.95^{BCD}	0.18	.905	<.001	<.001	<.001
14 p.p.	3.96^{BC}	4.39^{BC}	5.41^{BC}	5.37^{BC}	0.18				
28 p.p.	5.03^{Bb}	5.71^{ABab}	6.99^{Ba}	6.72^{ABab}	0.18				
70 p.p.	5.83^{Ab}	6.57^{Ab}	10.01^{Aa}	7.88^{Ab}	0.18				

LT, day of lactation.

^{ab}LSMeans within row with different superscripts differ ($p < .05$).

^{ABCD}LSMeans within column with different superscripts differ ($p < .05$).

*Standard Error of Means (*SEM*), averaged.

†Treatment: before calving cows were fed a concentrate proportion of 60%. P.p. the concentrate proportion increased from 30% to 50% within 3 weeks in all groups. CLA (*n* = 16) and CLA + Vit. E (*n* = 12) received 8.4 g *trans*-10, *cis*-12 conjugated linoleic acid/d (BASF Lutrell®). Vit. E (*n* = 15) and CLA + Vit. E groups received 2,327 IU Vit. E/d (BASF Lutavit® E 50). The control group (*n* = 16) as well as the Vit. E group received 88 g/d of a rumen-protected fat supplement.

and Thier (1993). Samples of 0.5 g of milk were mixed with 2 ml of a 10 g/L pyrogallol solution (in ethanol, absolute) and 300 µl of a saturated sodium hydroxide solution. After flushing with N_2, this mixture was heated for 30 min at 70°C in closed glass tubes. The vitamins were then extracted by addition of 2 ml *n*-hexane and 2 ml of bidestillated water. After centrifugation (5 min at 10°C and 3,500 *g*), an aliquot of the hexane phase was evaporated to dryness under nitrogen and redissolved in methanol containing 0.05% of butylated hydroxytoluene. The tocopherols and retinol were separated isocratically by HPLC using methanol/H_2O (95:5 v/v) as mobile phase, a C18 column (≥3 µm particle size, 4 mm length, 2 mm internal diameter, Phenomenex, Aschaffenburg, Germany) (pre-column) and a C-18-reversed phase column (Luna C18 (2), 150 × 4.6 mm; Phenomenex) and detected by fluorescence (Fluorescence Detector L-7480, LaChrom, Merck-Hitachi). Excitation and emission wavelengths were 295 and 325 nm for tocopherols, and 325 and 475 nm for retinol respectively. For determination of β-carotene, the upper hexane layer was injected directly on a diol-HPLC column (≥5 µm particle size, 4 mm length, 4 mm internal diameter, Merck, Darmstadt, Germany) (pre-column) and a Diol phase column (LichroCART Diol, 125 × 4.6 mm; Merck) using a mixture of *n*-hexane (99.95%) and acetonitrile (0.05%) as eluent (1 ml/min). The column oven (Column Oven, L7360, LaChrom, Merck-Hitachi) was kept at 30°C. β-Carotene was quantified by measuring the absorption at 455 nm (UV-VIS-Detector L4250, Merck-Hitachi).

The statistical analyses were performed using the SAS software package (version 9.4; SAS Institute, Cary, NC, USA). The procedure MIXED for repeated measures was used with a compound symmetry structure (Littell, Henry, & Ammerman, 1998). The fixed effects in the model were CLA, Vit. E and day of lactation as well as the interaction between them. Each cow within treatment was considered to be a random effect. For Vit. E analysis in blood serum the day of sampling was a repeated measure. For all blood parameters, the concentration on day 42 a.p. was used as a covariable. *p*-Values > .05 and ≤.10 were considered to be a trend, while *p*-values ≤ .05 were considered to be significant.

3 | RESULTS

Influences on lactation performance variables and body composition have been presented in Schäfers et al. (2017). Briefly, Vit. E counteracted the milk fat decreasing effect of CLA. Therefore, the effect, which CLA might have had on milk energy output, was reduced by treatment with Vit. E. The effects CLA had on milk fat content and thereby reducing the milk energy output were compensated by a decrease of DMI ($p = .022$). Consequently, the net energy balance and the lipomobilisation were not affected by either treatment. Cows did not develop a subclinical ketosis. Under this non-challenged condition the intervention with CLA and Vit. E did not produce any effect on ketosis parameters.

Blood serum concentrations of Vit. E are presented in Table 2. The observed interaction between treatment and time ($p < .001$) was driven by a lower increase from day 28 p.p. to day 70 p.p. in the CLA + Vit. E group compared to the Vit. E group.

Concentrations of cholesterol in blood serum are presented in Table 3. Only day of trial had an influence on these concentrations ($p < .001$). In the control group, the concentrations on day 28 and day 70 p.p. were 1.12–2.94 mmol/L higher than on the other days. In the CLA group, the concentrations on day 42 a.p. (2.23 mmol/L) and day 7 p.p. (2.09 mmol/L) were lower than on day 28 (4.18 mmol/L) and day 70 p.p. (5.50 mmol/L). The concentrations on day 7 a.p. and day 1 p.p. were 1.22–3.77 mmol/L lower than on day 14, day 28 and day 70 p.p.

TABLE 3 Concentration of cholesterol in blood serum (mmol/L) of the experimental groups at day 42 and 7 ante partum (a.p.), day 1, 7, 14, 28 and 70 post-partum (p.p.) (LSMeans). The concentration on day 42 a.p. was used as a covariable in the statistical analysis

	Treatment†					*p*-Value			
Day	Control *n* = 16	CLA *n* = 16	Vit. E *n* = 15	CLA + Vit. E *n* = 12	*SEM**	CLA	Vit. E	LT	CLA × Vit. E × LT
42 a.p.	2.43^{b}	2.23^{cd}	2.39^{c}	2.20^{bc}	0.12				
7 a.p.	2.20^{b}	1.77^{d}	2.14^{c}	2.19^{c}	0.12				
1 p.p.	1.77^{b}	1.73^{d}	1.64^{c}	1.14^{c}	0.12				
7 p.p.	2.07^{b}	2.09^{cd}	1.97^{c}	2.14^{c}	0.11	.556	.163	<.001	.076
14 p.p.	2.66^{b}	2.99^{c}	2.69^{bc}	2.14^{bc}	0.11				
28 p.p.	3.78^{a}	4.18^{b}	3.65^{b}	3.40^{b}	0.11				
70 p.p.	4.71^{a}	5.50^{a}	5.32^{a}	4.66^{a}	0.11				

LT, day of lactation.
^{abcd}LSMeans within column with different superscripts differ ($p < .05$).
*Standard Error of Means (*SEM*), averaged.
†Treatment: before calving cows were fed a concentrate proportion of 60%. P.p. the concentrate proportion increased from 30% to 50% within 3 weeks in all groups. CLA ($n = 16$) and CLA +Vit. E ($n = 12$) received 8.4 g *trans*-10, *cis*-12 conjugated linoleic acid/d (BASF Lutrell®). Vit. E ($n = 15$) and CLA + Vit. E groups received 2,327 IU Vit. E/d (BASF Lutavit® E 50). The control group ($n = 16$) as well as the Vit. E group received 88 g/d of a rumen-protected fat supplement.

The concentration on day 14 p.p. was lower than on day 28 and day 70 p.p., while the concentration on day 28 p.p. was lower than on day 70 p.p. In the Vit. E group, the concentrations on day 42 a.p., 7 a.p., 1 and 7 p.p. were 1.26–3.68 mmol/L lower than on day 28 and day 70 p.p.. The concentrations of day 14 p.p. and day 28 p.p. were lower than on day 70 p.p.. In the CLA + Vit. E group, cholesterol concentrations on day 42 a.p. until day 28 p.p. were 0.33–0.83 mmol/L lower than on day 70 p.p. Furthermore, concentrations on day 7 a.p. until day 7 p.p were 1.26–3.52 mmol/L lower than on day 28 p.p. No significant differences between groups on the same day of trial were found.

The calculated ratios of α-tocopherol to cholesterol in blood serum are presented in Figure 1. The ratios were influenced by Vit. E ($p < .001$) and day of trial ($p < .001$). Correlations between Vit. E and cholesterol (Figure 2) were 0.60 for all animals together ($p < .001$), 0.76 for the control group ($p < .001$), 0.48 for the CLA group ($p < .001$), 0.80 for the Vit. E group ($p < .001$) and 0.56 for the CLA + Vit. E group ($p < .001$) (Figure 2). Slope of regression lines of animals treated with CLA was lower ($p < .001$) than in both other groups.

Results of Vitamin analysis in milk are presented in Table 4. Retinol (μg/g milk) was influenced by day of lactation ($p = .004$). For β-carotene (μg/g milk), an interaction between treatment and day of lactation ($p < .001$) was observed, driven by a higher reduction from day 7 to day 28 of lactation in animals receiving CLA. Concentrations of α-tocopherol (μg/g milk) were influenced by Vit. E ($p < .001$) and day of lactation ($p < .001$). On day 7, the Vit. E group had higher concentrations than the control group ($p = .009$) and a trend for higher concentrations compared to the CLA group ($p = .065$). The CLA + Vit.E group showed a trend for higher concentrations compared to the control group ($p = .097$). CLA had an influence on concentrations of retinol (μg/g fat) ($p = .009$). Correlations between retinol intake with feed and retinol excretion with milk are presented in Figure 3. Correlations were 0.16 for all animals together ($p < .100$), 0.25 for the control group ($p < .198$),

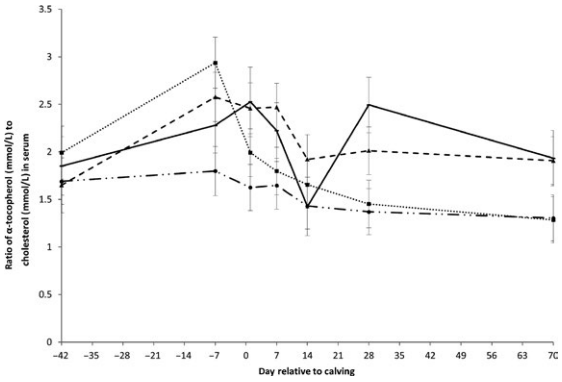

FIGURE 1 Ratios of α-tocopherol (mmol/L) to cholesterol (mmol/L) in serum (LSMeans ± *SEM*). Groups included a control group ($n = 16$) (●, solid line), a CLA group receiving 8.4 g of *trans*-10, *cis*-12 CLA/d ($n = 16$; ■, dotted line), a vitamin E group ($n = 15$) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group ($n = 12$) receiving both treatments (♦, long dashed line) from day −42 until 70 relative to calving

0.36 for the CLA group ($p < .044$), −0.20 for the Vit. E group ($p < .295$) and 0.33 for the CLA + Vit. E group ($p < .145$). Although the slope of the regression line of the Vit. E group is negative, differences between the correlation coefficients were not significant. Concentrations of β-carotene (μg/g fat) were neither influenced by treatment nor by day of lactation. Concentrations of α-tocopherol (μg/g fat) were influenced by CLA ($p = .034$) and Vit. E ($p < .001$). On day 7 of lactation, the Vit. E group had higher concentration ($p = .005$) compared to the control group. On day 28 of lactation, concentrations in the control group were reduced ($p < .001$) compared to the CLA + Vit. E group. Values of total vitamin excretion with the milk are presented in Table 5. Total excretion of retinol with the milk did not differ between groups or days. Excretion of β-carotene was influenced by CLA ($p = .021$). Vit. E did

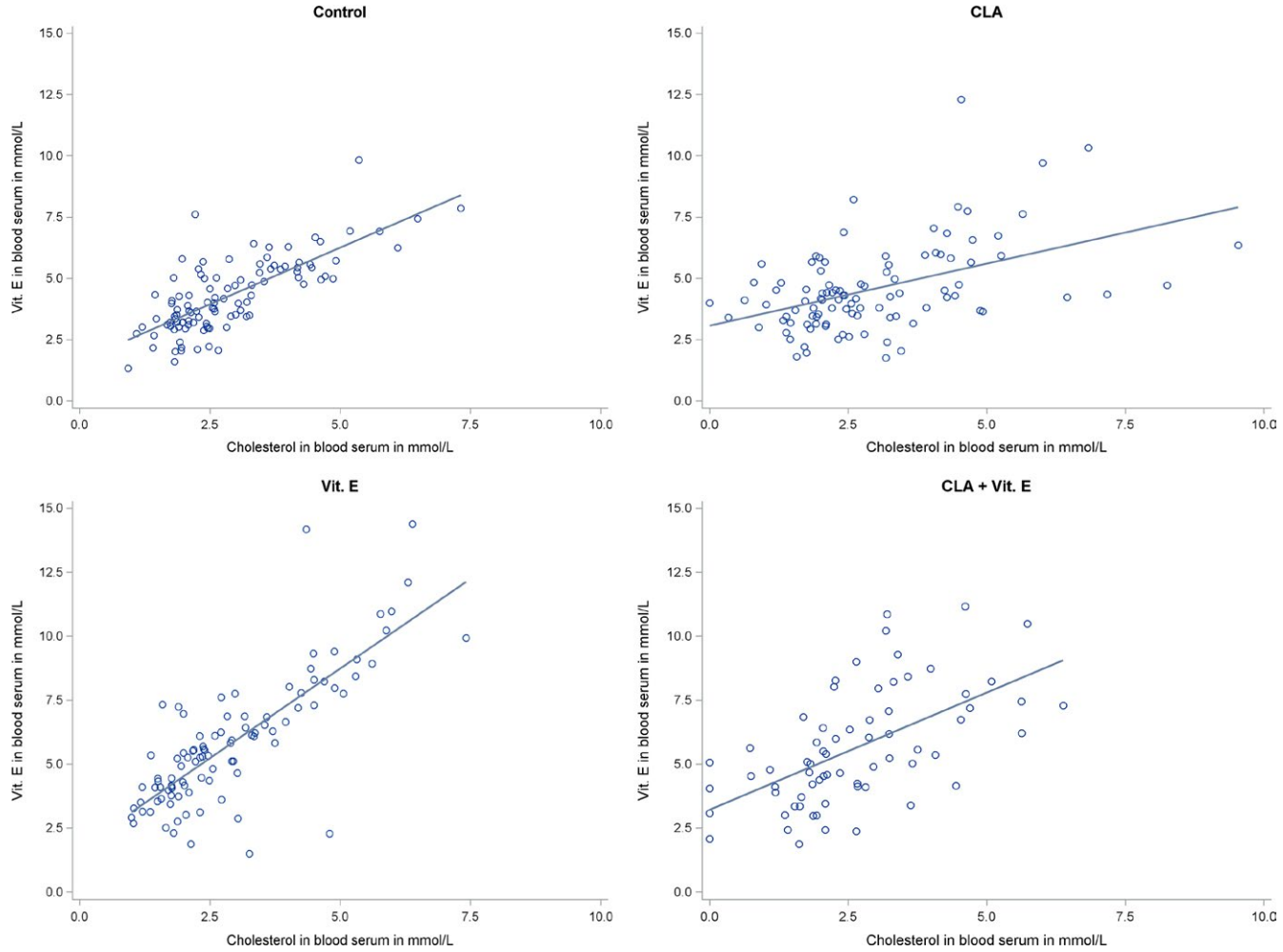

FIGURE 2 Correlations between cholesterol (mmol/L) and Vit. E (mmol/L) in blood serum

elicit an influence on the total excretion of α-tocopherol ($p < .001$). On day 7 p.p., animals in the Vit. E group had a higher excretion than animals in the control ($p = .032$) and the CLA group ($p = .028$). On day 28 p.p., the excretion in the Vit. E group was higher than in the CLA group ($p = .028$). Additionally, the CLA + Vit. E group showed a trend for a higher excretion ($p = .069$) compared to the CLA group.

Distribution of fatty acids in milk is presented in Table 6. Day of lactation increased ($p < .001$) the percentage of lauric acid (C12:0) in relation to total milk fat. However, while all groups tended to have a higher percentage of C12:0 on day 28 of lactation, these differences were not significant. The same holds true for myristic acid (C14:0). Percentage of pentadecylic acid (C15:0) was increased by day of lactation ($p < .001$). Differences were observed in the control ($p = .021$) and the CLA + Vit. E group ($p = .031$). Day of lactation had an increasing ($p < .001$) effect on the percentages of margaric acid (C17:0). Day of lactation reduced the percentage of stearic acid (C18:0) ($p < .001$). Significant differences were found in all groups except the CLA + Vit. E group. While day of lactation had a reducing influence on the percentage of *cis*- and *trans*-oleic acid (C18:1 n-9), differences between days were not significant. *Cis-9*, *trans-11* CLA was influenced by treatment with CLA and day of lactation. While percentages of *cis-9*, *trans-11* CLA tended to be higher in the control and the Vit. E group on day 28 p.p. than on day 7, percentages were higher in both groups receiving CLA. For *trans*-10, *cis*12 CLA the interaction between treatment and time ($p = .019$) was mainly driven by no change of percentage in the control group. Percentages of *trans*-10, *cis*12 CLA tended to be higher in all groups except the control group on day 28 than on day 7. On day 7 of lactation *trans*-10, *cis*12 CLA was higher in the CLA group than in the control ($p = .001$) and Vit. E ($p = .008$) groups. On day 28 of lactation *trans*-10, *cis*12 CLA was higher in the CLA group than in the control ($p < .001$) and Vit. E ($p = .005$) groups. The CLA + Vit. E group showed a higher *trans*-10, *cis*12 CLA percentage than the control group ($p = .040$). The correlation between CLA-intake and CLA-excretion was 0.52.

4 | DISCUSSION

Literature indicates that cows are susceptible to oxidative stress especially around parturition (Bernabucci, Ronchi, Lacetera, & Nardone, 2002; Castillo et al., 2005). To protect animals from oxidative stress,

TABLE 4 Concentration of vitamins in milk of the experimental groups at day 7 and day 28 of lactation (LSMeans)

	Treatment†					*p*-Value			
	Control *n* = 16	CLA *n* = 16	Vit. E *n* = 15	CLA + Vit. E *n* = 12	*SEM**	CLA	Vit. E	LT	CLA × Vit. E × LT
Retinol (μg/g milk)									
d 7	0.38	0.39	0.39	0.38	0.01	.783	.446	.004	.805
d 28	0.33	0.32	0.33	0.36	0.01				
β-Carotene (μg/g milk)									
d 7	0.19	0.20	0.21	0.18	0.01	<.001	.885	<.001	<.001
d 28	0.16	0.14	0.18	0.14	0.01				
α-Tocopherol (μg/g milk)									
d 7	0.77^{a}	0.84^{ab}	1.09^{b}	1.05^{ab}	0.03	.911	<.001	.001	.832
d 28	0.68^{ab}	0.62^{a}	0.91^{b}	0.95^{b}	0.03				
Retinol (μg/g fat)									
d 7	6.35	7.28	7.47	7.06	0.30	.009	.373	.109	.278
d 28	6.56	8.57	6.92	8.78	0.28				
β-Carotene (μg/g fat)									
d 7	3.16	3.82	3.89	3.23	0.23	.820	.763	.895	.651
d 28	3.27	3.73	3.71	3.54	0.22				
α-Tocopherol (μg/g fat)									
d 7	12.82^{a}	15.78^{ab}	20.85^{b}	19.49^{ab}	0.78	.034	<.001	.383	.458
d 28	13.62^{a}	17.00^{ab}	18.91^{ab}	23.17^{b}	0.74				

d, day; LT, day of lactation.

^{ab}LSMeans within row with different superscripts differ ($p < .05$).

*Standard Error of Means (*SEM*), averaged.

†Treatment: before calving cows were fed a concentrate proportion of 60%. Post-partum the concentrate proportion increased from 30% to 50% within 3 weeks in all groups. CLA ($n = 16$) and CLA + Vit. E ($n = 12$) received 8.4 g *trans*-10, *cis*-12 conjugated linoleic acid/d (BASF Lutrell®). Vit. E ($n = 15$) and CLA + Vit. E groups received 2,327 IU Vit. E/d (BASF Lutavit® E 50). The control group ($n = 16$) as well as the Vit. E group received 88 g/d of a rumen-protected fat supplement.

high levels of antioxidants such as α-tocopherol might be favourable. However, Goff and Stabel (1990) and Calderon, Chauveau-Duriot, Martin, et al. (2007) have shown that the levels of α-tocopherol decrease around calving, which is in line with our own results. Due to α-tocopherol levels in blood serum being higher in the Vit. E than in the other groups only on day 70 p.p. it can be concluded that oral supplementation of 2,327 IU Vit. E/d does not help to increase the antioxidant capacity of the blood during the critical periparturient phase. Moreover, supplementation with CLA + Vit. E does not increase blood levels of Vit. E at all. This might be attributed to a higher excretion rate of α-tocopherol with milk caused by CLA, which has also been observed by Gessner et al. (2015) and Zeitz et al. (2015). However, as a consequence Vit. E blood levels of the CLA group should be reduced compared to the control group. In our study and in Gessner et al. (2015), no difference was found although the serum levels of α-tocopherol in their control cows were lower (1,255 vs. 4,000 μg/L in 1st week of lactation). Bouwstra et al. (2008) used 3,000 IU of Vit. E and were able to almost prevent a decrease of Vit. E levels in the blood around calving. Consequently, we conclude that supplementation with 2,327 IU Vit. E/d was insufficient to reach a steady state of α-tocopherol levels in blood around calving and thereby facilitating the prevention of oxidative stress. According to Herdt and Smith (1996), it is necessary to calculate the Vit.E/cholesterol-ratio in order to correct for the transport capacity, because tocopherols are transported in blood bound to lipoproteins. While similar to Bouwstra et al. (2008) we did not observe any differences in cholesterol levels, we did not find an increased ratio in cows supplemented with Vit. E neither, which contradicts the result of Bouwstra et al. (2008) and might be attributed to a lower Vit. E dose used in this study. Bouwstra et al. (2008) reported correlations between Vit. E in blood and cholesterol of 0.73, which is higher than the correlations found in our study. As the slopes of the regression lines were lower in the CLA groups, it is suggested that CLA increases the transport capacity of very low density lipoproteins, resulting in higher Vit. E concentrations per lipoprotein.

Generally, our results for retinol and tocopherol in milk are in line with literature (Calderon, Chauveau-Duriot, Martin et al., 2007; Calderon, Chauveau-Duriot, Pradel et al., 2007; Gessner et al., 2015; Zeitz et al., 2015). Similar to Gessner et al. (2015) we observed a trend for higher tocopherol excretion in cows treated with CLA. However, a significant increase of tocopherol excretion in our study was only found in groups treated with Vit. E. Gessner et al. (2015) reported, that CLA increases retinol excretion with milk, which is not supported by

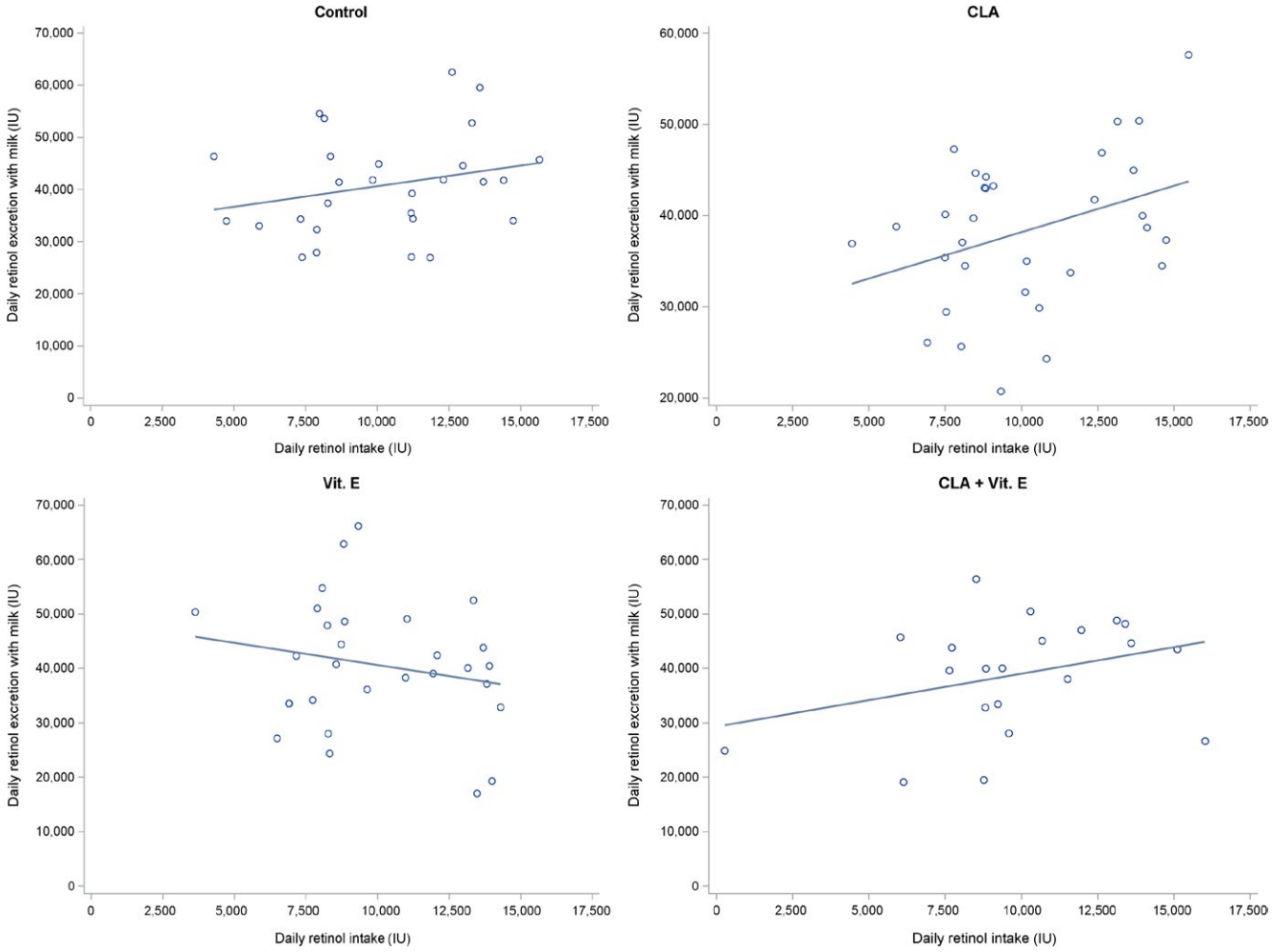

FIGURE 3 Correlations between retinol intake (IU) with feed and retinol excretion (IU) in milk

our results. However, Gessner et al. (2015) applied a daily dose of 4.3 g *cis*-9, *trans*-11 CLA and 3.8 g *trans*-10, *cis*-12 CLA, and the retinol concentration in their study was 1.8 mg/kg of concentrate, while we used concentrations between 0.33 mg/kg and 0.45 mg/kg of concentrate. Therefore, it might be possible that either the retinol intake of cows in this study was too low to show effects of CLA or the proportion of CLA-isomers influences vitamin excretion. On the other side, the tocopherol levels in the control milk of Gessner et al. (2015) were only ca. 58% of our control milk. This implies higher sensitivity of their cows towards treatments, which could modify milk tocopherol levels. Up to the present, the mechanism by which CLA increases vitamin excretion in the milk has not been clarified. Baumgard, Matitashvili, Corl, Dwyer, and Bauman (2002) as well as Harvatine and Bauman (2006) have reported that the *trans*-10, *cis*-12 CLA reduces the activity of lipoprotein lipase, which is responsible for the intake of fat-soluble vitamins from blood lipoproteins into mammary gland cells (Rigotti, 2007). Therefore, it is expected that supplementation with CLA results in decreased excretion of Vit. E in the milk. However, Thellman and Shireman (1985) have reported that the low density lipoprotein receptor (LDLR) also regulates intake of Vit. E from the blood. Because a high amount of tocopherols are transported bound to low density lipoproteins, it is possible that *trans*-10, *cis*-12 CLA enhances the capacity or expression of the LDLR and thereby increases the excretion of Vit. E in the milk. Gessner et al. (2015) interpreted the effect of higher excretion at indifferent plasma levels as either an improved absorption from the gut or stimulated mobilisation from adipose tissue, but both are difficult to explain without an increase in blood levels. An alternative interpretation could be a sparing effect of CLA on tocopherol levels in the mammary gland. We consider this explanation as more likely given the in vitro findings of Basiricò et al. (2015) and Basiricò, Morera, Dipasquale, Tröscher, and Bernabucci (2017). They demonstrated that both of the relevant CLA-isomers exert a protective effect in bovine mammary cell cultures which were exposed to oxidation. At least the results show that a reduction of de novo milk fat synthesis does not lead to a reduction in milk excretion of tocopherol, retinol or beta-carotene. However, further research is necessary to clarify the exact mechanism by which CLA influences the lipoprotein lipase and the LDLR.

We aimed to investigate the influence of the dietary addition of rumen-protected CLA on milk fatty acid composition. Although we

TABLE 5 Total excretion of vitamins in milk of the experimental groups at day 7 and day 28 of lactation (LSMeans)

	Treatment†					*p*-Value			
	Control *n* = 16	CLA *n* = 16	Vit. E *n* = 15	CLA + Vit. E *n* = 12	*SEM**	CLA	Vit. E	LT	CLA × Vit. E × LT
Retinol (mg)									
d 7	11.89	11.32	12.32	11.04	0.43	.254	.980	.432	.953
d 28	12.59	11.68	12.06	12.11	0.40				
β-Carotene (mg)									
d 7	6.02	5.70	6.34	5.08	0.32	.021	.867	.756	.904
d 28	6.23	5.06	6.36	4.92	0.31				
α-Tocopherol (mg)									
d 7	24.12^{b}	24.32^{b}	34.58^{a}	30.42^{ab}	1.19	.256	<.001	.849	.879
d 28	26.07^{ab}	22.97^{b}	33.05^{a}	32.61^{ab}	1.13				

d, day; LT, day of lactation.
^{ab}LSMeans within row with different superscripts differ ($p < .05$).
*Standard Error of Means (*SEM*), averaged.
†Treatment: before calving cows were fed a concentrate proportion of 60%. Post-partum the concentrate proportion increased from 30% to 50% within 3 weeks in all groups. CLA (*n* = 16) and CLA + Vit. E (*n* = 12) received 8.4 g *trans*-10, *cis*-12 conjugated linoleic acid/d (BASF Lutrell®). Vit. E (*n* = 15) and CLA + Vit. E groups received 2,327 IU Vit. E/d (BASF Lutavit® E 50). The control group (*n* = 16) as well as the Vit. E group received 88 g/d of a rumen-protected fat supplement.

clearly observed an influence of treatment with CLA on the CLA percentage in milk fat for both isomers, it was possible to detect differences to the control group only for the *trans*-10, *cis*-12 isomer. The levels of CLA (both isomers) vary between 0.44 and 0.69 g/100 g fatty acids. This is in line with Chouinard, Corneau, Barbano, Metzger, and Bauman (1999), who found levels of 0.68 g CLA/100 g fatty acids for cows not treated with CLA and Jahreis, Fritsche, and Steinhart (1997), who observed CLA levels of 0.30–0.97 g/100 g fatty acids. Treatment with CLA at a dose of 50 g/d resulted in a CLA content of 2.35 g/100 g fatty acids, while treatment with higher doses resulted in even higher CLA percentages in milk fat. Chouinard, Corneau, Barbano et al. (1999) suggest that the CLA-isomers are transferred to milk dose dependently. In another study, Chouinard, Corneau, Saebo, and Bauman (1999) showed that the efficiency, by which abomasally infused CLA-isomers are transferred to milk, is about 25% for all isomers. Although the used supplement was rumen-protected, it cannot be assured, that biohydrogenation did not occur. Another explanation, why the *cis*-9, *trans*-11-isomer in milk fat was not enhanced, might be that the endogenous synthetisation of CLA from *trans*-11 octadecenoic acid by the Δ9-desaturase was reduced and this reduction was not compensated by the amount of dietary CLA. Lee, Pariza, and Ntambi (1998) reported that expression of Δ9-desaturase mRNA was decreased in rat liver after dietary supplementation of CLA-isomers. However, this mechanism has not yet been shown in cow mammary gland tissue. Although we cannot explain, why treatment with CLA did not enhance *cis*-9, *trans*-11-isomer in milk fat, we conclude, that the 8.4 g/d of CLA, which were administered, might be too low to elicit an effect. This conclusion is supported by the trial of Pappritz et al. (2011) who did not observe an increase of the *cis*-9, *trans*-11-isomer in milk at a similar dose of 8 g/d of both CLA-isomers. Another hypothesis we wanted to test in this trial was that treatment with Vit. E might enhance CLA levels in milk fat above treatment with solely CLA and thus elicit synergistic effects by acting as a precursor for an important cofactor needed by desaturases. Our results indicate that treatment with Vit. E counteracted the effect of treatment with solely CLA, as percentages of *trans*-10, *cis*12 CLA in milk fat were lower in the CLA + Vit. E group than in the CLA group. This might be due to the influence, which Vit. E has on the *trans*-11 to *trans*-10 shift in the rumen (Pottier et al., 2006). Percentages of other fatty acids were not under influence of treatment with Vit. E. This is in line with the findings of Kay, Roche, Kolver, Thomson, and Baumgard (2005) and O'Donnell-Megaro, Capper, Weiss, and Bauman (2012) who observed no influences of Vit. E on milk fatty acid composition.

5 | CONCLUSION

Treatment with CLA is suitable to enhance the proportion of CLA in milk. However, in order to enhance the nutritive value of dairy products for humans, it is favourable to enhance especially the content of the *cis*-9, *trans*-11 isomer of CLA as this is probably responsible for the anticarcinogenic effect of CLA. With the treatment used in this study it was impossible to enhance that level. Therefore, we conclude that the dose of dietary CLA should be increased. Because treatment with Vit. E did not have an impact on milk fatty acid composition, it might be possible to change the oxidative status of the dairy cow without affecting milk properties and simultaneously protect fatty acids in milk against peroxidation. However, to enhance tocopherol levels in blood around parturition, the daily dose of Vit. E should be at least 3,000 IU. Feeding of CLA and Vit. E together can be used to enhance the Vit. E excretion with the milk and thereby prevent fatty acids from peroxidation without affecting tocopherol levels in blood. As a consequence

TABLE 6 Percentages of individual fatty acids of total milk fat of the experimental groups at day 7 and day 28 of lactation (LSMeans)

	Treatment†					*p*-Value			
	Control *n* = 16	CLA *n* = 16	Vit. E *n* = 15	CLA + Vit. E *n* = 12	*SEM**	CLA	Vit. E	LT	CLA × Vit. E × LT
C 12:0									
d 7	2.55	2.95	2.63	2.25	0.14	.586	.237	<.001	.618
d 28	3.39	3.79	3.43	3.44	0.13				
C 14:0									
d 7	9.05	9.84	9.43	8.48	0.32	.870	.295	<.001	.712
d 28	11.62	11.83	11.47	11.14	0.30				
C 15:0									
d 7	0.69^{A}	0.82	0.72	0.68^{A}	0.04	.243	.400	<.001	.800
d 28	1.03^{B}	1.06	0.96	1.07^{B}	0.04				
C 17:0									
d 7	0.86^{A}	0.82	0.82	0.86	0.02	.350	.866	<.001	.588
d 28	0.72^{B}	0.75	0.71	0.75	0.02				
C 18:0									
d 7	13.97^{A}	12.88^{A}	14.07^{A}	13.58	0.21	.886	.739	<.001	.685
d 28	11.47^{B}	12.04^{B}	12.13^{B}	12.14	0.20				
C 18:1 n9 c+t									
d 7	36.33	34.95	35.52	37.59	0.65	.444	.402	.002	.676
d 28	32.72	33.10	32.66	34.34	0.62				
cis-9, *trans*-12 CLA									
d 7	0.40	0.47^{A}	0.38	0.43^{A}	0.01	<.001	.071	<.001	.885
d 28	0.49	0.60^{B}	0.47	0.56^{B}	0.01				
trans-10, *cis*12 CLA									
d 7	0.04^{b}	0.07^{a}	0.04^{b}	0.05^{ab}	0.00	<.001	.305	.024	.019
d 28	0.04^{b}	0.09^{a}	0.06^{bc}	0.07^{ac}	0.00				

d, day; LT, day of lactation.

^{ab}LSMeans within row with different superscripts differ ($p < .05$).

^{AB}LSMeans within column with different superscripts differ ($p < .05$).

*Standard Error of Means (*SEM*), averaged.

†Treatment: before calving cows were fed a concentrate proportion of 60%. Post-partum the concentrate proportion increased from 30% to 50% within 3 weeks in all groups. CLA (*n* = 16) and CLA + Vit. E (*n* = 12) received 8.4 g *trans*-10, *cis*-12 conjugated linoleic acid/d (BASF Lutrell®). Vit. E (*n* = 15) and CLA + Vit. E groups received 2,327 IU Vit. E/d (BASF Lutavit® E 50). The control group (*n* = 16) as well as the Vit. E group received 88 g/d of a rumen-protected fat supplement.

combined treatment with CLA and Vit. E might elicit synergistic effects on the cow itself as well as on the quality of milk.

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6. Paper III

Influence of conjugated linoleic acids and vitamin E on biochemical, hematological, and immunological variables of dairy cows during the transition period

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Influence of conjugated linoleic acids and vitamin E on biochemical, hematological, and immunological variables of dairy cows during the transition period

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ABSTRACT

The objective of this experiment was to determine the effects of conjugated linoleic acid (CLA) and vitamin E as well as their interaction on biochemical and hematological variables and on leukocyte populations and their functionality. We assigned 59 German Holstein cows between the 2nd and 9th lactation to 4 dietary groups in a 2 × 2 factorial design with the factors CLA and vitamin E. Six weeks before calving the cows had a BCS of 3.7 to provoke a higher risk of developing ketosis, which might impair their immune function. Blood samples for analyses were taken on d −42, −14, −7, −3, 1, 3, 7, 10, 14, 21, 28, 35, 42, 56, and 70 relative to parturition. Furthermore, peripheral blood mononuclear cells were cultured on d −42, −7, 1, 7, 14, 28, and 70 relative to calving. Most variables were characterized by a high variation between d 7 antepartum and d 7 postpartum. Treatments did not elicit any effect, with the exception of vitamin E, which increased serum urea concentrations and decreased monocyte percentages. Haptoglobin, aspartate-aminotransferase, red blood cell count, leukocyte percentage and populations, as well as peripheral blood mononuclear cells were influenced by parity. In conclusion, the impairment of immune function caused by calving was more severe in cows in ≥3rd parity than in younger cows. However, neither vitamin E nor CLA supplementation was successful to stabilize parity or parturition related variance in hematological and immunological traits.

Key words: dairy cow, conjugated linoleic acid, vitamin E, immune function

INTRODUCTION

During the transition period, 3 wk before until 3 wk after calving (Grummer et al., 1995), dairy cows are subjected to various endocrinological and metabolic changes. According to Mallard et al. (1998), these changes are accompanied by a decreased immune response. As a consequence, production diseases show the highest incidence in early lactation (Goff and Horst, 1997; LeBlanc et al., 2006). As Lacetera et al. (2005) described, cows, especially over-conditioned cows, are prone to immune suppression and resulting health disorders. After parturition, transitional dairy cows are in a state of a negative energy balance (Grummer et al., 1995) due to the onset of lactation and a decreased DMI. This negative energy balance is accompanied by lipid mobilization, leading to elevated blood concentrations of fatty acids. Due to a relative small amount of oxaloacetate, fatty acids can only partly be metabolized by the liver and, consequently, ketone bodies are produced. Schulz et al. (2014) proved that over-conditioned cows at calving are more prone to lipomobilization and have higher fatty acid concentrations as a result. Contreras and Sordillo (2011) discovered that the function of neutrophils and leukocytes might be impaired due to increased concentrations of fatty acids and ketone bodies. Lacetera et al. (2004) observed in heifers that enhanced fatty acid concentrations decreased proliferation as well as secretion of antibodies and cytokines of lymphocytes. According to Scalia et al. (2006), high fatty acid concentrations are associated with respiratory burst activities, leading to a decreased cell viability and increased necrosis rates in PMNL in vitro. Furthermore, the chemotaxis of leukocytes is impaired by high concentrations of BHB in vitro (Suriyasathaporn et al., 1999). Hoeben et al. (1997) investigated the influence of normal and subketotic BHB concentrations on the respiratory burst activity of PMNL in vitro and observed that it is impaired by high BHB concentrations. It is

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consequently suggested by Hoeben et al. (1997) that elevated BHB concentrations might be partly responsible for the higher incidence of infections during the transition phase. Sordillo and Aitken (2009) proposed that oxidative stress might be another risk factor further impairing the immune system in dairy cows. Oxidative stress is defined as the imbalance between the production of different reactive oxygen species (**ROS**) and the antioxidant mechanisms in the organism (Betteridge, 2000). According to Miller et al. (1993), damage of lipids, proteins, polysaccharides, and DNA molecules might occur as a result of oxidative stress and might therefore be responsible for altered cell functions. On the other hand, the production of different ROS by the PMNL is a physiological protection mechanism against infectious diseases. According to Dahlgren and Karlsson (1999), this reaction is mediated by the NADPH oxidase; however, the ROS production in transition cows is elevated up to a certain level, where the antioxidant mechanisms might be depleted (Sordillo and Aitken, 2009). Additionally, it has been observed that high-conditioned cows are especially vulnerable to oxidative stress during the transition period (Bernabucci et al., 2005). Vitamin E has been proven to elicit antioxidant activities by reacting with peroxyl radical and thereby protecting polyunsaturated fats (Burton and Traber, 1990). According to Rimbach et al. (2002), vitamin E influences different inflammatory cell signaling pathways and acts as a ligand at the peroxisome proliferator activating receptor (**PPAR**)-γ, whereby it is involved in the expression of different antioxidative enzymes (Nakamura and Omaye, 2010). Bassaganya-Riera et al. (2002) and O'Shea et al. (2004) reported that CLA interact with PPAR-γ as well. According to Chen et al. (2012), supplementation with CLA leads to an enhanced storage of vitamin E in the tissue. Furthermore, CLA may increase the secretion of vitamin E via milk (Gessner et al., 2015) and raise the vitamin E transport capacity of cholesterol (Schäfers et al., 2017b). Studies in humans (Albers et al., 2003; Song et al., 2005) showed that CLA neither affects lymphocyte populations nor does it influence the proliferation of unstimulated or concanavalin A (**Con A**)-stimulated peripheral mononuclear blood cells (**PBMC**; Nugent et al., 2005). Kelley et al. (2002) reported no alteration after supplementation with both CLA isomers on the white blood cell profile in mice. Renner et al. (2013) observed that CLA inhibited proliferation of PBMC dose dependently.

Our objective was to evaluate ex vivo and in vivo the influence of CLA and vitamin E on biochemical and hematological variables, as well as on populations and functionality of immune cells from dairy cows during the transition period. As we have shown, CLA increases the transport capacity of lipoproteins, resulting in higher vitamin E concentrations per lipoprotein. Consequently, we hypothesized that a combined treatment with CLA and vitamin E would attenuate the reduction of vitamin E in the blood, which is caused by reduced cholesterol concentrations shortly after parturition. In addition, we aimed to compare the ability of pluriparous cows from different parities to cope with the stress caused by the event of parturition.

MATERIALS AND METHODS

The study was conducted at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institut in Braunschweig, Germany. The experiment was carried out in accordance with the German Animal Welfare Act approved by the LAVES (Lower Saxony State Office for Consumer Protection and Food Safety, Germany).

Experimental Design

The whole study design is presented in detail in Schäfers et al. (2017a). Briefly, 59 pluriparous German Holstein cows were allocated to 4 treatment groups 8 wk antepartum (**a.p.**). We used an animal model established by Schulz et al. (2014), consisting of a high concentrate proportion a.p., a high BCS at calving, and a decelerated increase of concentrate proportion postpartum (**p.p.**), a combination suitable to induce susceptibility to lipomobilization. The treatment groups received either 8.4 g of *trans*-10,*cis*-12 and 8.4 g of *cis*-9,*trans*-11 CLA/d (BASF Lutrell; CLA group, n = 16) or 2,327 IU of vitamin E/d (BASF Lutavit E 50; **VE** group, n = 15) or both supplements (CLA + VE, n = 12). The control group (**CON**; n = 16) as well as the vitamin E group received a control fat supplement for caloric balance. All supplements were given from d 42 a.p. until d 70 p.p. During the whole experiment, animals were provided ad libitum with a standardized partial mixed ration by self-feeding stations (type RIC, Insentec B.V., Marknesse, the Netherlands). The different concentrates were administered via computerized self-feeding stations (Insentec B.V.). The ingredients and chemical composition of the feedstuffs are presented in Table 1. The study was divided into 3 periods: period 1 from d 42 a.p. until the day of calving, period 2 from calving until d 21 p.p., and period 3 until d 70 p.p.

Experimental Animals

In the CON group, 10 cows were in the 2nd (**Pa 1**) and 6 cows in the 3rd or higher parity (**Pa 2**). In the

Table 1. Ingredients and chemical composition of concentrate and roughage during the experimental period from d 42 antepartum until d 70 postpartum

	Concentrate[1]					Roughage[2]	
Item	LAC[3]	CLA[4]	SF[5]	VE[6]	DRY[7]	Maize silage	Grass silage
Ingredient (%)							
Wheat	41	41	41	41	41		
Dried sugarbeet pulp	30.3	24.95	26.05	29.55	30.5		
Rapeseed meal	20	20	20	20	20		
Soybean meal	6.5	6.5	6.5	6.5	6.5		
CLA supplement	—	5.5	—	—	—		
Silafat	—	—	4.4	—	—		
Vitamin E	—	0.05	0.05	0.75	—		
Vitamin/mineral premix[8]	—	2.0	2.0	—	2.0		
Vitamin/mineral premix[9]	2.0	—	—	2.0	—		
Calcium carbonate	0.2	—	—	0.2	—		
Chemical analysis (g/kg of DM, unless noted)							
DM (g/kg)	879	884	883	886	875	340	302
Crude ash	64	67	57	66	59	36	112
CP	188	183	187	187	191	59	124
Ether extract	25	64	67	28	25	31	34
Crude fiber	107	93	95	102	98	199	283
NDF_{OM}[10]	267	243	262	258	272	399	496
Energy[11] (MJ/kg of DM)							
ME	12.9	13.4	13.6	12.9	13.0	10.9	9.8
NE_L	8.2	8.6	8.6	8.2	8.2	7.0	5.4
DL-α-tocopheryl acetate (IU/kg of DM)	—	217	188	2,453	—	—	—
trans-10,*cis*-12 CLA (g/kg of DM)	<0.01	4.7	<0.01	<0.01	—	—	—

[1]Concentrate allocation (original substance) per group before parturition: control group = 2 kg of SF, 1 kg of LAC; CLA group = 2 kg of CLA, 1 kg of LAC; VE group = 2 kg of SF, 1 kg of vitamin E; CLA + VE group = 2 kg of CLA, 1 kg of vitamin E. Concentrate allocation (original substance) per group after parturition: control group = 2 kg of SF, 1 kg of LAC; CLA group = 2 kg of CLA, 1 kg of LAC; VE group: 2 kg of SF, 1 kg of vitamin E; CLA + VE group: 2 kg of CLA, 1 kg of vitamin E.

[2]50% maize silage, 50% grass silage on a DM basis.

[3]Concentrate for lactation (LAC).

[4]CLA concentrate.

[5]Silafat (SF; BASF SE, Lampertheim, Germany) concentrate.

[6]Vitamin E concentrate.

[7]Concentrate for dry cows.

[8]Ingredients per kg of mineral feed: 50 g of Ca; 120 g of Na; 70 g of P; 50 g of Mg; 7 g of Zn; 4.8 g of Mn; 1.3 g of Cu; 100 mg of I; 50 mg of Se; 35 mg of Co; 800,000 IU of vitamin A; 100,000 IU of vitamin D_3.

[9]Ingredients per kg of mineral feed: 140 g of Ca; 120 g of Na; 70 g of P; 40 g of Mg; 6 g of Zn; 5.4 g of Mn; 1 g of Cu; 100 mg of I; 40 mg of Se; 25 mg of Co; 1,000,000 IU of vitamin A; 100,000 IU of vitamin D_3.

[10]NDFOM = NDF determined on an OM base.

[11]Calculation based on equations for calculation of energy content in feedstuffs published by the GfE (2001, 2008, 2009).

CLA group, 9 cows were in Pa 1 and 6 in Pa 2. For the VE group, this relationship was 10:5, whereas in the CLA + VE group 5 cows were in Pa 1 and 7 cows in Pa 2. All cows were daily clinically monitored by veterinarians. Body temperature was regularly measured rectally, and animals were inspected visually with regard to their appearance (skin turgor, body control, nose excretions) and gait. Consistency of feces was also observed. Furthermore, we monitored daily feed intake of each animal individually by means of the used feeding system. In total, for 15 animals (CON: n = 3; CLA: n = 3; vitamin E: n = 5; CLA + vitamin E: n = 4) subclinical mastitis was detected. Cows that had to be treated medicinally were excluded from the trial.

Sample Collection

Samples of roughage feed were taken twice weekly. Concentrate samples were taken once weekly for every group. Roughage and concentrate samples were pooled to a collective sample for periods of 4 wk.

Blood samples were taken on d −42 (initial sample), and then on d −14, −7, −3, 1, 3, 7, 10, 14, 21, 28, 35, 42, 56, and 70 relative to parturition. After morning milking and before main feeding, samples were taken from the jugular vein into serum and EDTA tubes. Additionally, blood samples were taken on d −42, −7, 1, 7, 14, 28, and 70 relative to calving into lithium-heparinized vacuum tubes (Vacuette, Greiner Bio-One

GmbH, Frickenhausen, Germany) to culture PBMC. All blood was used for flow cytometry and analysis of hematological variables. Serum and plasma samples were centrifuged (Varifuge 3.0R, Heraeus, Osterode, Germany; 2,123 × *g*, 15°C, 15 min) and stored at −80°C until analysis.

Analyses

Samples of partial mixed ration and concentrate were analyzed according to the standard methods defined by VDLUFA (1993; method numbers in brackets) for DM (3.1), crude ash (8.1), CP (4.1.2), ether extract (5.1.1), crude fiber (6.1.1) as well as NDF and tocopherol acetate according to a modified version of the tocopherol acetate method (Japanese Pharmacopoeia Committee, 2011). The CLA isomers were analyzed by HPLC as described in Schäfers et al. (2017b).

Serum samples were analyzed for albumin (**ALB**), aspartate-aminotransferase (**AST**), gamma-glutamyl-transferase (**GGT**), glutamate dehydrogenase (**GLDH**), protein, bilirubin, triglycerides, and urea by a photometric measuring system (Eurolyser VET CCA, Salzburg, Austria). Haptoglobin concentrations were determined using an ELISA according to Hiss et al. (2004) with a limit of detection of 0.07 mg/mL. The concentrations of adiponectin were analyzed using an ELISA as described by Mielenz et al. (2013) with a limit of detection of 0.03 ng/mL.

Hematological variables were analyzed in potassium-EDTA-whole blood with an automatic analyzer Celltac-α (MEK 6450; Nihon Kohden, Qinlab Diagnostik, Weichs, Germany). Both white and red blood cell profiles were generated. Total leukocyte count data (**WBC**), lymphocytes, monocytes, basophile, and neutrophile granulocytes were analyzed for the white blood cell profile, whereas the red blood cell profile consisted of red blood cell count (**RBC**), hemoglobin, hematocrit, mean corpuscular volume (**MCV**), mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration.

Isolation of PBMC was performed according to a method described by Renner et al. (2011). Briefly, heparinized whole blood was diluted 1:1 with PBS and then centrifuged at 603 × *g* for 35 min at room temperature using Biocoll separation solution (Biochrom L6113, Biochrom GmbH, Berlin, Germany). The PBMC were isolated as buffy coat and analyzed for their metabolic activity and the proliferation stimulated by Con A (Sigma-Aldrich, Steinheim, Germany) with an AlamarBlue reagent (**AB**; AbD Serotec, Oxford, UK). Each well of a 96-well plate was filled with 100,000 cells in quintuplicates with or without 0.5 μg of Con A /well. Plates were incubated at 37°C with 5% CO_2 for 69.5 h and then centrifuged at 200 × *g* for 5 min at room temperature (Hettich Universal 320, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). After centrifugation, 100 μL of the supernatants were removed from each well and 11 μL of AB (dilution ratio 1:10) were added. After further incubation for 2.5 h at 37°C, the reduction of nonfluorescent resazurin to fluorescent resorufin, which is performed by metabolically active cells, was measured photometrically (Tecan infinite M200, Grödig, Austria) at 540 nm of excitation and 590 nm of emission.

To phenotype the T-cell-populations, whole blood was either stained with monoclonal antibodies for CD4+ (mouse anti-bovine CD4:fluorescein isothiocyanate, Bio-Rad Laboratories Inc., Hercules, CA) and CD8+ (mouse anti-bovine CD8:phycoerythrin, Bio-Rad Laboratories Inc.) or with isotype controls (mouse IgG2a negative control:RPE or mouse IgG2b: fluorescein isothiocyanate negative control, Bio-Rad Laboratories Inc.). Samples were incubated at room temperature for 30 min. After incubation, lysis buffer (BD Biosciences, Franklin Lakes, NJ) was applied for 10 min to lyse the red blood cells. Samples were centrifuged (Heraeus Varifug 3.0R; 200 × *g*, 4°C, 5 min), then resuspended in HEPES-buffered saline and measured by the FACSCanto II flow cytometer (BD Biosciences). The different populations of lymphoid cells were identified using distinct side- and forward-scattering characteristics. Measuring was performed until at least 10,000 lymphocytes were stored in list mode data files. The BD FACSDiva software (BD Biosciences) was used to adjust the spillover of the fluorochromes fluorescein isothiocyanate and phycoerythrin. The quantity of each T-cell phenotype was estimated with the help of the percentages obtained by the flow cytometer.

The capacity of granulocytes to induce oxidative burst and the release of ROS was assessed by measuring the oxidation of nonfluorescent dihydrorhodamine 123 (**DHR**) to fluorescent rhodamine 123 (**R123**) by means of hydrogen peroxide with the flow cytometer. The fraction of PMNL, which converted DHR to R123 by the production of ROS, is represented by the R123+ population. The mean conversion of DHR per cell can be measured by the mean fluorescence intensity (**MFI**). Either 40 μ*M* DHR (Molecular Probes, Eugene, OR) or 40 μ*M* DHR and 20 n*M* phorbol myristate acetate (Sigma-Aldrich) were added to whole blood samples. Samples were then incubated at 37°C for 15 min. Lysis buffer (BD Pharm Lyse, BD Biosciences) was applied for 10 min to lyse red blood cells. Thereafter, cells were washed with HEPES-buffered saline. Measurement was done dually by the FACSCanto II (BD Biosciences) until at least 10,000 granulocytes were measured. Granu-

locytes were defined by their size and granularity by using side and forward scattering characteristics.

Calculations and Statistical Analysis

To calculate the proliferation of the PBMC in vitro, the stimulation index (**SI**) was used:

$$\text{SI} = (\text{fluorescence of cells stimulated with Con A})/(\text{fluorescence of unstimulated cells}). \quad [1]$$

The CD4+-to-CD8+ ratio was calculated according to the formula

$$\text{CD4:CD8} = (\text{percentage of CD4+})/(\text{percentage of CD8+}). \quad [2]$$

Trevisi et al. (2013) had described a high correlation of vitamin E and cholesterol as an indication of circulating lipoproteins. On that basis, Schäfers et al. (2017b) used ratios of vitamin E to cholesterol for calculating correlations to vitamin E levels in blood.

Statistical analyses were performed using the SAS software package (version 9.4; SAS Institute Inc., Cary, NC). The procedure MIXED for repeated measures was used with an autoregressive covariance structure (Littell et al., 1998). The fixed effects in the model were CLA, vitamin E, time, parity, and the interaction between them. Each cow within treatment was considered to be a random effect. The day or week of sampling was a repeated measure. For all variables, the value of the d −42 sample was used as a covariate. Data are reported as least squares means ± standard error of the mean; P-values >0.05 and ≤0.10 were considered to be a trend, whereas P-values ≤0.05 were considered to be statistically significant after Tukey post-hoc test.

RESULTS

Samples taken on d 42 a.p. displayed for all presented variables showed no significant differences between groups. Selected variables of clinical chemistry are presented in Figure 1. During the course of the trial, haptoglobin concentrations were lower in Pa 1 than in Pa 2 (0.70 ± 0.16 vs. 1.38 ± 0.17 mg/mL; $P = 0.006$) and were influenced by time. The concentrations of adiponectin (Table 2) were influenced by time only ($P < 0.001$), showing a drop toward day of calving. The GLDH activity (not presented) was 14.0 ± 8.94 U/L; for this variable, we did not detect any influence of time, treatment, or parity. Over the course of the trial, AST activity (Table 2) was higher in Pa 2 than in Pa 1 (97.7 ± 6.01 vs. 81.3 ± 5.39 U/L, $P = 0.049$) and was influenced by time ($P < 0.001$), showing peaks at d 7 and 14 p.p. The GGT activity in Pa 1 was 27.6 ± 1.14 U/L, whereas cows in Pa 2 showed mean activities of 31.2 ± 1.26 U/L. Although we observed an influence of time ($P < 0.001$) for GGT activity, the difference between parities was only a trend ($P = 0.054$). Pearson correlation coefficients between GGT and cholesterol were significant for all groups except the CLA + VE group (control group: r = 0.27, $P = 0.005$; VE group: r = 0.31, $P = 0.002$; CLA group: r = 0.32, $P = 0.001$). Concentrations of triglycerides (Table 2) dropped during the course of the trial ($P < 0.001$); we observed the lowest concentrations of triglycerides on d 7 p.p. The protein concentration (not presented) on d 42 a.p. was 69.7 ± 1.46 g/L, it dropped to 65.9 ± 1.67 g/L at d 1 p.p. and increased thereafter to 77.0 ± 1.44 g/L on d 70 p.p., being influenced by time ($P < 0.001$). The concentrations of ALB (Table 2) were influenced by time ($P = 0.023$); ALB concentrations increased from d 42 a.p. to reach a peak at d 1 p.p. and decreased again beginning on d 7 p.p.. The urea concentrations (Table 2) were higher in cows treated with vitamin E than in those not treated with vitamin E (25.65 ± 1.28 vs. 20.19 ± 1.15 mg/dL, $P = 0.003$) and were influenced by time ($P < 0.001$). Expressions of high urea concentration a.p. and low concentrations p.p. were caused by the employed animal model, which included dietary protein excess a.p. and low protein p.p. Bilirubin concentrations (Figure 1) showed an interaction between time, treatment, and parity ($P = 0.005$), which is reflected by cows in Pa 2, except for the control group peaking on d 1 p.p.; however, the peak occurred on d 3 p.p for cows in Pa 1. Pearson correlation coefficients between vitamin E in blood and bilirubin were not significant and slope of the regression lines did not differ between treatment groups. Pearson correlation coefficients between GGT and bilirubin were significant only for the CLA (r = 0.21, $P = 0.037$) and CLA + VE group (r = 0.38, $P < 0.001$).

Figure 2 shows the red blood cell profile. For Pa 1, RBC was 5.93 ± 0.07 × 10^6/μL over the course of the trial, whereas cows in Pa 2 showed a mean RBC of 5.40 ± 0.08× 10^6/μL. Time ($P < 0.001$) and parity ($P = 0.005$) had an influence on RBC. In cows in Pa 1, HGB concentration was 9.93 ± 0.10 g/dL, whereas we observed a HGB concentration of 10.2 ± 0.11 g/dL in cows in Pa 2. However, the influence of parity was only a trend ($P = 0.062$), whereas time had a significant influence ($P < 0.001$). Progressions of hematocrit were analogous, with time eliciting an influence ($P < 0.001$) and parity showing a trend ($P = 0.075$). The MCV values in animals treated with vitamin E were higher (61.8 ± 0.47 vs. 53.5 ± 0.41 fL) and showed a

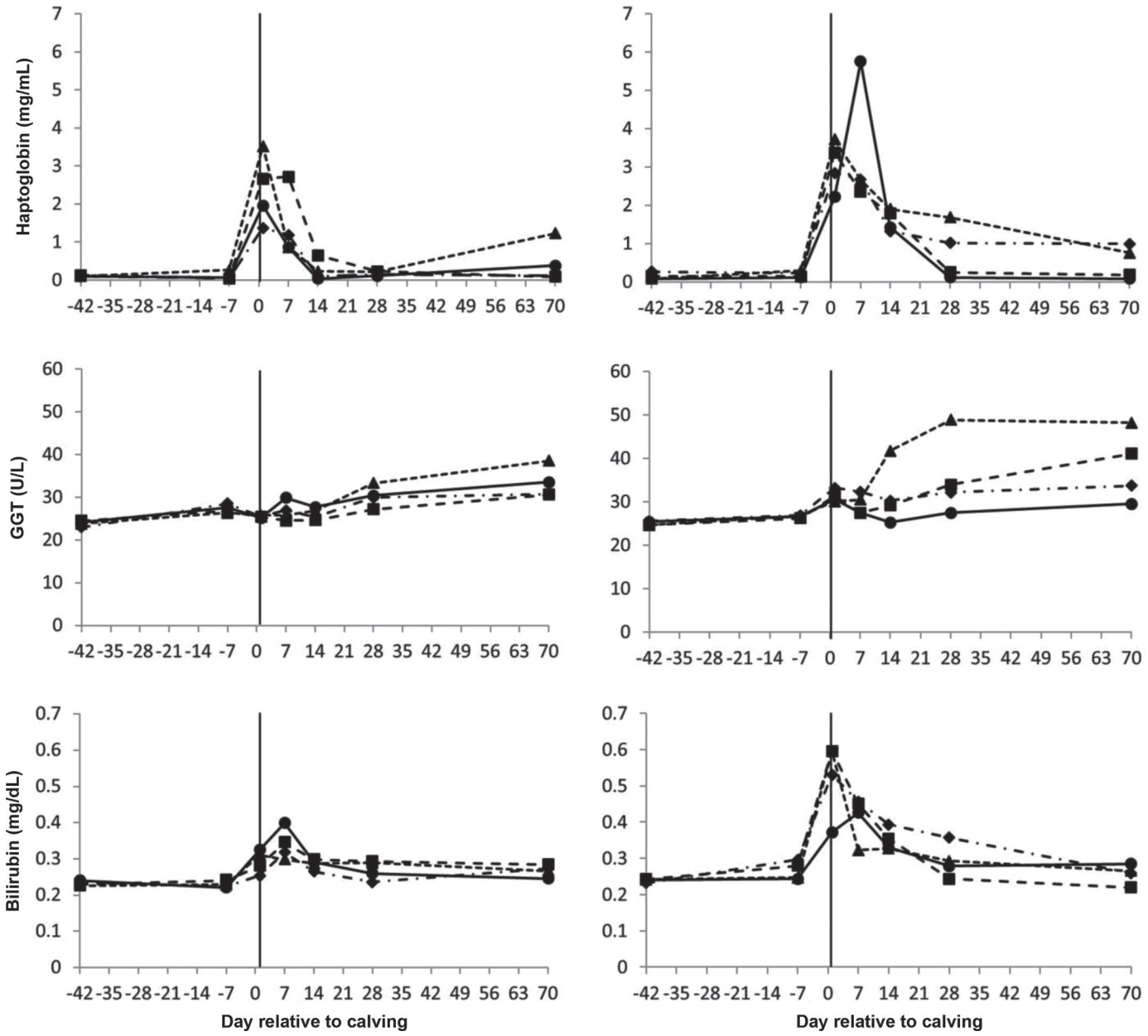

Figure 1. Variables of clinical chemistry of cows in 2nd (left side) and ≥3rd (right side) parity (LSM). Groups included a control group (n = 16; ●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, long dashed line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dash-dotted line) from d −42 until 70 relative to calving. In the control group, 10 cows were in 2nd and 6 in 3rd or higher parity. In the CLA group, 9 cows were in 2nd and 6 in 3rd or higher parity. For the vitamin E group, this relationship was 10:5, whereas in the CLA + vitamin E group 5 cows were in 2nd and 7 cows in 3rd or higher parity. GGT = gamma-glutamyl-transferase.

more profound dip after calving in Pa 2 compared with animals in Pa 1. This is reflected by an interaction between time, treatment and parity ($P < 0.001$). The mean corpuscular hemoglobin values of the VE group in Pa 2 were elevated on d −3, 1, 21, 42, 56, and 70, causing an interaction between time, treatment, and parity ($P = 0.010$). During the course of the trial the mean corpuscular hemoglobin concentration was 30.9 ± 0.17 g/dL, with the CLA + VE group in Pa 1 showing elevated values on d 28 and 35 p.p., and thereby causing an interaction between time, treatment and parity ($P = 0.050$).

The white blood cell profile is presented in Figure 3; WBC increased toward calving ($P < 0.001$) and tended to be higher in animals treated with vitamin E than in those not treated with vitamin E ($8.45 \pm 0.31 \times 10^3$ vs.

7.90 ± 0.31× 10^3/μL; $P = 0.097$). Cows in Pa 1 had the tendency toward higher values than those in Pa 2 (8.51 ± 0.31 vs. 7.84 ± 0.31 × 10^3/μL; $P = 0.056$). Percentage of lymphocytes dropped toward calving ($P < 0.001$) and was higher in Pa 1 than in Pa 2 (37.58 ± 0.74 vs. 34.27 ± 0.83%; $P = 0.005$). Percentage of monocytes in cows treated with vitamin E was decreased compared with those not treated with vitamin E (2.90 ± 0.26 vs. 3.69 ± 0.31%; $P = 0.047$), whereas percentage of granulocytes increased toward calving ($P < 0.001$).

Figure 4 shows the evaluation of the AB assay. In Pa 2, fluorescence of unstimulated cells was decreased compared with Pa 1 (6,478 ± 317 vs. 7,825 ± 273; $P = 0.002$). Fluorescence of stimulated PBMC of control group cows in Pa 2 showed a drop on d 7 a.p., which is reflected by an interaction between time, treatment, and parity ($P = 0.001$). We observed an influence of time ($P < 0.001$) and parity ($P = 0.004$) on the SI. Correlation coefficient between fatty acids and SI was significant only for the VE group (r = −0.19, $P = 0.040$).

Population of lymphocytes is presented in Figure 5. The CLA group in Pa 1 showed a peak in percentage of CD4+-lymphocytes on d 1 a.p., causing an interaction between time, treatment, and parity ($P = 0.039$). Cows

Table 2. Concentration of adiponectin (ADI), aspartate aminotransferase (AST), triglycerides (TRI), albumin (ALB), and urea in blood serum of the experimental groups in 2nd (Pa 1) and 3rd and higher parities (Pa 2) during period 1 (d 42 antepartum until calving), period 2 (d 1–21 postpartum), and period 3 (d 22–70 postpartum; LSM)[1]

		Treatment[2]					*P*-value[3]				
Item		Control n = 16	CLA n = 16	Vitamin E n = 15	CLA + vitamin E n = 12	SEM	CLA	Vitamin E	T	Pa	C × VE × T × Pa
ADI (mg/mL)											
Period 1	Pa 1	29.14	29.62	31.04	30.25	0.58	0.325	0.971	<0.001	0.909	0.215
	Pa 2	31.14	29.14	30.51	30.03						
Period 2	Pa 1	27.41	28.72	27.59	24.11	0.55					
	Pa 2	30.73	26.30	29.60	27.76						
Period 3	Pa 1	28.67	30.33	26.94	28.17	0.57					
	Pa 2	26.28	25.36	28.88	27.52						
AST (U/L)											
Period 1	Pa 1	70.27	61.28	61.19	63.77	5.91	0.972	0.738	<0.001	0.092	0.577
	Pa 2	66.56	68.14	64.60	72.67						
Period 2	Pa 1	119.60	85.58	84.20	84.30	5.12					
	Pa 2	119.88	133.03	139.88	119.53						
Period 3	Pa 1	79.24	81.74	83.38	89.53	5.58					
	Pa 2	82.98	92.75	71.58	94.40						
TRI (mg/dL)											
Period 1	Pa 1	15.91[a]	15.87	14.24	14.23[ab]	0.59	0.893	0.273	<0.001	0.831	0.702
	Pa 2	13.76[ab]	15.61	14.10	16.02[a]						
Period 2	Pa 1	12.05[ab]	7.76	9.66	9.36[ab]	0.50					
	Pa 2	8.80[ab]	10.76	7.27	9.15[ab]						
Period 3	Pa 1	8.42[b]	9.50	10.34	7.84[ab]	0.56					
	Pa 2	9.84[ab]	10.69	9.32	8.05[b]						
ALB (g/L)											
Period 1	Pa 1	34.63	34.00	34.03	31.61	0.42	0.072	0.954	0.023	0.076	0.963
	Pa 2	36.34	33.55	36.13	35.17						
Period 2	Pa 1	33.96	33.91	34.59	34.07	0.37					
	Pa 2	36.17	32.97	35.26	34.87						
Period 3	Pa 1	35.09	34.91	35.50	33.85	0.40					
	Pa 2	36.11	35.51	35.61	36.05						
Urea (mg/dL)											
Period 1	Pa 1	26.42	26.17	31.70	31.68	1.26	0.296	0.008	<0.001	0.991	0.415
	Pa 2	29.99	29.47	34.19	25.85						
Period 2	Pa 1	16.62	16.27	23.26	27.29	1.11					
	Pa 2	18.97	18.85	32.03	19.86						
Period 3	Pa 1	14.49	15.79	24.45	18.78	1.19					
	Pa 2	13.16	16.96	18.81	14.55						

[a,b]Least squares means with different superscripts within columns differ ($P < 0.05$).

[1]The concentration on d 42 antepartum was used as a covariable in the statistical analysis.

[2]Treatment: Before calving cows were fed a concentrate proportion of 60%. Postpartum, the concentrate proportion increased from 30 to 50% within 3 wk in all groups. CLA (n = 16) and CLA + vitamin E (n = 12) received 8.4 g of *trans*-10,*cis*-12 CLA/d (BASF Lutrell; Ludwigshafen, Germany). Vitamin E (n = 15) and CLA + vitamin E groups received 2,327 IU of vitamin E/d (BASF Lutavit E 50). The control group (n = 16) as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

[3]T = time; Pa = parity; C × VE × T × Pa = interaction between CLA, vitamin E, time, and parity.

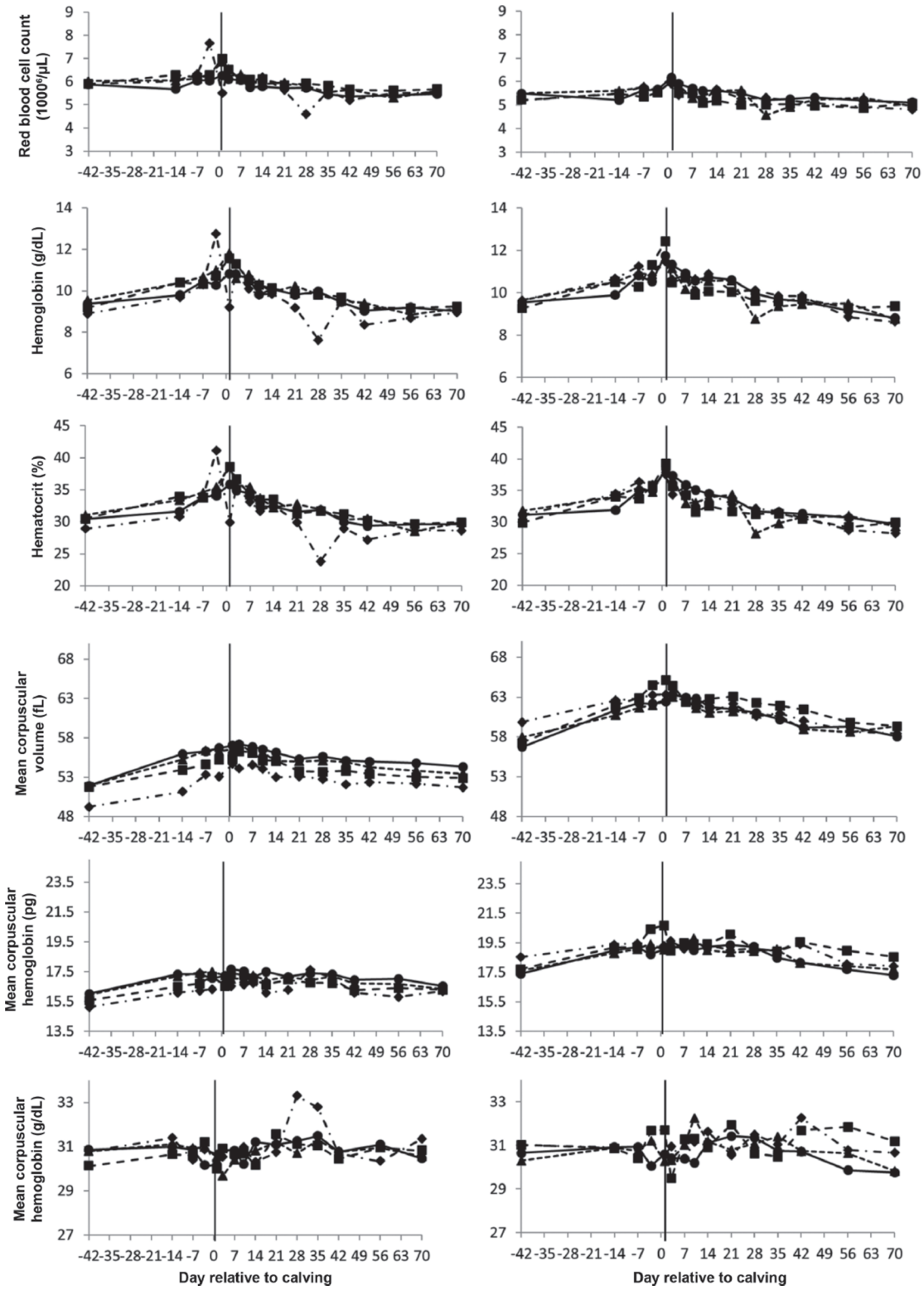

Figure 2. Red blood cell profile of whole blood of cows in 2nd (left side) and ≥3rd (right side) parity (LSM). Groups included a control group (n = 16; ●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, long dashed line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dash-dotted line) from d −42 until 70 relative to calving. In the control group, 10 cows were in 2nd and 6 in 3rd or higher parity. In the CLA group, 9 cows were in 2nd and 6 in 3rd or higher parity. For the vitamin E group, this relationship was 10:5, whereas in the CLA + vitamin E group 5 cows were in 2nd and 7 cows in 3rd or higher parity.

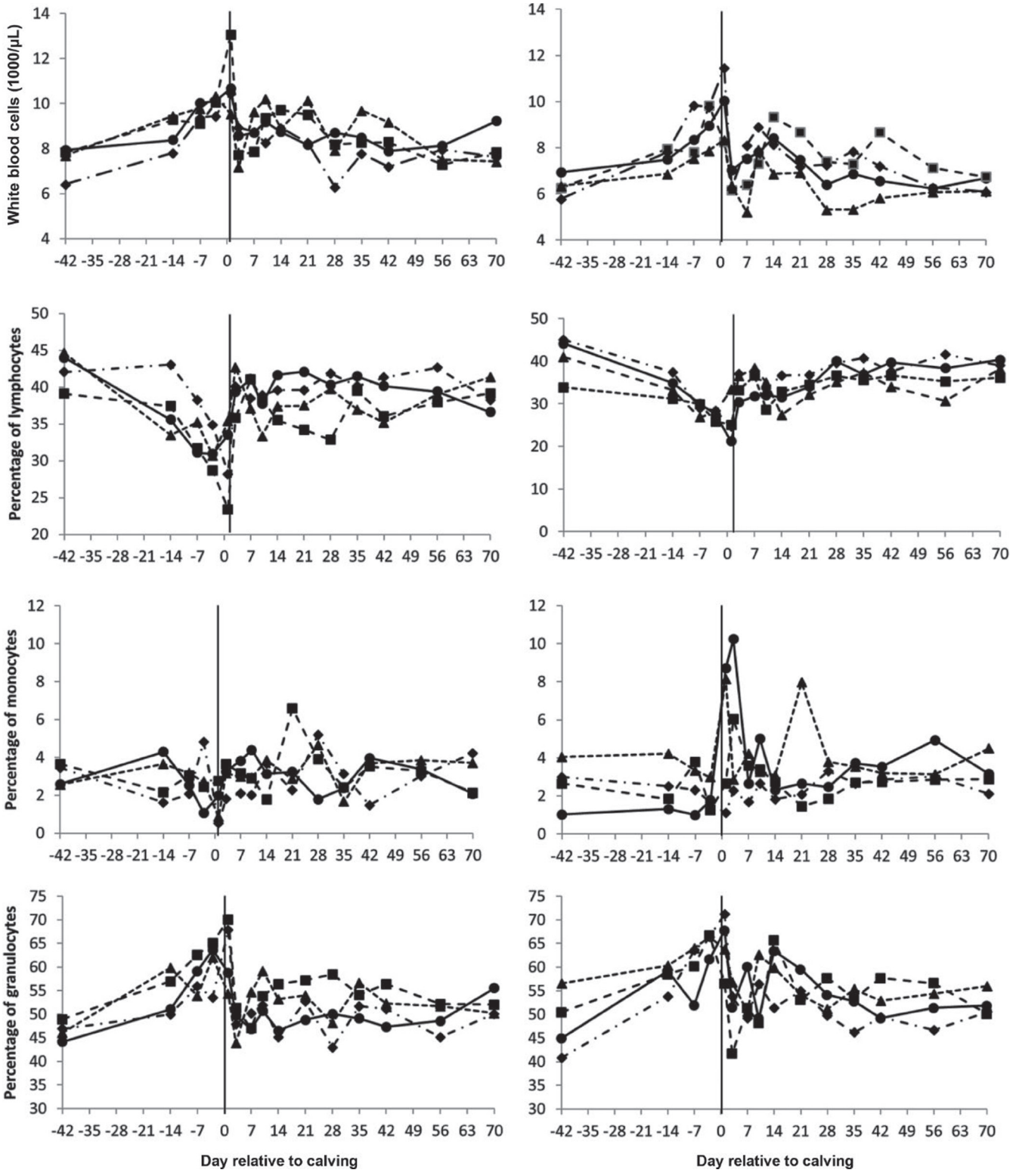

Figure 3. White blood cell profile of whole blood of cows in 2nd (left side) and ≥3rd (right side) parity (LSM). Groups included a control group (n = 16; ●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, long dashed line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dash-dotted line) from d −42 until 70 relative to calving. In the control group, 10 cows were in 2nd and 6 in 3rd or higher parity. In the CLA group, 9 cows were in 2nd and 6 in 3rd or higher parity. For the vitamin E group, this relationship was 10:5, whereas in the CLA + vitamin E group 5 cows were in 2nd and 7 cows in 3rd or higher parity.

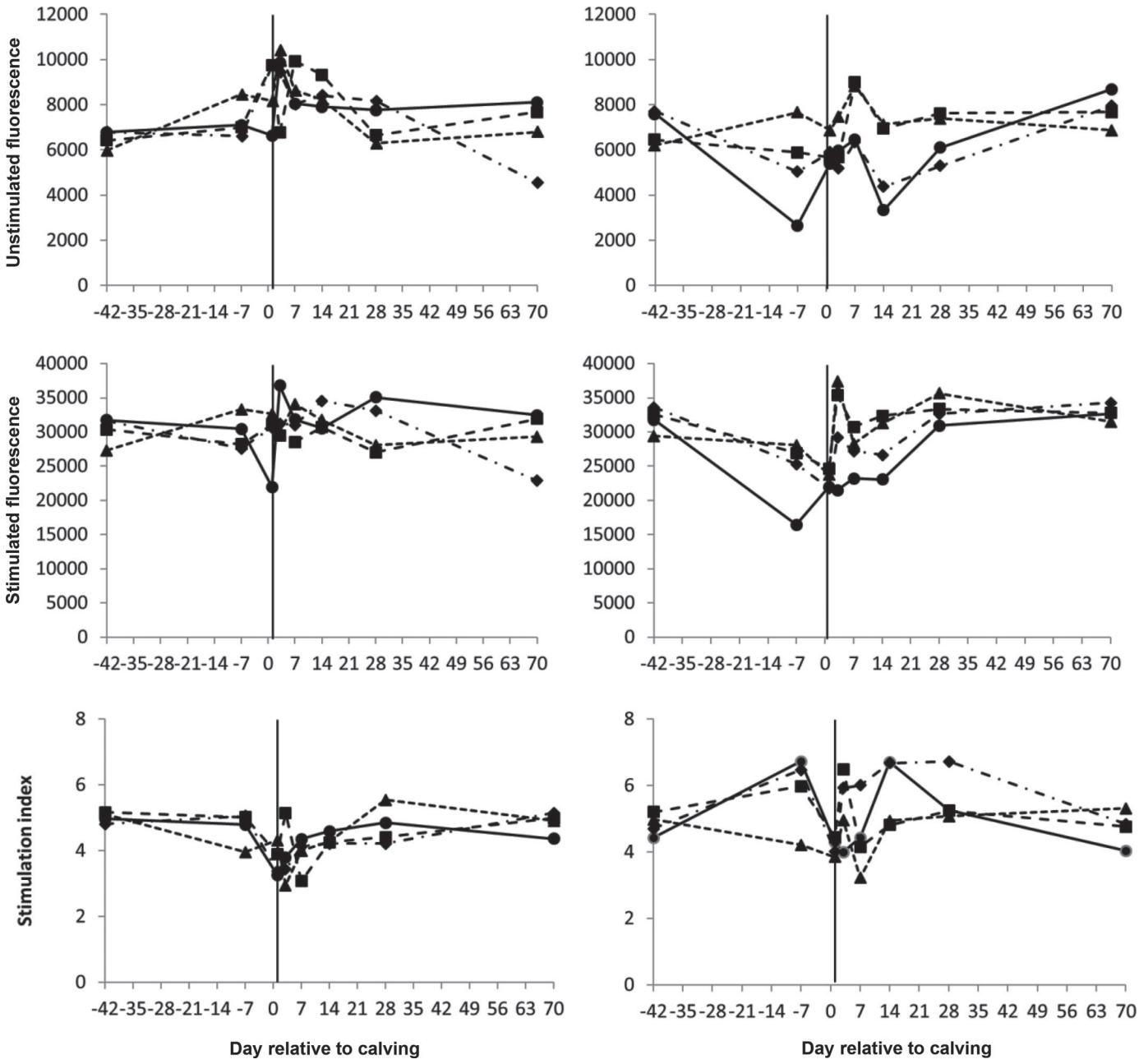

Figure 4. Activity of unstimulated and concanavalin A-stimulated peripheral mononuclear blood cells (PBMC) and resulting stimulation index of cows in 2nd (left side) and ≥3rd (right side) parity (LSM). Groups included a control group (n = 16; ●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, long dashed line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dash-dotted line) from d −42 until 70 relative to calving. In the control group, 10 cows were in 2nd and 6 in 3rd or higher parity. In the CLA group, 9 cows were in 2nd and 6 in 3rd or higher parity. For the vitamin E group, this relationship was 10:5, whereas in the CLA + vitamin E group 5 cows were in 2nd and 7 cows in 3rd or higher parity.

in Pa 1 had lower percentages of CD8+-lymphocytes than older cows (10.93 ± 0.63 vs. 13.11 ± 0.71%, respectively; $P = 0.022$). We detected no influence of time, treatment, or parity on the ratio of CD to lymphocytes.

Figure 6 shows the basal and stimulated proportion and fluorescence intensity of the R123+ population of PMNL. Basal R123+ proportion in Pa 1 was increased compared with Pa 2 (10.4 ± 0.56 vs. 8.2 ± 0.63%, respectively; $P = 0.012$) and decreased by 78% from d 42 a.p. to d 1 p.p. ($P < 0.001$). The MFI of basal R123+ cells dropped 32% from d 42 to 3 a.p. ($P < 0.001$). Treatment and parity were without effect. Correlation

coefficients between MFI of basal R123+ cells and vitamin E in serum were only significant for the VE group ($r = -0.25$, $P = 0.012$). Stimulated R123+ proportion peaked on d 1 a.p. ($P = 0.038$) without influence of treatment or parity; MFI of stimulated R123+ proportion increased 29% from d 42 to 1 a.p. ($P < 0.001$).

DISCUSSION

The objective of our trial was to investigate the influences of treatment with either CLA or vitamin E or a combination thereof on biochemical and hematological variables as well as on populations of immune cells and

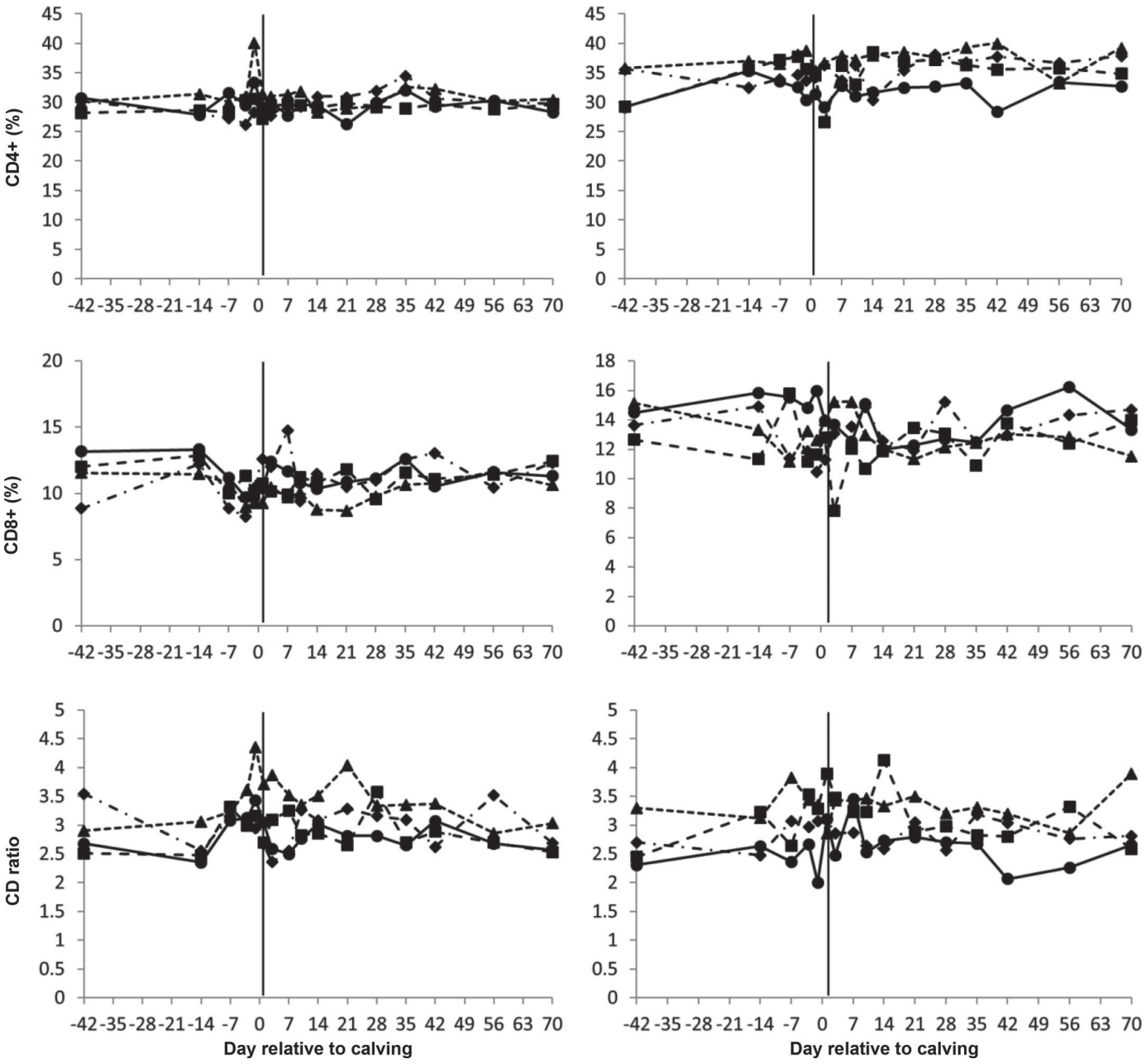

Figure 5. Mean proportion of $CD4^+$ and $CD8^+$ subpopulations of lymphocytes as well as the CD ratio of whole blood of cows in 2nd (left side) and ≥3rd (right side) parity (LSM). Groups included a control group (n = 16; ●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, long dashed line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dash-dotted line) from d −42 until 70 relative to calving. In the control group, 10 cows were in 2nd and 6 in 3rd or higher parity. In the CLA group, 9 cows were in 2nd and 6 in 3rd or higher parity. For the vitamin E group, this relationship was 10:5, whereas in the CLA + vitamin E group 5 cows were in 2nd and 7 cows in 3rd or higher parity.

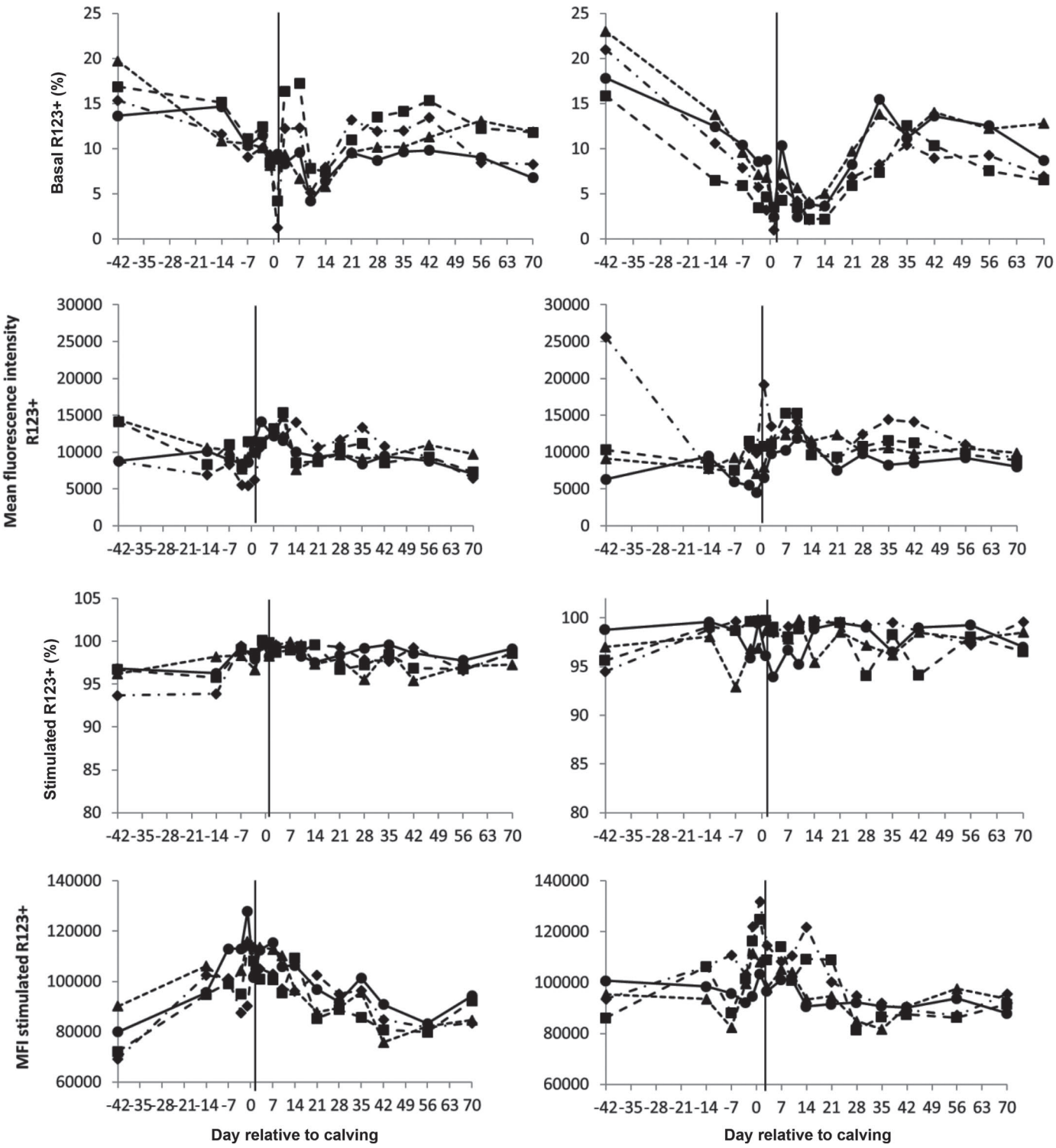

Figure 6. Mean basal and stimulated proportion and fluorescence intensity (MFI) of rhodamine (R)123+ population of total PMNL of cows in 2nd (left side) and ≥3rd (right side) parity (LSM). Groups included a control group (n = 16; ●, solid line), a CLA group receiving 8.4 g of *trans*-10,*cis*-12 CLA/d (n = 16; ■, long dashed line), a vitamin E group (n = 15) receiving 2,327 IU of vitamin E/d (▲, short dashed line), and a group (n = 12) receiving both treatments (◆, long dash-dotted line) from d −42 until 70 relative to calving. In the control group, 10 cows were in 2nd and 6 in 3rd or higher parity. In the CLA group, 9 cows were in 2nd and 6 in 3rd or higher parity. For the vitamin E group, this relationship was 10:5, whereas in the CLA + vitamin E group 5 cows were in 2nd and 7 cows in 3rd or higher parity.

their function in higher-conditioned dairy cows during the transition period (mean BCS at d −42 was 3.7). Gillund et al. (2001) showed that cows with high body condition before parturition are at a particularly high risk p.p. for lipomobilization and, consequently, ketosis. Therefore, we chose the animal model proposed by Schulz et al. (2014), according to which cows sensitive to ketosis should be selected for the trial. This model was shown by Drong et al. (2016) to be suitable to investigate the efficacy of treatments on health variables of transitional dairy cows.

The present trial's serum concentrations of BHB, fatty acids, and glucose, as well as variables of lactation performance, were described in Schäfers et al. (2017a). According to Holtenius and Holtenius (1996), the type-II ketosis is characterized by an increase of BHB and fatty acids during the first 3 wk in early lactation, which in combination with stress might lead to a fatty liver. In our trial, however, cows did not develop fatty livers (Schäfers et al., 2017a). Contrary to the type-II ketosis, type-I ketosis occurs after 3 to 6 wk of lactation, with cows able to use ketone bodies to partially compensate for the decreased energy intake. Consequently, these animals do then not show symptoms related to liver stress. The cows in our trial were higher-conditioned (Schäfers et al., 2017a) and fed according to the chosen animal model, but they did not develop type-II ketosis (Holtenius and Holtenius, 1996). High concentrations of fatty acids and ketone bodies in early lactation accompanied with a fatty liver might be important for the development of diseases in transition dairy cows (Roche et al., 2009; Esposito et al., 2014). The activity of AST, GGT, and GLDH measured blood is suitable for assessing liver cell integrity (Bertoni and Trevisi, 2013). Sevinc et al. (2001) reported that elevated activities of AST and GGT are linked to fatty liver. During the present trial, the activities of these enzymes were in all groups within physiological range according to Kraft and Dürr (2005); therefore, we can conclude that liver cell integrity was not impaired. Consequently, it was not possible to show any effect of treatment on variables of clinical chemistry related to liver health.

The peak bilirubin concentration in our trial observed shortly after parturition exceeded the physiological concentration of 0.35 mg/dL. Increased bilirubin concentrations might be attributed to prehepatic, hepatic, or posthepatic causes. Bertoni and Trevisi (2013) suggest that increased bilirubin concentrations around parturition are caused by inflammation, which is responsible for a reduced production of liver proteins. We assumed that, among the reduced liver proteins, enzymes responsible for excretion of bilirubin into bile were reduced as well and that, consequently, this excretion might be impaired. According to Bobe et al. (2004), increased bilirubin might also be an indicator of a decreased bile flow within the liver. Correlations between GGT and bilirubin were significant for the groups treated with CLA, whereas correlations between GGT and cholesterol were significant for all groups except the CLA + VE group. This might indicate a decreased bile flow, which we, however, did not particularly measure. As concentrations were within physiological values 14 d after parturition for cows in Pa 1 and 28 d after parturition for cows in Pa 2, we concluded that that the effect of inflammation around parturition on the production of liver proteins was more severe in older than in younger cows.

Hematological variables were influenced by day and, hence, by parturition rather than by treatment. A hemoconcentration caused by a reduced water intake around calving might be responsible for the peaks in hemoglobin and hematocrit. According to Shperling and Danon (1990), the MCV in humans increases with age. This increase is probably caused by a decreased lifespan of red blood cells, leading to an increased production of red blood cells. As young red blood cells have a higher volume, the MCV consequently increases as well. Generally, the values were within physiological ranges according to Kraft and Dürr (2005) for all groups during our trial. Therefore, we came to the conclusion that neither treatment nor parity elicited a negative effect on the red blood profile.

The increase in WBC toward calving is in line with literature (Zerbe et al., 2000; Drong et al., 2017). According to Quiroz-Rocha et al. (2009), the WBC of cows in 3rd or higher parity should be enhanced compared with cows in the 2nd parity before calving; this finding was not confirmed by the present results. Similar to Kelley et al. (2002) and Albers et al. (2003) in humans, as well as Song et al. (2005) in mice, we detected no influence of treatment with CLA on the white blood cell profile. Meydani et al. (1997) reported that vitamin E supplementation did not affect total quantity of B and T lymphocytes in humans, which is in accordance with our findings; however, supplementation with CLA and vitamin E might elicit a different effect in cows challenged by ketosis.

The subpopulations and functionality of immune cells were influenced by parturition and not by treatment or parity. Metabolic activity of PBMC was lowest around parturition, which is in accordance with the findings of Ishikawa (1987), Detilleux et al. (1994), Lessard et al. (2004), and Renner et al. (2012). The ability of PBMC to respond to stimulation showed in all groups the lowest values on d 7 p.p., which is in accordance with the results by Hussen et al. (2011) and Renner et al. (2013). In the present trial, we observed no difference in SI increase after parturition, and that no effect of CLA

supplementation could be found may be attributed to several factors. Though we did not analyze the fatty acid profile in PBMC, we assumed that a dose of 8.4 g of CLA/d for 112 d was sufficient to include *trans*-10,*cis*-12 CLA into the fatty acid profile. Hussen et al. (2011) and Renner et al. (2012) reported a change of fatty acid profile at 5.7 g of CLA/d for 105 d; Renner et al. (2012) also observed negative correlations between fatty acids and SI. In the present study, correlations between fatty acids and SI were not significant; therefore, we might conclude that fatty acid concentrations were not high enough to provoke a disrupting effect on PBMC. Peak fatty acid concentration in our study was 0.79 mmol/L at d 7 p.p. Lacetera et al. (2004) reported a decreased proliferation of PBMC from fatty acid concentrations of 1 mmol/L and above. Park and Pariza (1998) observed that commercial calf serum as used in the AB assay can contain significant amounts of *trans*-10,*cis*-12 CLA itself, which might mask the effect of the exogenous CLA supplementation. According to Nugent et al. (2005), the effect of CLA on PBMC might also be attributed to the *cis*-9,*trans*-11 isomer. Those authors observed an influence of CLA when cows received 1.4 g/d of *cis*-9,*trans*-11 CLA in an 80:20 mixture. The PBMC of cows that received 1 g/d of *cis*-9,*trans*-11 CLA in a 50:50 mixture were not influenced; however, cows in our trial received a higher dose of 8.4 g/d of *cis*-9,*trans*-11 CLA in a 50:50 mixture. Therefore, it might be possible that not the daily amount of *cis*-9,*trans*-11 CLA is crucial, but the ratio of both isomers.

Contrary to Hussen et al. (2011), who detected a decrease of CD4+ lymphocytes from d 21 p.p. on in animals treated with 5.7 g of CLA daily, we did not detect any changes in lymphocyte subpopulations. The observed distinct nadir of the R123+ proportion of granulocytes from 2 to 10% shortly after calving is in line with results described in literature (Dosogne et al., 1999; Rinaldi et al., 2008); and Drong et al. (2017) emphasized the importance of parturition for the oxidative system of the cow. The R123+-populations in cows in 3rd or higher parity were increased only from d 28 p.p. on. Consequently, we concluded that the ability of PMNL to elicit an oxidative burst reaction might be impaired in older cows compared with younger cows. This effect of aging is well described in literature for human PMNL (Niwa et al., 1989). As the oxidative burst of granulocytes is an important defense mechanism against pathogens, older cows might be more sensitive to infections. We did not observe any influence of supplementation. Ndiweni and Finch (1996) found in vitro that vitamin E increased chemotactic and phagocytic abilities as well as the production of superoxide of bovine PMNL. Politis et al. (1995, 2004) reported that doses of 3,000 IU/d of vitamin E increase the function of bovine PMNL. Gyang et al. (1984) compared the phagocytizing ability of cows supplemented with 1,000 IU of vitamin E and selenium to that of selenium-deficient cows and detected no difference in function of PMNL. Therefore, we can conclude that supplementation with 2,354 IU of vitamin E does not increase the function of PMNL to phagocyte pathogens and that a daily supplementation of at least 3,000 IU might be necessary. Kang et al. (2007) reported that *trans*-10,*cis*-12 CLA upregulates the PPARγ-expression in porcine PBMC, which leads to an increased production of tumor necrosis factor-α and consequently improves the phagocytic capacity of PMNL. Furthermore, Kang and Yang (2008) observed that supplementation of *trans*-10,*cis*-12 CLA enhances the phagocytic capacity and oxidative burst activity in PMNL of dogs. Because vitamin E is a ligand at PPARγ, we assumed that supplementation of CLA and vitamin E might have an effect on the function of PMNL. As this is not the case, further studies are necessary to clarify stimulation mechanisms of bovine PMNL.

CONCLUSIONS

The aim of this study was to investigate the effects of treatment with CLA or vitamin E or a combination thereof on biochemical, hematological and immunological variables of dairy cows during the critical transition phase. Elevated BHB and fatty acid concentrations play an important role in the disruption of immune functions around calving. Though we applied an established animal model, which has been proven to increase the susceptibility for lipomobilization and ketosis, cows in the present trial did not show ketotic concentrations of BHB and fatty acids. Therefore, the potential to observe treatment effects might have been limited. However, we can conclude that the supplemented dose of vitamin E was not sufficient to counteract the immune suppression caused even in healthy cows by the event of parturition. Furthermore, we concluded that the immune function of older cows was more sensitive to the event of calving. Older cows might be more susceptible to inflammatory events, which might be caused by their impaired ability to produce liver proteins in the state of inflammation caused by the event of parturition.

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7. General Discussion

Genetic improvement of dairy cows results in increased lactation performances, but cannot be matched by an appropriate increase of DMI and energy intake in the transition period, resulting in a negative EB. Consequently, nutrition strategies in dairy cows must aim at reducing this imbalance. Baumgard et al. (2000) have proven, that *trans*-10, *cis*-12 isomer can be successfully supplemented to reduce milk fat synthesis. Because the highest energy expenditure in lactogenesis is accounted to fat synthesis, supplementation of *trans*-10, *cis*-12 CLA might be applied to decrease the negative EB in early lactation. However, reported effects of CLA on the energy balance in literature are inconsistent, since CLA might decrease DMI and increase total milk yield, which might lead to redistribution of energy and consequently not improve energy balance. Literature indicates that supplementation of CLA results in higher proportions of CLA in milk fat. Since both isomers of CLA might be beneficial for human health, a higher proportion of CLA in milk fat might enhance the nutritive value of milk products. However, unsaturated fatty acids are susceptible for oxidation. Since Vit. E is a strong antioxidant, increased concentrations of Vit. E in milk might be suitable to prevent fatty acids from oxidation. In addition, supplementation of Vit. E in transition dairy cows might be applied to reduce the oxidative stress and immune suppression caused by the event of calving. The present experiment aimed to investigate if a supplementation of rumen-protected CLA and Vit. E or a combination thereof had an influence on the energy metabolisms, body fat mobilization, lactation performance, vitamin content and fatty acid distribution of milk and the immune system of dairy cows in the transition period and early lactation. Therefore, an animal model based on a combination of a high concentrate proportion a.p., a high BCS a.p. and a deceleration in concentrate proportion increase p.p. was applied to evoke lipomobilisation and following ketosis type II p.p. The concentrate proportion of 60% a.p. resulted in an energy density of the ration of 7.43 MJ NE_L/kg DM in period 1. P.p. the energy density was 7.11 MJ NE_L/kg DM in period 2 (30% concentrate proportion) and 7.19 MJ NE_L/kg DM in period 3 (50% concentrate proportion), which was slightly lower in period 1 and 3, while being higher in period 2 than the 7.46, 7.01 and 7.55 MJ NE_L/kg DM used by Drong et al. (2016a). Mean BCS (Figure 4) 6 weeks a.p. was 3.7 and BCS increased towards 4.0 at calving due to the high energy density of the ration.

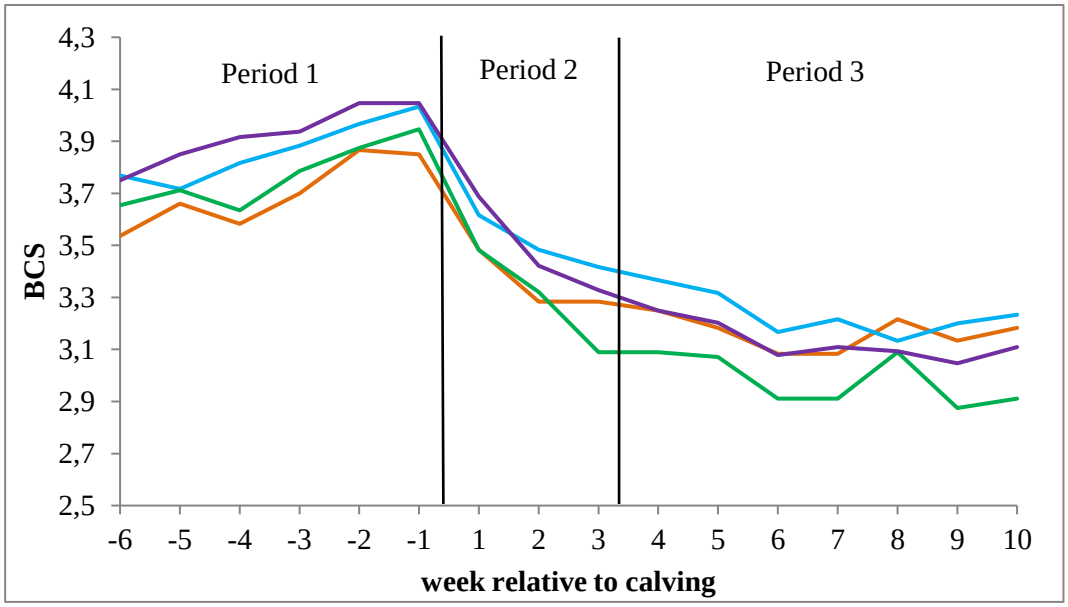

Figure 4: Course of the BCS (means) during the experiment. Purple line: control group (n=16); blue line: CLA group (n=16); orange line: Vit. E group (n=15); green line: CLA + Vit. E group. CLA and CLA + vitamin E received 8.4 g of *trans*-10, *cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E and CLA + Vit. E groups received 2,327 IU of Vit. E/d (BASF Lutavit E 50). The control group as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

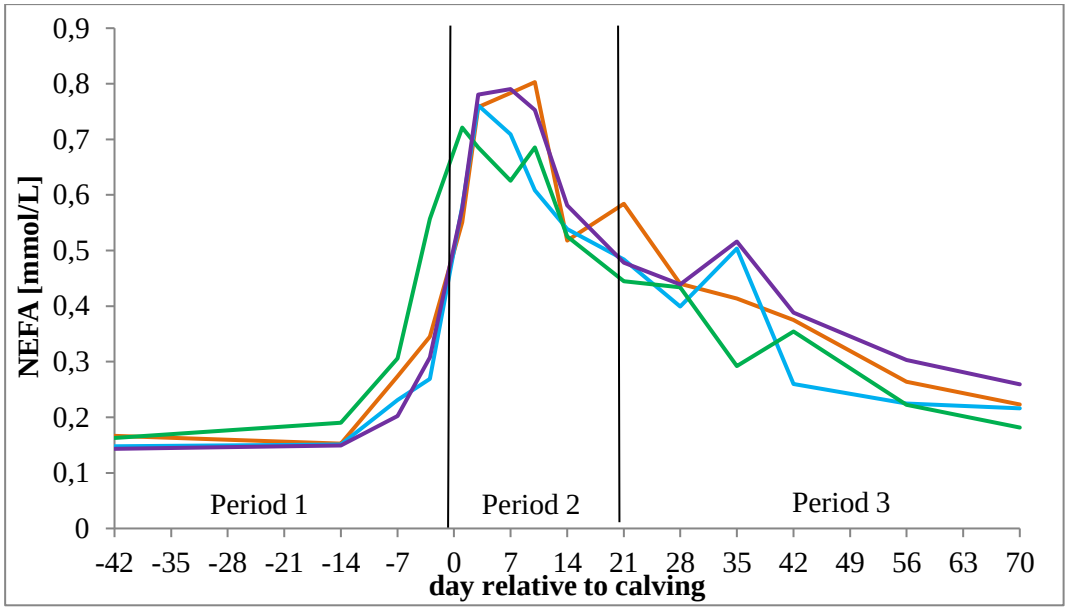

Figure 5: Course of the NEFA (LSMeans) during the experiment. Purple line: control group (n=16); blue line: CLA group (n=16); orange line: Vit. E group (n=15); green line: CLA + Vit. E group (n=12). CLA and CLA + vitamin E received 8.4 g of *trans*-10, *cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E and CLA + Vit. E groups received 2,327 IU of Vit. E/d (BASF Lutavit E 50). The control group as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

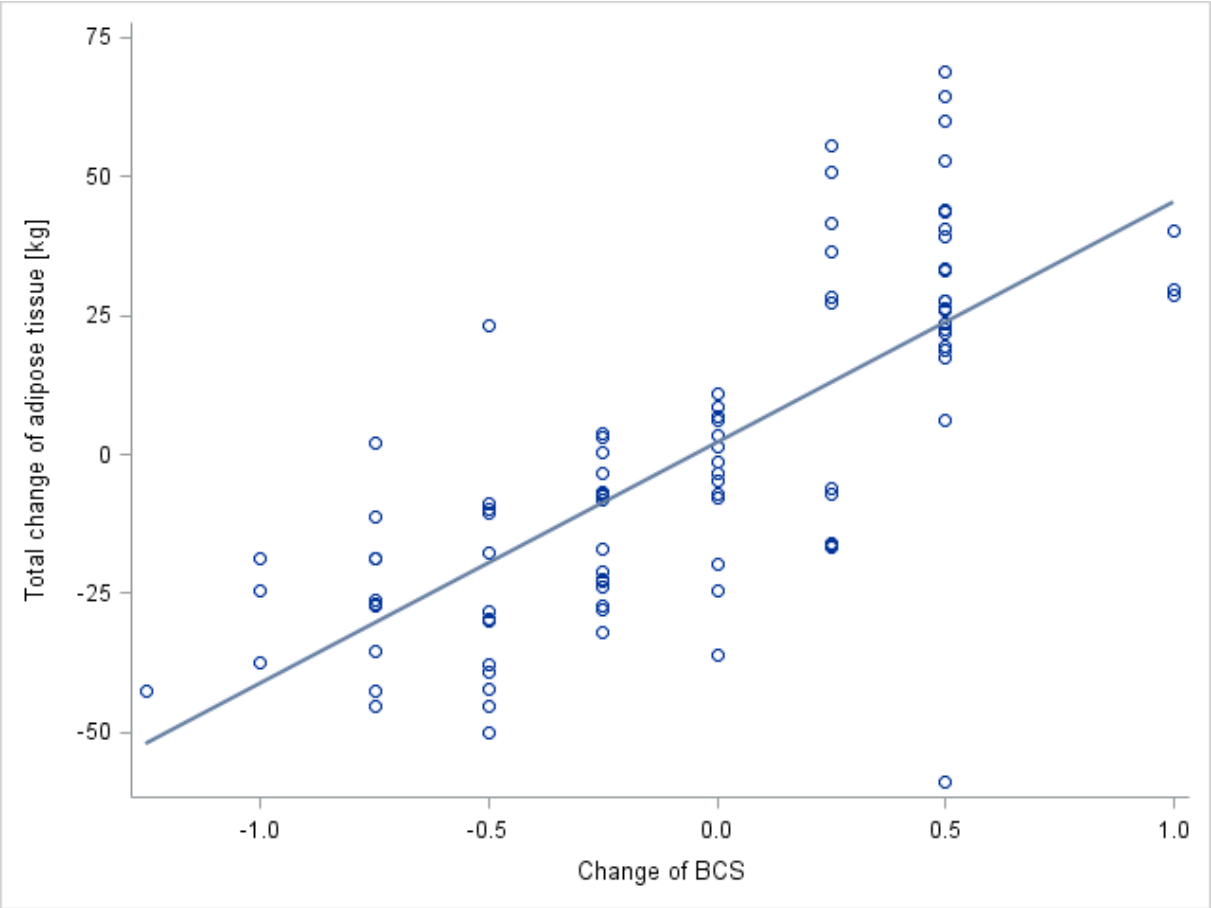

Figure 6: Correlation between change of BCS and total change of adipose tissue [kg]

In period 2 a distinct drop towards 3.3 followed, which is reflected by a mobilization of adipose tissue (**Paper I**, Figure 3) and consequently increase of NEFA (Figure 5) in early lactation. Change of BCS and total change of adipose tissue show a strong correlation of 0.74 (Figure 6, $P < 0.001$).

The correlation between daily change of adipose tissue and serum concentrations of NEFA (Figure 7) is -0.72 ($P < 0.001$) for all groups together with no difference between groups. This negative correlation reflects the increased NEFA in phases of high lipomobilisation.

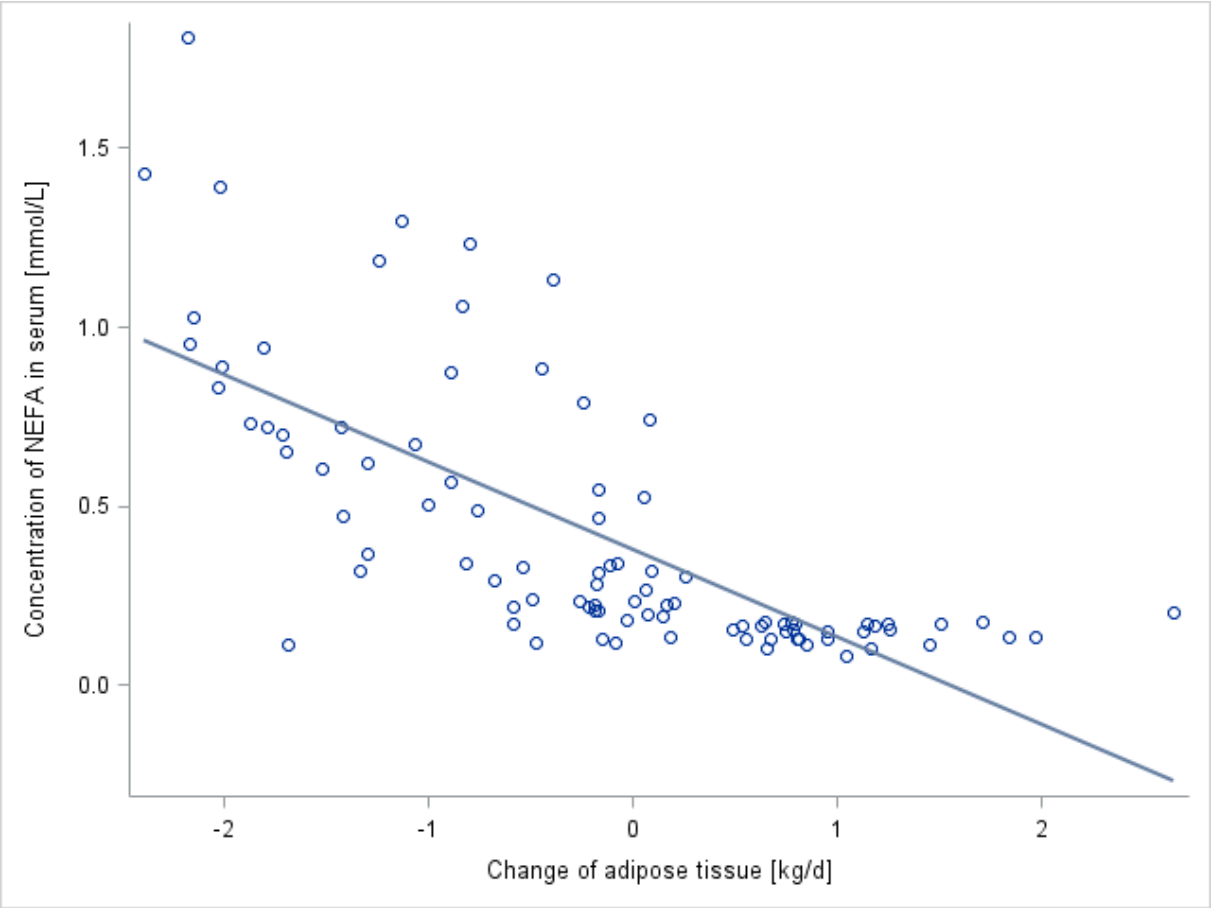

Figure 7: Correlation between change of adipose tissue [kg/d] and concentration of NEFA in serum [mmol/L]

Therefore, it is concluded that the preconditions of the animal model were achieved. During the course of the trial, subclinical ketosis occurred in 63% of the control cows, 87% of the CLA group, 80% of the Vit. E group, and 67% of the CLA + Vit. E group. However, whereas Schulz et al. (2014) and Drong et al. (2016a) reported BHB-concentrations > 1.2 mmol/L in higher conditioned cows during period 2, BHB- concentrations in this trial remained < 1.2 mmol/L during period 2 (**Paper I**, Table 5), which might be attributed to a less pronounced negative EB p.p. Peak BHB-concentrations were achieved during period 3 in 3 treatment groups (**Paper I**, Figure 4). Furthermore, liver lipid content was back down to reference range (Fürll et al., 2002) on day 28 p.p. in three groups and in all groups on day 70 p.p. (**Paper I**, Table 6). Consequently, it can be stated that cows did not develop a ketosis type II but rather a type I ketosis. This might have affected the influences of the supplementations.

7.1 Effects of CLA and Vit. E on energy metabolism and lactation performance

One aim of the current trial was to investigate the influence of CLA and Vit. E on energy metabolism, lactation performance and resulting lipomobilisation. As presented in **Paper I,** DMI and consequently net energy intake of cows supplemented with CLA was reduced. Reduction of DMI amounted to 8% in the CLA group compared to 4% in the CLA + Vit. E group, resulting in an adequate net energy intake decrease. This finding indicates that Vit. E counteracts the DMI-reducing effect of CLA. Comparison with literature (**Paper I**) illustrated that the influence of CLA supplementation is dose-dependent. A possible explanation for these findings might be the effects, which CLA and Vit. E elicit on rumen microbiota. According to Henderson (1973) the long-chain fatty acids inhibit growth of rumen bacteria at high doses. This finding is also emphasized by Maczulak et al. (1981), who observed that specifically the growth of cellulolytic bacteria is inhibited. According to Maia et al. (2007) linoleic acid damages cell-integrity and different poly-unsaturated fatty acids also inhibit growth of cellulolytic fungi. Consequently, it might be assumed, that CLA doses above a certain threshold would also inhibit growth and function of cellulolytic microbiota. A consequence of this inhibition might be a decrease of digestibility of fiber, which would result in a reduced rate of rumen passage. However, differences in rate of rumen passage between animals supplemented and non-supplemented with CLA were not assessed during this trial. Hino et al. (1993) reported that Vit. E was able to alleviate the growth inhibition of cellulolytic bacteria caused by unsaturated fatty acids in vitro. However, when investigating the mechanisms by which CLA reduces DMI, it is important to consider, that DMI was also reduced when CLA was abomasally infused as done by (Baumgard et al., 2000), indicating that depression of DMI might at least partly be independent of ruminal flora. The explanation for a decrease might be that the increased flow of poly-unsaturated fatty acids to the duodenum reduces DMI as was reported by Bremmer et al. (1998) and Benson et al. (2001), probably by decreasing ruminal motility and rumination time (Nicholson and Omer, 1983; Grovum, 1984). However, this cannot be clarified without the comparison of the fatty acid pattern of duodenal fluid from animals supplemented and non-supplemented with CLA. Whereas milk yield was numerically higher in animals supplemented with CLA (**Paper I**), the differences between groups were not significant, which is in line with the findings reported in literature as only Kay et al. (2006), Piamphon et al. (2009) and Moallem et al. (2010) observed a significant milk yield increase caused by CLA. However, daily doses in these trials ranged from 2 to 8 g and were thus lower than the dose used in the present trial. Consequently, DMI and net energy intake were not

reduced, which might allow for an increase of milk yield. In spite of the decreased DMI observed in this trial, net energy balance was less negative in CLA-supplemented cows during period 3, which might be attributed to a lower milk energy output caused by the reduction of milk fat yield. In order to evaluate, whether CLA has lipolytic or antilipogenic effects as presented in the background section in dairy cows, mobilization of adipose tissue was evaluated and was without any difference (**Paper I**). The findings of the present trial indicate that CLA actually has lipolytic or antilipogenic effects in cows, because despite the improved net energy balance, lipomobilisation was not reduced in CLA-supplemented cows.

7.2 Effects of CLA and Vit. E on the composition of milk fat and vitamins retinol, β-carotene and α-tocopherol in milk

Another aim of the present trial was to investigate the effect of both supplementations on the composition of milk fat and the antioxidative vitamins retinol, β-carotene and α-tocopherol in milk. Different nutritional strategies to alter the composition of milk fat and increase CLA content are described in literature (Table 1). It must be noted, that the feeding of fish oil has been shown to elicit a more pronounced effect to increase the percentage of CLA compared to feeding different plant oils (Chouinard et al., 2001). The effects on CLA content are even higher when fish oils are combined with feed which contains C18:2 (n-6), e.g. soybeans (Abu-Ghazaleh et al., 2002; Whitlock et al., 2002; Abu-Ghazaleh et al., 2003). However, these diets caused a reduction of milk protein content, which might probably be attributed to a reduced energy intake (Coulon and Rémond, 1991) caused by a lower DMI. Because a further reduction of DMI in dairy cows during the transition period should be avoided, the combination of fish oil and feed containing high amounts of C18:2 (n-6) might not be favorable in that situation and a direct supplementation of CLA might be preferred.

Table 1: Feeding strategies to alter milk fatty acid pattern and the effects on the proportion of short-chain (SC), medium-chain (MC) and long-chain (LC) fatty acids in milk of dairy cows

Treatment	Effects on proportion of fatty acids compared to the corresponding control groups			CLA % of milk fatty acids	Literature
	SC	MC	LC		
Encapsulated soya oil [630 g/d]	►	▼	▲	*	Mattos and Palmquist (1974)
Unprotected soya oil [600 g/d]	▼	▼	▲	*	Schingoethe et al. (1983)
Calcium salts of palm oil [650 g/d]	►	▼	▲	*	Chilliard et al. (1993)
Peanut oil [5.3% of DM]	no control group			1.33%	Kelly et al. (1998a)
Linseed oil [5.3% of DM]				1.67%	
Sunflower oil [5.3% of DM]				2.44%	
Pasture only	▼	▼	▲	1.09%	Kelly et al. (1998b)
Duodenal infusion of rapeseed oil [630 g/d]	►	▼	▲	*	Chilliard et al. (2000)
Fish oil [1% of DM]	▼	►	▲	1.71%	Donovan et al. (2000)
Fish oil [2% of DM]	▼	►	▲	2.53%	
Fish oil [3% of DM]	▼	►	▲	2.12%	
Fish oil [15 g/kg DM] + sunflower oil [30 g/kg DM]	►	►	►	4.68%	Jones et al. (2005)
Steam-flaked full fat soybeans [78 g/kg DM]	*	▼	▲	1.06%	Li et al. (2009)
Extruded full fat soybeans [78 g/kg DM]	*	▼	▲	1.05%	
Rapeseed oil [10 % of diet]	▼	▼	▲	1.36%	Rego et al. (2009)
Linseed oil [10 % of diet]	▼	▼	▲	1.92%	
Sunflower oil [10 % of diet]	▼	▼	▲	1.82%	
8.4 g/d rumen-protected *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA	*	►	►	0.48 – 0.69%	present study

* No information provided

► no difference ▼ decreased ▲ increased

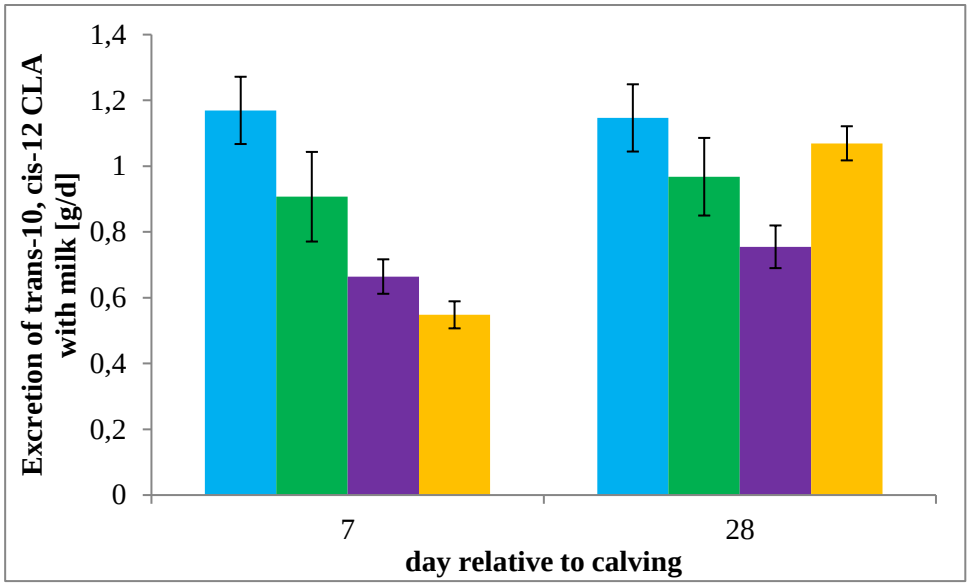

Figure 8: Daily excretion of *trans*-10, *cis*-12 CLA with milk of the experimental groups (means ± standard deviation). Purple: control group (n=16); blue: CLA group (n=16); orange: Vit. E group (n=15); green: CLA + Vit. E group (n=12). CLA and CLA + vitamin E received 8.4 g of *trans*-10, *cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E and CLA + Vit. E groups received 2,327 IU of Vit. E/d (BASF Lutavit E 50). The control group as well as the vitamin E group received 88 g/d of a rumen-protected fat supplement.

Calculating the carry-over from feed to milk (daily excretion of CLA-isomer with milk*100/ daily intake of CLA-isomer with feed [g]), revealed that 14% of the *trans*-10, *cis*-12 isomer were tranferred in the CLA group on d 7 and d 28 p.p. The CLA + Vit. E group showed numerically albeit not statistically significant lower carry-over of 11% and 12% on d 7 and d 28 p.p., respectively (Figure 8). When comparing these results to reports from literature concerning lipid-encapsulated CLA-supplements, carry-over ranged from 4.6% (Moallem et al., 2010) to 7.9% (Perfield et al., 2004a), whereas higher carry-over of 10.1% (Baumgard et al., 2002) to 27.1% (Baumgard et al., 2000; Perfield et al., 2004b) was typically observed in cows supplemented with an abomasal infusion of CLA. However, it must be noted, that in the present trial cows not supplemented with CLA had a proportion of 0.04% to 0.06% *trans*-10, *cis*-12 CLA on total milk fatty acids, resulting in a daily excretion of 0.5 to 1.1 g *trans*-10, *cis*-12 CLA with the milk, whereas previous studies reported *trans*-10, *cis*-12 CLA proportions of <0.01% and consequently daily excretions <0.01 g (Perfield et al., 2004a; Pappritz et al., 2011b). This difference might probably be attributed to the analytical methods used. When correcting the carry-over from feed to milk of animals supplemented with CLA for the values analyzed in non-supplemented cows, carry-over of 0% to 6% is observed, which is in line with previous studies. The low carry-over compared to abomasal infusion indicates, that the

lipid-encapsulation might not be sufficient to protect the CLA from ruminal degradation. Furthermore, the effect of CLA supplementation on CLA-excretion with milk fat might be depending on dose and time of supplementation. Pappritz et al. (2011b) observed no effect on the percentage of all *cis*-9, *trans*-11 CLA and both isomers together, when feeding either 8 or 16 g/d and supplementation began after calving, whereas both isomers were increased when feeding 6 or 16 g/d later in lactation (Pappritz et al., 2011a). However, it must be stated that only 5 and 3 animals were included in the intervention groups in that trial. Chouinard et al. (1999) abomasally infused 4 cows with 50, 100 or 150 g/d CLA with a supplement containing 23.7% of *cis*-9, *trans*-11 CLA. This amounts to a daily dose of 11.9, 23.7 or 35.6 g *cis*-9, *trans*-11 CLA. The *cis*-9, *trans*-11 isomer in milk fat increased in a linear way. However, cows were in late lactation, too (258 ± 43 d). In the present trial CLA proportions of 0.44 to 0.69% were observed, which is comparable to 0.68% in the control group of Chouinard et al. (1999a). These findings indicate, that supplementation with CLA increases the proportion of *cis*-9, *trans*-11 CLA later in lactation, while not affecting it in early lactation. A probable explanation for this difference might be the alteration of fatty acid pattern by BHB-concentrations. Melendez et al. (2016) investigated the relationship between BHB-concentrations and fatty acid proportions in milk and observed that cows with plasma BHB-concentrations > 0.7 mmol/L, which often occur in early lactation, have lower proportions of CLA in milk fat. This observation is confirmed when analysing the data obtained in the present trial (Table 2). It must be noted, that the proportion of the *cis*-9, *trans*-12 CLA in cows with BHB-concentrations > 0.7 mmol/L supplemented with CLA is comparable to that of cows not supplemented with CLA and having BHB-concentrations ≤ 0.7 mmol/L, whereas the proportion of the *trans*-10, *cis*-12 is independent of BHB and only influenced by the supplementation. According to Melendez et al. (2016), high BHB-concentrations might increase the proportion of short- and medium-chain fatty acids in milk fat, because butyrate is used as a precursor.

Table 2: Percentages of individual fatty acids of total milk fat of cows with BHB-concentrations ≤ 0.7 mmol/L and > 0.7 mmol/L (LSMeans)

Fatty acid	BHB ≤ 0.7 mmol/L n=33		BHB > 0.7 mmol/L n=73		*P*-value		
	CLA[1] n=15	Control[2] n=18	CLA n=36	Control n=37	CLA	BHB	CLA*BHB
C 12:0	3.45	3.10	3.17	2.99	0.262	0.398	0.712
C 14:0	11.61	11.03	10.19	10.19	0.568	0.032	0.574
C 15:0	1.16[a]	0.99[ab]	0.85[b]	0.79[b]	0.053	< 0.001	0.334
C 17:0	0.80	0.78	0.79	0.77	0.430	0.698	0.728
C 18:0	12.08	12.14	13.17	13.15	0.957	0.005	0.898
C 18:1 n9 c+t	33.66	33.38	34.99	34.63	0.752	0.210	0.965
C 18:2 n c+t	2.46	2.48	2.45	2.57	0.324	0.531	0.414
cis-9, *trans*-12 CLA	0.62[a]	0.48[bc]	0.49[b]	0.42[c]	<0.001	<0.001	0.092
trans-10, *cis*12 CLA	0.08[a]	0.05[b]	0.07[a]	0.04[b]	<0.001	0.176	0.631

[abc] LSMeans within row with different superscripts differ (P < 0.05)

[1]: Cows were supplemented with 8.4 g of *trans*-10, *cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany)

[2]: Cows were supplemented with 88 g/d of a rumen-protected fat supplement.

Whereas an increase of C14:0 and C15:0 was observed in the present trial, short-chain fatty acids were not analyzed in milk. Though it cannot be confirmed if BHB-concentrations elicit an influence on short-chain fatty acids, high BHB-concentrations reduced the percentage of *cis*-9, *trans*-12 CLA in the milk fat of animals supplemented with CLA. As presented in the background section, interventions increasing the proportion of CLA increase the proportion of other mono- and poly-unsaturated fatty acids as well. These unsaturated fatty acids are more susceptible to oxidation than saturated fatty acids (Wood et al., 2004). Consequently, it might be favourable to increase the amount of antioxidants in milk. As discussed in the background

section, Vit. E has strong anti-oxidative effects. Therefore, an increased Vit. E content in the milk might contribute to the stability of unsaturated fatty acids and thereby prevent the milk from sensory alterations. On the other side, Vit. E acts as a precursor for α-tocopherolquinone, which is needed by fatty acid desaturases, it might thereby elicit synergistic effects with CLA in increasing the proportion of unsaturated fatty acids. However, this was not observed in this trial. Quite the contrary, Vit. E reduced the proportion of *trans*-10, *cis*-12 CLA, probably by decreasing the *trans*-11 to *trans*-10 shift (Pottier et al., 2006) through intercepting the impairment of growth and function of *trans*-11 producing bacteria (Hino et al., 1993). However, the populations of rumen microbiota were not investigated in the present trial. In addition, milk is an important source for Vit. A and E in human nutrition which emphasizes the importance of the excretion of these vitamins with milk. Jensen et al. (1999) suggest that the excretion ability of Vit. A and E is partly inherited and there is an absolute maximum due to the necessary active transport across membranes. In consequence, according to Jensen et al. (1999) higher milk fat yield and higher milk yield should result in a decreased concentration of these vitamins. Consequently, it should be expected that the concentration per g fat should be higher in cows supplemented with CLA and per g milk should be lower in cows supplemented with CLA. However, as presented in **Paper II**, this hypothesis cannot be confirmed. The correlation between milk yield and excretion of α-tocopherol was 0.26 (P = 0.006) and that between milk yield and excretion of β-carotene was 0.12 and not significant. However, it is important to note, that in the trial of Jensen et al. (1999) the mean maximum excretion amounted to 32.4 mg/d for α-tocopherol and 27.5 mg/d for β-carotene, whereas the results obtained in this trial show a lower mean excretion of 28.2 and 5.8 mg/d. Therefore, to investigate the relationship of milk and milk fat yield and excretion of Vit. E and Vit. A, it might be necessary to increase Vit. E supplementation and supplement Vit. A as well.

7.3 Effects of CLA and Vit. E on oxidative stress and immune function

A higher supplementation of Vit. E might also be necessary to reduce the oxidative stress caused by the event of parturition (**Paper II**). Whereas Vit. E in blood was increased in the Vit. E group on d 70 p.p., the Vit. E in blood of the CLA + Vit. E group did not increase at all, suggesting that CLA might lead to an enhanced excretion of Vit. E with the milk. However, when assessing total excretion of Vit. E with the milk (**Paper II**, Table 5), CLA did not elicit any influence and both groups supplemented with CLA show numerically, albeit not significantly, lower excretion than the control group on d 28 p.p. In consequence, another mecha-

nism must be responsible for no observed improvement of blood Vit. E levels in the CLA + Vit. E group. Risérus et al. (2002) reported that *trans*-10, *cis*-12 CLA increased oxidative stress by increasing lipid peroxidation in obese humans. However, in that trial, Vit. E concentrations in blood were not affected by supplementation. In the present study, direct markers for lipid peroxidation were not analyzed and consequently, from the results obtained in this trial, it cannot be clarified, why supplementation with CLA + Vit. E did not increase Vit. E in blood.

From the results presented in **Paper III** it appeared that older cows are more sensitive to the disruption of immune function by the event of calving. When assessing the number of days, on which cows were medically treated (Table 3), no influence of parity was detected, whereas supplementation with Vit. E had a reducing effect on days of treatment. It must however be noted, that days medically treated does not necessarily fully reflect days of illness. Consequently, it might be possible, that supplementation with Vit. E does not reduce days, on which cows are ill, but attenuate the symptoms of diseases. This effect might be associated with the tendency for higher white blood cell counts in cows supplemented with Vit. E (**Paper III**) probably caused by an enhancing effect of Vit. E on function of the immune system, resulting in a more pronounced reaction to diseases. However, cows were only observed for 16 weeks and health status was not assessed according to a standardized procedure. Therefore, this finding should be treated with caution and further research should be conducted on the influence of Vit. E on incidence of diseases in order to obtain reliable results.

As inflammatory processes might not necessarily cause clinical symptoms and thus treatment, but nevertheless impair lactation and reproductive performance, it is necessary to identify these subjects. The liver functionality index (**LFI**) according to Bertoni et al. (2006) is a measurement to identify cows which suffer from inflammatory diseases, or whose liver function is impaired after 4 weeks of parturition, but who do not show clinical symptoms.

Table 3: Days of medical treatment per group of cows in the 2nd (Pa 1) and ≥ 3rd (Pa 2) parity (LSMeans)

	Treatment[1]					*P*-value			
	Control n=16	CLA n=16	Vit. E n=15	CLA + Vit. E n=12	SEM	CLA	Vit. E	Pa	CLA*Vit. E*Pa
Pa 1	12	8	3	3	2	0.886	0.048	0.879	0.667
Pa 2	6	11	6	4	2				

Pa: Parity

[1]Treatment: CLA and CLA + vitamin E were supplemented with 8.4 g of *trans*-10, *cis*-12 CLA/d (BASF Lutrell, Lampertheim, Germany). Vitamin E and CLA + Vit. E groups were supplemented with 2,327 IU of Vit. E/d (BASF Lutavit E 50). The control group as well as the Vit. E group were supplemented with 88 g/d of a rumen-protected fat supplement.

LFI is used to reflect the changes occurring in concentrations of albumin, cholesterol and bilirubin between d 3 p.p. and d 28 p.p., which are standardized according to the changes of these three variables observed in healthy cows at the same lactation stage. The LFI is calculated according to the following formula:

$$LFI = \frac{Alb-17.71}{1.08} + \frac{Chol-2.57}{0.43} - \frac{Bil-6.08}{2.17}$$

Where Alb and Chol are the albumin and cholesterol subindices, which are calculated as 0.5*concentration on d 3 p.p. + 0.5*(concentration on d 28 p.p. – concentration on d 3 p.p.) and Bil is the subindex for bilirubin, which is calculated as 0.67*concentration on d 3 p.p. + 0.33*(concentration on d 28 p.p. – concentration on d 3 p.p.). In case of no sampling on d 3 values from the sample of d 1 were taken. Four animals were excluded from analysis because of missing values on d 3 as well as d 1. The LFI ranged from -14.6 to 6.3 with a mean of 0.7 ± 3.2, the 25th percentile being -0.27 and the median being 1.08. Positive LFI, which were obtained by 37 out of 55 cows in this trial are considered to be favorable. When evaluating the influence of supplementations or parity on the LFI, no effects were detected. The same is true, when quartiles of LFI were analyzed. Eckersall and Conner (1988) stated, that haptoglobin is the most sensitive positive acute phase protein in cows. Consequently, cows suffering from acute inflammations and thus having low LFI should have increased haptoglobin concentrations. However, the correlation between haptoglobin and LFI in this trial (Figure 9) was –0.02 and was not significant ($P = 0.867$). When comparing haptoglobin-concentrations of the lowest and the highest quartile of LFI, no differences were found (Figure 10). Trevisi et al. (2012) reported that cows with lower LFI have increased concentrations of NEFA and bilirubin from d 28 a.p. as well, indicating that these subjects might be more susceptible to an impaired liver function.

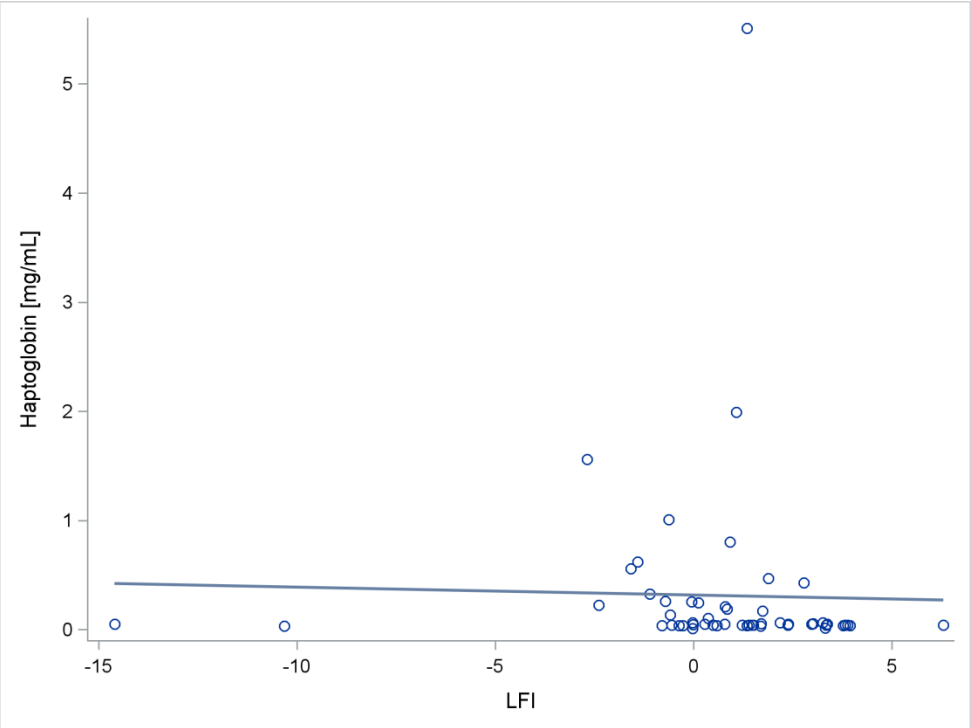

Figure 9: Correlation between the liver functionality index (LFI) and haptoglobin

Interestingly, 6 cows having a LFI in the 4th quartile were treated for either mastitis or metritis, whereas only 5 cows in the lowest quartile were treated and correlation between LFI and days treated was not significant. For NEFA (not shown) neither an interaction nor an influence of LFI was detected. In addition, the correlation between LFI and milk yield during the trial was -0.04 and not significant. When comparing 6 cows with the lowest to 6 cows with the highest LFI mean milk yield was 35.5 ± 4.5 L/d and 35.2 ± 4.2 L/d. In consequence, the LFI would have not been suitable to detect subjects prone for inflammatory problems or an impairment of liver function 4 weeks p.p. Taken together, these findings emphasize the conclusion drawn from the results presented in **Paper III**, which indicate that the immune system of cows was not adequately challenged to investigate the effects of the supplementations during this trial.

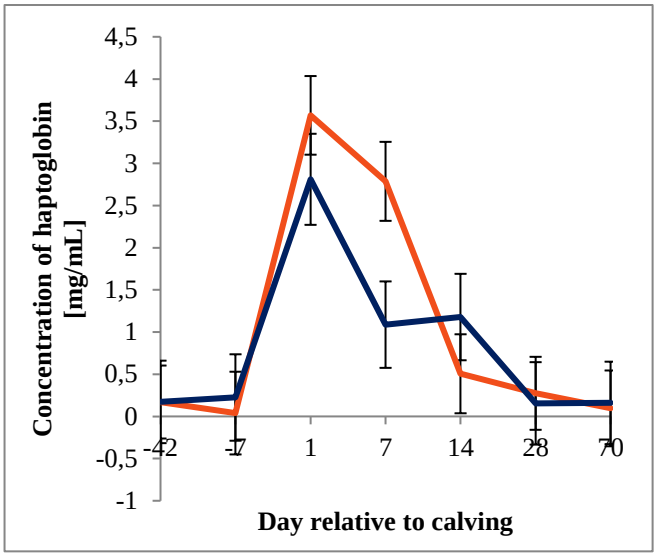

Figure 10: Concentration of haptoglobin during the trial in cows in the 1st (red line) and 4th (dark blue line) quartile of liver functionality index (LFI)(LSMeans ± SEM)

8. Conclusion

In this trial supplementation with CLA induced reduction of milk fat content, milk fat yield and milk energy concentration as expected. It must be noted, that these reductions were observed in animals supplemented with the combination of CLA + Vit. E in period 3 only and were less pronounced compared to animals supplemented with CLA. This finding might be due to counteracting effects of CLA and Vit. E on the activity of Δ9-desaturase. However, the reducing effect of CLA on milk energy output was partly compensated by a decrease of DMI and subsequently decreased net energy intake. Therefore, net energy balance was not increased. As a consequence, an effect of CLA on lipomobilisation was not observed. Neither supplements affected NEFA- and BHB-concentrations. Therefore, supplements were not suitable to reduce the incidence of subclinical ketosis in this trial.

CLA did not decrease the proportion of short-chain saturated fatty acids in milk. However, it was possible to induce an increase of the proportion of *trans*-10, *cis*-12 CLA, which might positively influence the nutritional properties of milk. Simultaneously, Vit. E might be used to protect milk fatty acids against oxidation without affecting the fatty acid pattern. Doses of at least 3,000 IU/d Vit. E should be applied to prevent the drop of serum Vit. E-concentration caused by parturition.

As expected, the event of parturition affected the immune function, which is reflected by a decreased ability of PBMC to respond to stimulation. A more pronounced disruption is observed in animals in $\geq 3^{rd}$ parity, although no effect on actual health was found. Supplementation of CLA, Vit. E or the combination thereof was not successful to prevent this decrease of immune function.

Overall, it can be stated as a conclusion that effects of the supplementations might be masked by cows not developing a type II ketosis. Further trials are necessary to clarify the mechanisms behind the interaction on milk fat and the mechanism by which supplements might influence the immune system.

9. Summary

Energy demand of the dairy cow in the transition period, which comprises late gestation and early lactation, increases due to the growth of the fetus and the onset of milk production. This increase cannot be matched by an adequate feed intake around parturition and in early lactation. Therefore, cows perceive a period of being in a negative energy balance with subsequent mobilization of adipose tissue. Pronounced lipomobilisation results in high concentrations of non-esterified fatty acids (**NEFA**) as well as betahydroxybutyrate (**BHB**) and is associated with metabolic disorders as the fatty liver syndrome and a disruption of immune function. Consequently, the highest incidence of production diseases is observed during the first weeks of lactation. High incidence of production diseases results in reduction of lactation and reproductive performance. Therefore, dairy farms might experience economic losses. As a high milk yield is preferable and breeders concentrate on further increase thereof, strategies in nutrition of dairy cows aim at reducing the negative energy balance, high lipomobilisation and associated health disorders. One approach is the reduction of energy expenditure caused by lactation. The *trans*-10, *cis*-12 isomer of conjugated linoleic acid (**CLA**) has been observed to be capable to induce a reduction of milk fat content by decreasing the de novo synthesis of fatty acids. Because fat is the major contributor to energy in milk, a substantial reduction of milk fat content might spare energy and thereby reduce the negative energy balance. This decrease of negative energy balance might be reflected in a reduction of lipomobilisation. Therefore, the flood of NEFA to the liver and resulting production of ketone bodies should be reduced. Vitamin E (**Vit. E**) is a strong antioxidant and literature indicates that supplementation of Vit. E above the recommendation might counteract the oxidative stress caused by the event of parturition and increased NEFA- as well as BHB-concentrations in serum. However, whereas CLA is suitable to reduce milk fat content, milk fat yield might not be reduced due to a redistribution of energy to an increase in milk yield. Furthermore, in literature effects of CLA on dry matter intake (**DMI**) and lipomobilisation are inconsistent and might be depending on dose and time of supplementation. In addition, CLA and Vit. E might interact with each other via influencing the activity of the stearoyl-CoA desaturase (**Δ9-desaturase**) and acting on the peroxisome proliferator activated receptor gamma. Therefore, this trial aimed to clarify the influences that single and combined supplementations of CLA and Vit. E exert on the energy metabolism, lactation performance, milk fatty acid profile and immune system of dairy cows during the transition period. For this purpose 64 pluriparous German Holstein

dairy cows were allocated in a 2x2 factorial design to 4 groups. Cows received either 8.4 g/d of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA (CLA group) or 2,327 IU Vit. E (Vit. E group) or both supplements (CLA + Vit. E group) from day 42 antepartum (**a.p.**) until day 70 postpartum (**p.p.**), which resulted in 3 intervention groups. A control group as well as the Vit. E group received a control fat supplement for caloric balance. A partly mixed ration consisting of 50% grass and 50% maize silage and differing concentrate proportions was fed ad libitum during the whole trial. The trial was divided into 3 periods: period 1 from 42 d a.p. until calving (60% concentrate), period 2 from calving until d 21 p.p. (increasing from 30% to 50% concentrate) and period 3 from d 21 until 70 p.p. (50% concentrate). Samples of feed, blood, liver and milk as well as ultrasonic measures of adipose tissue were collected during the trial to assess performance as well as biochemical and haematological attributes. Cows did not develop a type II ketosis and did not suffer from fat accumulation in the liver. Though milk fat content in cows supplemented with CLA was reduced by up to 26% and milk fat yield was reduced up to 23% resulting in a reduction of milk energy output by CLA, net energy balance was not improved. This might be attributed to a reduction of DMI of up to 11% in CLA-supplemented animals resulting in a reduction of net energy intake by 6% to 11%. However, CLA did not affect lipomobilisation and residual energy intake (**RSEI**) was numerically but not significantly lower in CLA-supplemented cows. This indicates an increase of efficiency by CLA. However, further trials are needed to verify this finding. Reduction of milk fat content and yield as well as RSEI in cows supplemented with the combination of CLA + Vit. E was less distinct, which might be attributed to counteracting effects of CLA and Vit. E on the Δ9-desaturase. Supplementation with CLA increased the proportion of the *cis*-9, *trans*-12 isomer, whereas both supplementations lead to a numerically higher proportion of the *trans*-10, *cis*-12 isomer in milk fat. Concentration of Vit. E in milk was increased by up to 53% in cows supplemented with Vit. E. Concentration of Vit.E in relation to milk fat was increased by CLA supplementation, whereas CLA did numerically reduce total excretion of Vit. E with milk. Serum status of Vit. E remained unaffected by either treatment. Biochemical and immunological variables were affected by calving, with the event of parturition affecting cows in 3rd or higher parity more than cows in 2nd parity. This is reflected by a delayed increase of rhodamine 123 positive proportion of granulocytes p.p. Results suggest that both supplementations did not improve the immune function. However, cows in this trial did not develop a type II but rather a type I ketosis, in which cows are able to utilize ketone bodies and partially compensate the reduction of energy intake. Therefore, the effects of supplementation with CLA and Vit. E observed in this trial might not be applicable to cows in the state of having a

type II ketosis. Further research is necessary to assure that the results obtained are convincing for animals with a type II ketosis as well.

10. Zusammenfassung

Während der peripartalen Phase bestehend aus später Trächtigkeit und früher Laktation steigt der Energiebedarf von Milchkühen aufgrund des Wachstums des Fötus sowie der einsetzenden Milchproduktion. Zwischen diesem Anstieg des Energiebedarfs und einer adäquaten Futteraufnahme um die Abkalbung herum und in der Frühlaktation kann eine Diskrepanz auftreten. Aufgrund dessen befinden Milchkühe sich in einer Periode der negativen Energiebilanz, welche eine Mobilisierung von Fettgewebe zur Folge hat. Ausgeprägte Lipomobilisation bewirkt einen Anstieg der Konzentration unveresterter Fettsäuren (**NEFA**) sowie β-Hydroxybutyrat (**BHB**) im Blut und ist assoziiert mit verschiedenen metabolischen Störungen wie dem Fettlebersyndrom und einer verminderten Immunfunktion. Folglich wird die höchste Inzidenz von Produktionskrankheiten in der Frühlaktation beobachtet. Da hohe Inzidenz von Produktionskrankheiten zu reduzierter Laktations- und reproduktiver Leistung führen, können Milchbauern ökonomische Verluste erleiden. Aufgrund einer erwünschten hohen Milchleistung und züchterischen Bemühungen um eine weitere Steigerung dieser zielen Strategien in der Fütterung von Milchkühen darauf ab, die negative Energiebilanz, hohe Lipomobilisation und die damit verbundenen Gesundheitsstörungen zu reduzieren. Ein Ansatz hierzu ist die Reduzierung des durch die Laktation verursachten Energieverbrauchs. Es wurde beobachtet, dass das *trans*-10, *cis*-12-Isomer von konjugierter Linolsäure (**CLA**) eine Verringerung des Milchfettgehalts durch die verringerte de-novo-Synthese von Fettsäuren induzieren kann. Fett stellt den Hauptenergiebeitrag in der Milch ist; daher könnte durch eine erhebliche Verringerung des Milchfettgehalts Energie gespart und in Folge eine negative Energiebilanz verringert werden. Diese Abnahme der negativen Energiebilanz könnte sich in einer Reduktion der Lipomobilisation widerspiegeln, was wiederum zur Reduktion des Anstromes von NEFA zur Leber und der resultierenden Produktion von Ketonkörpern führen sollte. Vitamin E (**Vit. E**) ist ein starkes Antioxidans und Literatur zeigt, dass die Ergänzung von Vit. E oberhalb der Empfehlung dem oxidativen Stress, der durch die Abkalbung verursacht wird, und erhöhten NEFA- sowie BHB-Konzentrationen im Serum entgegenwirken könnte. Obwohl CLA sich als geeignet erwies, den Milchfettgehalt zu reduzieren, könnte der Milchfettertrag aufgrund einer Umverteilung von Energie zu einer Erhöhung der Milchleistung möglicherweise nicht reduziert werden. Darüber hinaus sind die Wirkungen von CLA auf die Aufnahme von Trockenmasse (**DMI**) und Lipomobilisation in der Literatur inkonsistent und könnten von der Dosis und der Zeit der Supplementierung abhängig sein. Zusätzlich könnten CLA und Vit. E über

die Beeinflussung der Aktivität der Stearoyl-CoA-Desaturase (**Δ9-Desaturase**) miteinander interagieren und auf den Peroxisom-Proliferator-aktivierten Rezeptor Gamma einwirken. Daher sollte mit dieser Studie geklärt werden, welchen Einfluss einzelne und kombinierte Supplementierungen von CLA und Vit E auf den Energiestoffwechsel, die Laktationsleistung, das Milchfettsäureprofil und das Immunsystem von Milchkühen während der Übergangsphase ausüben. Zu diesem Zweck wurden 64 pluripare Milchkühe der Rasse Deutsche Holstein in einem 2x2 faktoriellen Design vier Gruppen zugeordnet. Kühe erhielten entweder 8,4 g/d *cis*-9, *trans*-11 und *trans*-10, *cis*-12 CLA (CLA-Gruppe) oder 2.327 IE Vit. E (Vit. E-Gruppe), beide (CLA + Vit. E-Gruppe) oder keine der Supplementierungen (Kontrollgruppe) von Tag 42 antepartum (**a.p.**) bis Tag 70 postpartum (**p.p.**). Die Kontrollgruppe sowie die Vit. E-Gruppe erhielt ein Kontrollfett zur Kalorienbilanz. Eine Teilmischration, bestehend aus 50% Gras und 50% Maissilage und unterschiedlichen Konzentratanteilen wurde während des gesamten Versuchs ad libitum gefüttert. Der Versuch war in 3 Perioden unterteilt: Periode 1 von Tag 42 a.p. bis zur Abkalbung (60% Konzentrat), Periode 2 von der Abkalbung bis Tag 21 p.p. (Anstieg von 30% auf 50% Konzentrat) und Periode 3 von Tag 21 bis Tag 70 p.p. (50% Konzentrat). Proben von Futter, Blut, Leber und Milch sowie Ultraschallmessungen des Fettgewebes wurden während der Studie genommen, um die Leistung sowie biochemische und hämatologische Variablen zu bewerten. Kühe entwickelten keine Typ-II-Ketose und litten nicht unter Fettansammlung in der Leber. Obwohl der Milchfettgehalt bei Kühen, die mit CLA supplementiert waren, um bis zu 26% reduziert wurde und der Milchfettertrag um bis zu 23% reduziert wurde, was zu einer Verringerung der Energieabgabe mit der Milch durch CLA führte, wurde die Nettoenergiebilanz nicht verbessert. Dies könnte einer Verringerung der DMI von bis zu 11% bei CLA-supplementierten Tieren zugeschrieben werden, was zu einer Verringerung der Nettoenergieaufnahme um 6% bis 11% führte. Allerdings beeinflusste CLA die Lipomobilisation nicht und die residuale Aufnahme an Nettoenergie Laktation (**RSEI**) war bei CLA-supplementierten Kühen zahlenmäßig, aber nicht signifikant niedriger. Dies zeigt eine Effizienzsteigerung durch CLA an. Es sind jedoch weitere Versuche erforderlich, um diese Feststellung zu bestätigen. Reduzierung von Milchfettgehalt und -ertrag sowie RSEI bei Kühen, die beide Supplementierungen erhielten, war weniger ausgeprägt, was auf die entgegenwirkenden Wirkungen von CLA und Vit. E auf die Δ9-Desaturase zurückzuführen sein könnte. Die Supplementierung mit CLA erhöhte den Anteil des *cis*-9, *trans*-12 Isomers, während beide Supplementierungen zu einem zahlenmäßig höheren Anteil des *trans*-10, *cis*-12 Isomers führten. Die Konzentration von Vit. E in der Milch wurde bei Kühen, die mit Vit E. supplementiert waren, um bis zu 53% erhöht. Die Konzentration von Vit. E in Bezug auf

Milchfett wurde durch CLA-Supplementierung erhöht, wohingegen CLA die Gesamtausscheidung von Vit. E mit der Milch numerisch reduzierte. Der Serumstatus von Vit. E blieb von beiden Behandlungen unberührt. Biochemische und immunologische Variablen wurden durch die Abkalbung beeinflusst, wobei die Abkalbung Kühe in der dritten oder einer höheren Parität stärker beeinflusste als Kühe in der zweiten Parität. Dies zeigte sich in einem verzögerten Anstieg des Rhodamin 123 positiven Anteils der Granulozyten p.p.. Die Ergebnisse legen nahe, dass beide Ergänzungen die Immunfunktion nicht verbesserten. Kühe in dieser Studie entwickelten jedoch keine Typ-II-, sondern eine Typ-I-Ketose, bei der Kühe Ketonkörper nutzen und die Reduzierung der Energieaufnahme somit teilweise kompensieren können. Daher sind die in dieser Studie beobachteten Wirkungen der Supplementation mit CLA und Vit. E möglicherweise nicht auf Kühe übertragbar, die eine Typ-II-Ketose haben. Weitere Untersuchungen sind notwendig, um sicherzustellen, dass die erhaltenen Ergebnisse auch für Tiere mit einer Typ-II-Ketose zutreffend sind.

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Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.
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