The effect of conjugated linoleic acid (CLA) supplements on the oxidative and antioxidative status of periparturient and lactating dairy cows

Thesis
Submitted in partial fulfilment of the requirements for the degree
- Doctor of Veterinary Medicine -
Doctor medicinae veterinariae
(Dr. med. vet.)

by
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Hannover 2014
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Date of oral examination: 11th of March 2014

This study was funded by the German Research Funding Organisation (Deutsche Forschungsgemeinschaft).
Meiner Familie
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<td>8-oxo-Gua</td>
<td>8-hydroxy-7,8-dihydroguanine</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2’-azino-di-(3-ethyl-benzthiazoline sulphonate)</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxytoluene</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CLA</td>
<td>conjugated linoleic acid</td>
</tr>
<tr>
<td>CLA 50</td>
<td>group of animals who received 50 g of a supplement containing different isomers of conjugated linoleic acids</td>
</tr>
<tr>
<td>CLA 100</td>
<td>group of animals who received 100 g of a supplement containing different isomers of conjugated linoleic acids</td>
</tr>
<tr>
<td>Control</td>
<td>group of animals who received no conjugated linoleic acids</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DEPPD</td>
<td>N,N-diethyl-para-phenylene diamine</td>
</tr>
<tr>
<td>DIM</td>
<td>days in milk</td>
</tr>
<tr>
<td>DM</td>
<td>dry matter</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMPD</td>
<td>N,N-dimethyl-para-phenylene diamine</td>
</tr>
<tr>
<td>DPPH</td>
<td>2,2-diphenyl-1-picrylhydrazyl radical</td>
</tr>
<tr>
<td>DSB</td>
<td>double strand breaks</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5’-dithiobis(2-nitrobenzoic) acid</td>
</tr>
<tr>
<td>ESR</td>
<td>electron spin resonance</td>
</tr>
<tr>
<td>FapyGua</td>
<td>2,6-diamino-4-hydroxy-5-formamidopyrimidine</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>ferrous</td>
</tr>
<tr>
<td>Fe&lt;sup&gt;3+&lt;/sup&gt;</td>
<td>ferric</td>
</tr>
<tr>
<td>FRAP</td>
<td>ferric reducing ability of plasma</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

\( g \) gravitational constant
G group
G x DIM group times days in milk interaction
G x Lact group times number of lactations interaction
GC gas chromatography
GPx glutathione peroxidase
GSH glutathione
GS-SG a dimer formed of two reduced glutathione molecules
HCl hydrochloric acid
\( \text{H}_2\text{O}_2 \) hydrogen peroxide
HPLC high pressure liquid chromatography
\( \text{H}_2\text{SO}_4 \) sulphuric acid
IU international units
kg kilogram
l litre
Lact number of lactations
LDL low-density lipoprotein
LS means least square means
MDA malondialdehyde
mg milligram
Mio million
ml millilitre
\( \mu \text{l} \) microlitre
mmol millimole
\( \mu \text{mol} \) micromole
mol mole
MnSOD manganese-superoxide dismutase
<table>
<thead>
<tr>
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<th>Definition</th>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>n</td>
<td>number of animals</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NEB</td>
<td>negative energy balance</td>
</tr>
<tr>
<td>NEFA</td>
<td>non-esterified fatty acid</td>
</tr>
<tr>
<td>nm</td>
<td>nanometre</td>
</tr>
<tr>
<td>nmol</td>
<td>nanomole</td>
</tr>
<tr>
<td>p</td>
<td>probability</td>
</tr>
<tr>
<td>P1</td>
<td>period one</td>
</tr>
<tr>
<td>P2</td>
<td>period two</td>
</tr>
<tr>
<td>P5</td>
<td>five per cent percentile</td>
</tr>
<tr>
<td>P95</td>
<td>ninety-five per cent percentile</td>
</tr>
<tr>
<td>pH</td>
<td>decimal logarithm of the reciprocal of the hydrogen ion activity</td>
</tr>
<tr>
<td>PMR</td>
<td>partial mixed ration</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RC-DEPPD</td>
<td>radical cation of N,N-diethyl-para-phenylene diamine</td>
</tr>
<tr>
<td>RCS</td>
<td>reactive chlorine species</td>
</tr>
<tr>
<td>RFM</td>
<td>retained fetal membranes</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCC</td>
<td>somatic cell count</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SH groups</td>
<td>sulfhydryl groups</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
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<td>SSB</td>
<td>single strand breaks</td>
</tr>
<tr>
<td>TAS</td>
<td>total antioxidant status</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>TBA</td>
<td>thiobarbituric acid</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>TEAC</td>
<td>trolox equivalent antioxidant capacity</td>
</tr>
<tr>
<td>Tg</td>
<td>thymine glycol</td>
</tr>
<tr>
<td>TMR</td>
<td>total mixed ration</td>
</tr>
<tr>
<td>TRAP</td>
<td>total radical-trapping antioxidant parameter</td>
</tr>
<tr>
<td>ZnCuSOD</td>
<td>zinc-copper-superoxide dismutase</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
</tbody>
</table>
1 Introduction

The transition period of dairy cows ranges from about three weeks before to ten weeks after parturition. This period is characterised by a substantial, hormone-controlled re-partitioning of nutrients, including important antioxidants (GOFF and STABEL 1990), from the uterus towards the mammary gland, to adjust from needs of foetal growth during pregnancy to needs of milk production for the new-born calf during early lactation. Due to the rapid increase in energy requirements with the onset of lactation and a lagged raise in dry matter intake, high yielding dairy cows commonly develop a negative nutritive energy balance (NEB), which is counterbalanced by a mobilisation of body energy reserves, in particular fat from adipose tissues. In case of excessive lipomobilisation, ketosis and fatty liver may develop, which are also seen as major risk factors for other production diseases, such as abomasal displacement, mastitis or metritis (DRACKLEY 1999).

Enhanced metabolism to meet energy and nutrient requirements of milk production may lead to a rise in production of reactive oxygen species (ROS). Reactive oxygen or nitrogen species (RNS) are molecules, which readily attack and extract hydrogen atoms from biological macromolecules, such as lipids and proteins, and thus cause structural changes and loss of function. Antioxidants are molecules or enzymes able to donate hydrogen atoms without negative consequences to their structure or function, such as α-tocopherol, retinol, and glutathione peroxidase or superoxide dismutase. An imbalance between ROS or RNS and antioxidants favouring the first and disfavouring the latter, is commonly referred to as oxidative stress (SIES 1993).

It is well established that dairy cows suffer from oxidative stress and increased lipid peroxidation during the periparturient period (CASTILLO et al. 2005; BIONAZ et al. 2007), accompanied by decreased plasma antioxidants (BOUWSTRA et al. 2010b) and decreased plasma antioxidative enzyme activity, as established for the superoxide dismutase (GAAL et al. 2006).

Conjugated linoleic acids (CLA) are a group of positional and geometric isomers of linoleic acid, containing a double bond. CLAs have attracted specific interest as supplements in dairy cattle nutrition, due to metabolic effects of some CLA isomers such as the
trans-10,cis-12 CLA isomer, which reduces the amount of milk fat produced (BAUMGARD et al. 2000). Decreased de novo fat synthesis in the mammary gland may improve energy balance of transition dairy cows (KRAMER et al. 2013). Additionally, antioxidative properties for CLA, fed to mice or hens, were shown (HA et al. 1990; QI et al. 2011). Antioxidants can lower oxidative stress, by re-establishing the equilibrium between oxidative and antioxidative substances, thus influencing the health of periparturient dairy cows in a positive way (MILLER and BRZEZINSKA-SLEBODZINSKA 1993; BOUWSTRA et al. 2010b). On the other hand, pro-oxidative properties of trans-10,cis-12 CLA were observed in human trials (BASU et al. 2000b; RISERUS 2002).

Thus, the objective of this study was to investigate long-term effects of supplementation of a commercial CLA product, consisting of a mixture of trans-10,cis-12 and cis-9,trans-11 CLA isomers, on oxidative and antioxidative profiles during the transition period and mid-lactation of dairy cows and heifers.
2 Literature

2.1 Oxidative stress

Reactive oxygen species is a collective term that includes both oxygen radicals and certain non-radicals, which act as oxidising agents and are easily converted into radicals (HALLIWELL 1996a). Free radicals and ROS (see Table 1) are formed under physiological and pathological conditions. Possible sources of ROS in an organism are mitochondria, where superoxide radicals can escape the respiratory chain, or the respiratory burst of immune defence, where superoxide or nitric oxide radicals are synthesised as cytotoxic weapons (BABIOR 1987). Next to free radicals and ROS, other molecules with oxidative properties are produced during metabolism, such as RNS or reactive chlorine species (RCS; Table 1) (EDER 2013). Superoxide radicals can be converted to hydrogen peroxide and are then further broken down to oxygen and water. If available, hydrogen peroxide reacts with free transition metals in the Fenton reaction, to form the highly reactive hydroxyl radical (MINOTTI and AUST 1987). Physiological functions of ROS or RNS include immune defence, in case of nitric oxide regulation of blood pressure (HALLIWELL 1996b), important co-factors for enzymes and gene regulation (HALLIWELL 1996a).

An imbalance, in favour of oxidative substances and disfavouring the antioxidative defence mechanisms of an organism, is commonly referred to as oxidative stress (SIES 1993). During oxidative stress radicals or ROS are able to damage biological macromolecules, such as lipids, proteins or DNA, possibly affecting and disrupting physiological metabolism (TREVISAN et al. 2001). Next to the damage done to lipids and other macromolecules, ROS might also alter cellular membranes or other components leading to changes in physiological pathways and maybe even causing pathology (MILLER and BRZEZINSKA-SLEBODZINSKA 1993; TOYOKUNI 1999).

Oxidative stress may be caused by external conditions, as well as by metabolism of the organism itself. A rise of ROS and antioxidative enzyme activity, caused by heat stress, was shown in grazing goats (DI TRANA et al. 2006). Similar results were obtained in a study of
heat-stressed dairy cattle (BERNABUCCI et al. 2002). Nutrition is an important influence on oxidative stress. A deficit of antioxidants (refer to 2.2) can lead to an imbalance of the delicate equilibrium and high intake of polyunsaturated fatty acids (PUFA) can cause a rise in lipid peroxidation in humans (FANG et al. 2002). According to DI TRANA et al. (2006) heat stress was the more important factor, causing moderate oxidative stress in dairy goats, rather than nutritional factors.

Exercise has been shown to cause oxidative stress and lipid peroxidation with a high turnover of vitamin E in a human trial (MASTALOUDIS et al. 2001). Since high muscular activity, especially on an irregular basis, results in oxidative stress (POWERS and JACKSON 2008; RADAK et al. 2008), it is supposable that events such as herding and changing barns associated with rearrangements in hierarchy may result in oxidative stress in dairy cattle.

**Table 1** - Overview of reactive oxygen, nitrogen and chlorine species (ROS, RNS and RCS, respectively) with broad classification of their reactivity (EVANS and HALLIWELL 2001)

<table>
<thead>
<tr>
<th>Reactive oxygen species (ROS)*</th>
<th>Non-radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radicals</td>
<td></td>
</tr>
<tr>
<td>Superoxide, $O_2^-$</td>
<td>Hydrogen peroxide, $H_2O_2$</td>
</tr>
<tr>
<td>Hydroxyl, $OH$</td>
<td>Hydrochlorous acid, HOCl</td>
</tr>
<tr>
<td>Peroxy, RO$_2^-$</td>
<td>Ozone, $O_3$</td>
</tr>
<tr>
<td>Alkoxy, RO$_3^-$</td>
<td>Singlet oxygen, $^1\Delta g$</td>
</tr>
<tr>
<td>Hydroperoxy, HO$_2$</td>
<td>Hypobromous acid, HOBr</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Reactive nitrogen species (RNS)†</th>
<th>Non-radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Radicals</td>
<td></td>
</tr>
<tr>
<td>Nitric oxide, NO</td>
<td>Nitrous acid, HNO$_2$</td>
</tr>
<tr>
<td>Nitrogen dioxide, NO$_2$</td>
<td>Nitroxy radical, NO$^-$</td>
</tr>
<tr>
<td>Nitroxy anion, NO$^-$</td>
<td>Dinitrogen tetroxide, N$_2$O$_4$</td>
</tr>
<tr>
<td>Peroxyanilide, ONCO$^-$</td>
<td>Dinitrogen trioxide, N$_2$O$_3$</td>
</tr>
<tr>
<td>Nitroxyl cation, NO$^+$</td>
<td>Peroxyanilide, ONCO$^-$</td>
</tr>
<tr>
<td>Nitryl chloride, NO$_2$Cl</td>
<td>Alkyl peroxy-nitrates, ROONO$^-$</td>
</tr>
<tr>
<td>Nitroxy anion, NO$^-$</td>
<td>Nitroxy anion, NO$^-$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reactive chlorine species (RCS)</th>
<th>Non-radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hypochlorous acid, HOCl†</td>
</tr>
<tr>
<td></td>
<td>Nitrile chloride, NO$_2$Cl</td>
</tr>
</tbody>
</table>

* ROS is a collective term which includes both oxygen radicals and certain non-radicals that are oxidizing agents and/or are easily converted into radicals.
† RNS includes both nitrogen containing radicals and non-radicals. Peroxy-nitrile can be regarded as a ROS or RNS.
‡ Some species, e.g. HOCl are both ROS and RCS. These species have widely varying reactivities: OH reacts rapidly with almost everything, whereas $H_2O_2$, $O_2$, NO only react quickly with a few molecules. RO$_2$, RO, HOCl, HOBr, NO$_2$Cl, NO$_2$, ONDO$^-$ and $O_3$ have intermediate reactivities.
2.1.1 Oxidative damage to lipids

Lipid peroxidation is a radical chain reaction, which in the majority of cases is started with a strong oxidising agent, such as the hydroxyl radical, produced either directly in cell organelles or via the Fenton reaction. Polyunsaturated fatty acids are more readily oxidised than monounsaturated or saturated fatty acids (GUTTERIDGE and HALLIWELL 1990). The remaining lipoperoxyl radical reacts with another oxygen or lipid to produce lipid hydroperoxides (TREVISAN et al. 2001), which are unstable. Due to instability, the lipid hydroperoxides produce peroxy and alkoxy radicals, which are further decomposed via iron-catalysed hydrolysis to a mixture of toxic aldehydes, hexanal, acrolein, malondialdehyde (MDA) and 4-hydroxynonenal (ARMSTRONG and BROWNE 1994; BARRERA 2012). Before hydrolysis, lipid hydroperoxides are capable of extracting a hydrogen atom of other PUFA, thus keeping up the chain reaction, and are therefore able to damage most body cells (HALLIWELL and CHIRICO 1993). The breakdown products, including MDA, are referred to as “oxidative stress second messengers”, due to their prolonged half-life and their ability to diffuse from their site of formation, compared to free radicals (BARRERA 2012). Peroxyl radicals, resulting from lipid peroxidation, can cause protein or DNA oxidation by extraction of hydrogen atoms (KRYSTON et al. 2011). Malondialdehyde itself may also react with DNA and form MDA-DNA adducts with mutagenic potential (MARNETT 1999).

2.1.2 Oxidative damage to proteins

Proteins can be damaged in multiple ways by oxygen radicals. The protein backbone is susceptible to oxidation, forming a carbon-centred radical (BERLETT and STADTMAN 1997). This radical undergoes multiple further reactions, possibly starting a chain reaction similar to lipid peroxidation or causing protein fragmentation, either by the α-amidation pathway or the diamide pathway (HUNT et al. 1988; STADTMAN and LEVINE 2003).

Next to polypeptides, amino acids themselves may be oxidised either alone or within a polypeptide, causing changes to tertiary protein structures and thus inhibiting protein function. Susceptibility of amino acids depends on their chemical properties. Aromatic residues are more likely to be oxidized than aliphatic residues (DEAN et al. 1997).

Tryptophan is readily oxidised to N'-formylkynurenine, oxindole-3-alanine and four different isomers of hydroxytryptophan, of which N'-formylkynurenine and 5-hydroxytryptophan occur...
physiologically in limited amounts (MASKOS et al. 1992). Oxidation of tyrosine results in formation of a tyrosyl radical. For this reaction strong oxidants, such as the hydroxyl radical, peroxynitrite or nitrogen dioxide are needed (GIULIVI et al. 2003). Two tyrosyl radicals may react to form an intra- or interprotein crosslink, named bityrosine, sometimes referred to as dityrosine (HEINECKE et al. 1993; HUGGINS et al. 1993).

Amino acids containing a sulfhydryl group (SH group) are very susceptible to oxidation, forming disulphide bridges or in the case of methionine a structure named methionine sulfoxide (BERLETT and STADTMAN 1997). Sulfhydryl groups are widely spread in proteins and used in antioxidative systems (refer to 2.2). Protein peroxidation by hydroperoxides, including lipid hydroperoxides, can lead to cellular dysfunction (SCHERER and DEAMER 1986; RAHMANTO et al. 2010), including disturbed function of enzymes and receptor or carrier proteins (EVANS and HALLIWELL 2001).

### 2.1.3 Oxidative damage to DNA

Reactive oxygen species may damage DNA double strands, leading to single strand breaks (SSB), double strand breaks (DSB), oxidised deoxyribose or abasic sites. A strand break starts with oxidation of deoxyribose, leaving a carbon-centred radical. In the presence of oxygen the carbon-centred radical forms a peroxyl radical, which rearranges and extracts hydrogen atoms from sugar moieties, causing a SSB (KRYSTON et al. 2011). This SSB or the DSB always cause complete loss of genetic information (DEMPLE and HARRISON 1994). Abasic sites occur naturally, due to spontaneous hydrolysis of the N-glycosidic bond, connecting a purine base to the 2’-deoxyribonucleotides, but occur much more frequently under oxidative stress (ATAMNA et al. 1999).

Another possible way of damaging DNA is oxidation of the bases. Guanine is readily oxidised at the C8 atom to form the 8-hydroxy-7,8-dihydroguanyl radical, which quickly rearranges to 8-hydroxy-7,8-dihydroguanine (8-oxo-Gua) or 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). Oxidations of thymine or cytosine at positions five or six of the ring structure can cause several base lesions, the two most abundant structures are 5,6-dihydroxy-5,6-dihydrothymine, also called thymine glycol (Tg) and 5,6-dihydroxy-5,6-dihydrocytosine. Some of these products are reliable markers of oxidative DNA damage, i.e. Tg and 8-oxo-7,8-dihydro-2’-deoxyguanosine (KRYSTON et al. 2011).
2.2 Antioxidative defence mechanisms

To limit the amount of damage done by free radicals and ROS a complex antioxidative defence system developed, to keep a steady equilibrium between oxidative and antioxidative substances. Antioxidants are molecules or enzymes capable of inhibiting the production of radicals or ROS, scavenging them from biological systems or disarming them to less reactive substances (Cadenas 1997).

The antioxidative defence system can be divided into low molecular mass antioxidants and antioxidative enzymes. Next to those, there are also repair mechanisms for DNA and proteins and substances sequestrating transition metal ions to inhibit the Fenton reaction. (Evans and Halliwell 2001). Possible ways of action against oxidative damage include decreasing local oxygen concentration, preventing initiation of peroxidation by scavenging substances capable of extracting hydrogen atoms and quenching or scavenging singlet oxygen. Binding transition metal ions, thus inhibiting the Fenton reaction or removing peroxides by turning them into unreactive, non-radical products are two further possible ways of preventing oxidative damage. Chain breaking antioxidants, such as α-tocopherol, stop oxidative damage by reacting with radicals keeping up chain reactions, such as the peroxyl radical (Halliwell and Gutteridge 1990).

2.2.1 Low molecular mass antioxidants

Vitamin E, a collective term used for tocopherol isomers including α-tocopherol, is a strong antioxidant (VAN DEN BERG et al. 1995; YU 2001), acting as a radical scavenger and thereby protecting cell membranes from the lipid peroxidation chain reaction. The α-tocopheroxyl radical formed, is sufficiently stable due to its mesomerism-stabilised form, and unable to continue the chain reaction (Burton and Traber 1990). The water-soluble ascorbic acid (vitamin C) effectively protects human plasma from lipid peroxidation by acting as a radical scavenger (Frei et al. 1988). Vitamin C has the ability, as well as ubiquinol-10 and bilirubin, to restore α-tocopherol from the α-tocopheroxyl radical, thus prolonging the lipoprotective properties of α-tocopherol (Burton and Ingold 1986). Vitamin A, a collective term for retinol, retinal and retinoic acid, and its precursor β-carotene act as antioxidants, by quenching singlet oxygen and peroxyl radicals (Palozza and Krinsky 1991). Vitamin A and β-carotene are able to enhance the antioxidative effect of α-tocopherol,
thus markedly reducing MDA formation (PALOZZA and KRINSKY 1992). Other low molecular antioxidants include ubiqinol-10 (TOMASETTI et al. 1999), protecting lipids from peroxidation (STOCKER et al. 1991), uric acid (NIETO et al. 2000), albumin, preventing copper-dependent hydroxyl radical formation (HALLIWELL 1988), and bilirubin, restoring α-tocopherol from its radical form (NEUZIL and STOCKER 1994).

Glutathione (GSH) is a very important antioxidative tripeptide of glutamic acid, cysteine and glycine, which is found in all types of cells in high concentrations. The SH groups of two cysteine residues are easily reduced under oxidative stress conditions, building a dimer of two glutathione molecules (GS-SG), thus protecting cells effectively from oxidative attack (WILD and MULCAHY 2000). Additionally, it is also an important co-factor of glutathione peroxidase (GPx) (CNUBBEN et al. 2001).

Several transition metals, such as copper, zinc and manganese and the non-metal element selenium, play an important role in the antioxidative defence system. Glutathione peroxidase exists in two isoforms, one of which is selenium-dependent (BRZEZINSKA-SLEBODZINSKA et al. 1994). Manganese, copper and zinc are needed for different types of superoxide dismutase (SOD) (EVANS and HALLIWELL 2001). Copper, incorporated into ceruloplasmin, is necessary for antioxidant function of ceruloplasmin (SPEARS and WEISS 2008). Zinc induces metallothionein synthesis, a metal binding protein, capable of scavenging hydroxide radicals (PRASAD et al. 2004).

### 2.2.2 Antioxidative enzymes

Biological systems have developed a vast number of antioxidative enzymes. Hence, in this chapter only a short insight will be given.

Superoxide dismutase in its different isoforms is the first line defence against superoxide radicals, escaping the electron-transport chain, by catalysing the reaction of superoxide to oxygen and hydrogen peroxide (FEE et al. 1975; HALLIWELL and CHIRICO 1993). The isoforms of SOD use different transition metals as cofactors, the zinc-copper-superoxide dismutase (ZnCuSOD) exists in an intracellular and an extracellular form (HALLIWELL and GUTTERIDGE 1990), whereas the manganese-superoxide dismutase (MnSOD) is found in mitochondria (HALLIWELL 1996a). Hydrogen peroxide created by SOD, or leaking from electron-transport chain, is further broken down by GPx or catalase (CAT) (LYKKESFELDT
Glutathione peroxidase uses the hydrogen atoms of two GSH molecules to form a GS-SG dimer and turn hydrogen peroxide into two molecules of water, whereas CAT turns two molecules of hydrogen peroxide into water and oxygen. Whilst some antioxidants, mostly low molecular antioxidants, are used up, enzymes can be restored after antioxidant activity. Two examples of restoring enzymes are glutathione reductase and dehydroascorbic acid reductase, recycling glutathione and vitamin C, respectively (LYKKESFELDT and SVENDSEN 2007).

Ceruloplasmin, released from the liver as an acute phase protein, is a multi-copper oxidase enzyme, and 95% of the plasma copper are bound to ceruloplasmin (GRUYS et al. 2005). It prevents contact between copper and oxygen, thus avoiding production of free radicals. On the other hand, ceruloplasmin is able to transfer copper to copper-dependent enzymes, such as SOD or cytochrome C oxidase (FLORIS et al. 2000). Next to that, ceruloplasmin has ferrooxidase activity – it oxidises Fe$^{2+}$ to Fe (III), while reducing oxygen to water, thus inhibiting the iron ion dependent lipid peroxidation (HALLIWELL and GUTTERIDGE 1990).

2.3 Oxidative stress and antioxidants in dairy cattle

The periparturient period is a very critical time for the dairy cow, since most metabolic diseases and even infectious diseases occur within a few weeks around calving (GOFF and HORST 1997). Dairy cows experience a drastic change in metabolism around parturition. Daily dry matter (DM) intake decreases up to 30% (GOFF and HORST 1997; GRUMMER et al. 2004) and at the same time energy demand rises due to the onset of lactation, leading to NEB. This enhances metabolism severely, resulting in a raised production of ROS and RNS (BIONAZ et al. 2007). It is well established that dairy cows suffer from increased oxidative stress in the periparturient period, measured as a periparturient rise in thiobarbituric acid reactive substances (TBARS) including MDA (BERNABUCCI et al. 2005; CASTILLO et al. 2005).

Neutrophil function is impaired during the periparturient period (KEHRLI JR et al. 1989) more drastically in cows with low anteprtum feed intake and cows with high non-esterified fatty acid (NEFA) concentrations (HAMMON et al. 2006). The periparturient cow faces
immunosuppression and a correlation with oxidative stress is widely discussed (LEBLANC 2008; SPEARS and WEISS 2008).

These are multiple factors, making high yielding dairy cows most susceptible to diseases such as mastitis or retained foetal membranes (RFM), as well as other typical periparturient diseases (SORDILLO and AITKEN 2009; CHAPINAL et al. 2012). Twenty-five per cent of mastitis occurs within four days after parturition (RAJALA-SCHULTZ et al. 1999). Cows with clinical mastitis show lower activity of antioxidative enzymes and higher blood MDA concentrations than healthy controls (JHAMBH et al. 2013). Due to mastitis the total herd milk production may be reduced by up to 5%, determined by somatic cell count (SCC) amongst other parameters (BARTLETT et al. 1990). The SCC is directly related to milk MDA concentrations (SURIYASATHAPORN et al. 2009). Cows with RFM have lower total antioxidant status (TAS) concentrations and higher blood MDA concentrations. (HEIDARPOUR et al. 2012). KANKOFER et al. (2010) found a different time pattern for ferric reducing ability of plasma (FRAP) concentrations between cows with RFM and healthy control animals, which numerically lower FRAP concentrations in diseased animals. In a previous study KANKOFER et al. (2005) found higher FRAP concentrations in placenta tissue samples of RFM compared to healthy control animals, suggesting a relocation of FRAP into the placental tissues of cows with RFM. The author suggested further that cows with RFM had a higher demand of antioxidants, to cope with oxidative stress, occurring locally in the placenta, probably having an influence on the proper release of the placental membranes.

In the study of AL-QUDAH (2009) correlations between pneumonia and increased oxidative stress markers, MDA and lipid hydroperoxides, were observed. In the acute stage of pneumonia, a rise of SOD and CAT activity was experienced, whereas in the chronic stage SOD, CAT, GPx activity and GSH concentrations decreased, suggesting depletion.

Antioxidant substances in the blood of periparturient dairy cows are lower compared to pregnant or lactating cows. SHARMA et al. (2011) showed that GSH was significantly lower in early lactating cows than in pregnant animals. KANKOFER et al. (2010) measured the lowest blood vitamin C concentrations at the day of parturition. Significantly lower α-tocopherol, retinol and zinc concentrations at one day postpartum were measured by GOFF and STABEL (1990), which may impair neutrophil function (HOGAN et al. 1992) and increases the risk for mastitis (LEBLANC et al. 2004). Some plasma antioxidant enzymes,
such as the calcium-dependent paraoxonase, work less efficiently in the periparturient period, due to nutrient shortages (TURK et al. 2004). Overall the antioxidative capacity is reduced in the periparturient period (TURK et al. 2013).

2.4 Measuring oxidative stress

There are several approaches to the measurement of oxidative stress (Figure 1). The first approach is to measure the oxidative or reductive potency of a sample, mostly serum or plasma. Other possibilities include the measurement of the susceptibility of the sample to oxidation by determining reactions rates or lag phases. The third approach is the determination of the composition of the sample, including end products of oxidation, such as MDA, Tg, N\textsuperscript{\textdegree}-formylkynurenine or bitirosine; antioxidants, antioxidative enzymes and their activity or the amount of ROS (DOTAN et al. 2004).

Figure 1 – an overview of different methods to measure of oxidative stress in biological fluids (DOTAN et al. 2004)

MDA = malondialdehyde, F2-Isop = F2-isoprostanes, CD = conjugated dienes, LOOH = lipid hydroperoxides, GSH = glutathione, DNA = deoxyribonucleic acid, SCSA = sperm chromatin structure assay, Comet = single cell microgel/gel electrophoresis, Tunnel = terminal uridine nick end-labelling assay, 8-OH-dG = 8-hydroxydeoxyguanosine, 8-oxo-dG = 7-hydroxy-8-oxo-20-deoxymethyl-20-deoxyuridine, ROS = reactive oxygen species, ESR = electron spin resonance, TRAP = total radical trapping parameter, FRAP = ferric reducing ability of plasma, ORAC = oxygen radical absorbance capacity, TEAC = Trolox equivalent antioxidant capacity, Max CD = maximum conjugated dienes, Vit C = vitamin C, Vit E = vitamin E, SOD = superoxide dismutase, GPX = glutathione peroxidase
2.4.1 Reductive potency
Potency tests, as described by DOTAN et al. (2004), assess the reductive capacity of body fluids, as well as the capacity of these fluids to inhibit oxidation by measuring the reduction potential. One way of measuring the potency is to determine the FRAP or TAS. The FRAP assay (BENZIE and STRAIN 1996) as used by KANKOFER et al. (2010), describes the ability of plasma to reduce a Fe$^{3+}$ complex to a bright blue coloured Fe$^{2+}$ complex. The FRAP is influenced by plasma concentrations of vitamin C, uric acid, bilirubin, total protein and albumin, as important co-antioxidants (BOUWSTRA et al. 2010a). A method to measure TAS was first described by MILLER et al. (1993) and subsequently used in multiple studies (CASTILLO et al. 2006; GAAL et al. 2006). The TAS is based on the incubation of 2,2´-azino-di-(3-ethyl-benzthiazoline sulphonate) (ABTS), peroxidase and hydrogen peroxide together with a plasma sample. The antioxidants in the plasma inhibit the conversion of ABTS into its radical form, which has a strong blue-green colour. Other methods as the TAS or FRAP are the trolox equivalent antioxidant capacity (TEAC) or the total radical-trapping antioxidant parameter (TRAP) (LYKKESFELDT and SVENDSEN 2007).

2.4.2 Oxidisability
Measurement of the susceptibility of substrates to oxidation, referred to as the oxidisability, is a method mainly used in biochemical investigations. These methods determine the lag phase until oxidation is traceable and the propagation or the maximal rate of oxidation (FREI et al. 1988; VAN DEN BERG et al. 1995).

2.4.3 Composition of the sample
2.4.3.1 Peroxidation end products
Measuring the concentration of peroxidation end products is a widely used method for the assessment of oxidative stress. It has to be distinguished between peroxidation products of lipid peroxidation (MDA, isoprostanes, lipid hydroperoxides) and others, such as protein or DNA peroxidation. The most common way to determine lipid peroxidation is to measure the amount of TBARS, one of which is MDA. During the cheap and simple determination of TBARS, the sample is heated with thiobarbituric acid (TBA). Malondialdehyde forms a pink chromogen with TBA, which can be detected photometrically (HALLIWELL and CHIRICO 1993). However, during the heating process more MDA is formed and other aldehydes
present in the sample, can react with TBA to produce chromogens (LYKKESFELDT and SVENDSEN 2007).

It has been suggested that determination of isoprostanes is a more reliable marker for lipid peroxidation and should be favoured over the TBA test. Commercial kits are available for the isoprostane assay (MARNETT 1999; LYKKESFELDT and SVENDSEN 2007). Other measurable lipid peroxidation products include conjugated dienes or lipid hydroperoxides.

Protein peroxidation can be measured by assaying concentrations of $N'$-formylkynurenine, bityrosine or of SH groups (BERLETT and STADTMAN 1997) in plasma samples. Multiple markers and assays exist for DNA-peroxidation, such as Tg or 8-oxo-7,8-dihydro-2' -deoxyguanosine (DOTAN et al. 2004).

### 2.4.3.2 Antioxidants

The low molecular mass antioxidants, described in 2.2.1, are commonly measured with high pressure liquid chromatography (HPLC) or gas chromatography (GC) combined with mass spectrometry (MS) (DOTAN et al. 2004). Some of the low molecular mass antioxidants are measured in combination with their redox partner, such as GSH and GS-SG or ascorbic acid and dehydroascorbic acid, and interpreted as ratios. Decreased ratios stand for decreased antioxidative status (LYKKESFELDT and SVENDSEN 2007). Concentrations of $\alpha$-tocopherol are sometimes calculated as the $\alpha$-tocopherol:cholesterol mass ratio, since tocopherols are lipid soluble and carried by lipoproteins in the blood (HERDT and SMITH 1996).

### 2.4.3.3 Antioxidant enzyme activity

Activity of enzymes, commonly used as indicators of oxidative stress, such as GPx and SOD activity can be assayed in commercial kits (BERNABUCCI et al. 2002). Ceruloplasmin was measured by KANKOFER et al. (2010) with a photometric detection after adding $p$-phenylenediamine to the sample.

### 2.4.3.4 Production of reactive oxygen species

The production of ROS in the sample can be measured by luminescence techniques or electron spin resonance (ESR). Hydroperoxides are measured indirectly by the conversion of $N,N,diethyl-para$-phenylene diamine (DEPPD) into the radical cation form (RC-DEPPD),
which was first described by ALBERTI et al. (2000). Alkoxy and peroxyl radicals originating from the iron catalysed decomposition of hydroperoxides can, next to other oxidising agents in the sample, convert DEPPD into RC-DEPPD. The RC-DEPPD has a strong pink colour and can be detected photometrically.

2.4.4 Methods chosen for this study

For a better understanding of the antioxidative status of animals at different time points, \( \alpha \)-tocopherol and retinol were determined from serum samples via HPLC and the FRAP was analysed in plasma samples. Concerning the oxidative status, five different parameters were determined in the present study. The serum content of hydroperoxides, by conversion of DEPPD, was measured to give an overview of oxidative stress. As an indicator of lipid peroxidation, the concentration of TBARS was analysed. Indicators of protein peroxidation were SH groups, \( N' \)-formylkynurenine and bityrosine.

2.5 Conjugated linoleic acids

Conjugated linoleic acid (CLA) is the collective term for geometrical and positional isomers of linoleic acid, which have been discussed as a group of fatty acids with different metabolic effects and antioxidative properties (IP et al. 1991). One metabolic effect is that the trans-10,cis-12 isomer of CLA effectively decreases fat percentage in the milk (BAUMGARD et al. 2000) by inhibiting milk synthesis in the mammary gland. PERFIELD II et al. (2002) suggested that during situations where animals cannot consume sufficient energy to meet requirements, the decrease in milk fat percentage could be advantageous for the metabolism of the periparturient cow. It could be assumed that this protective effect on metabolism would result in decreased ROS production.

Effects of CLA on milk fat content and metabolism of dairy cows have been thoroughly investigated and discussed. Most trials were short-term, lasting no more than five days and were held with either lactating cows (BAUMGARD et al. 2000; BAUMGARD et al. 2001; GIESY et al. 2002; VISWANADHA et al. 2003) or during the transition period (MOORE et al. 2004; SELBERG et al. 2004). Long-term trials, lasting up to twenty weeks, were held by BERNAL-SANTOS et al. (2003) and PERFIELD II et al. (2002) over the transition period
until mid-lactation. In both, long-term and short-term trials, a milk fat depression caused by trans-10, cis-12 CLA (BAUMGARD et al. 2000) was observed.

Biochemical experiments have shown that in vitro CLA had antioxidative properties and was an effective radical scavenger (YU 2001). Research on the differences between the two isomers cis-9,trans-11 CLA and trans-10,cis-12 CLA delivered contrasting results. LEUNG and LIU (2000) stated that trans-10,cis-12 CLA had stronger radical scavenging properties, whereas a follow-up study of YU et al. (2002) showed that a mixture of both was most effective. Studies in rats, mice and hens also showed antioxidative effects (HA et al. 1990; IP et al. 1991; QI et al. 2011). Further in vitro studies followed, delivering contradicting results, they disproved any antioxidative properties and showed a possible pro-oxidative effect of CLA (VAN DEN BERG et al. 1995; CHEN et al. 1997). Pro-oxidative properties of CLA have been described in human trials (BASU et al. 2000b; RISERUS 2002).

FLINTOFF-DYE and OMAYE (2005) suggested that antioxidative or pro-oxidative properties of CLA were dependent on micro-environmental factors, similar properties were shown for β-carotene, which worked better as an antioxidant at low oxygen-tension (PALOZZA and KRINSKY 1991). VAN DEN BERG et al. (1995) tried to establish conditions close to biological systems, using a model system with phospholipid membranes, and found no antioxidative or pro-oxidative properties of their mixture of CLA isomers.

Since the supplementation with commercial CLA mixtures is a commonly used technique to achieve milk fat depression in order to improve NEB during the periparturient period of dairy cows, with no knowledge on possible effects on antioxidative or oxidative status, the aim of this study was to investigate effects of long-term supplementation of a commercial CLA isomer mixture, containing trans-10,cis-12 CLA and cis-9,trans-11 CLA in equal amounts, on the antioxidative and oxidative profiles of peripartal and lactating dairy cows.
3 Materials and methods

3.1 Animals and feeding

The trial was conducted in a dairy herd at the Institute for Animal Nutrition of the Friedrich-Loeffler-Institute in Braunschweig, Germany. Details on housing, feeding and production parameters of studied cows in this trial were already presented by PAPPRITZ et al. (2011b).

Briefly, animals were housed in a free stall with a slatted floor and cubicles bedded with a sawdust-covered rubber mattress. Forty-five German Holstein cows, pluriparous and primiparous, were separated in three randomised groups, taking into consideration the body weight and for pluriparous animals also the number of lactations and the milk yield during the last lactation. The control group included ten cows and four heifers, the CLA 50 group eleven cows and four heifers, whereas the CLA 100 group included eleven cows and five heifers.

Before parturition all animals were pasture fed, heifers for three months prior to calving, cows only up to five weeks between dry off and study start. Three weeks antepartum all animals received the same total mixed ration (TMR) diet, with 37 % “dry cow concentrate” and 63 % silage (60 % maize and 40 % grass silage) until parturition. Offered amounts were calculated to meet a body condition score (WILDMAN et al. 1982) of 3.5 at the estimated calving date. After parturition a partial mixed ration (PMR) was fed ad libitum (Table 2) and provided in self-feeding stations (TYPE RIC, Insentec, B.V., Marknesse, Netherlands). The PMR was made up of 63 % silage (see above) and 37 % “lactation concentrate”. Additionally, lactating animals received 4 kg of concentrate, which included commercial fat supplements, from transponder feeding stations (TYPE RIC, Insentec, B.V., Marknesse, Netherlands).

The CLA fat supplement (Lutrell® pure, BASF SE, Ludwigshafen, Germany; Table 3) contained 10.42 % of trans-10,cis-12 CLA and 10.54 % cis-9,trans-11 CLA, while the control supplement (Silafat®, BASF SE, Ludwigshafen, Germany) contained only stearic acid (C_{18:0}).

Animals assorted to the control group received four kilograms of the concentrate containing the control supplement, whereas animals in the CLA 50 group were fed two kilograms each of the concentrates containing either supplement, thus receiving 50 g of CLA supplement and
five grams of each of the isomers mentioned above. The CLA 100 group received four kilograms of concentrate containing the CLA supplement, thus 100 g of CLA supplement and ten grams of either isomer. Supplementation started at calving date and was stopped at d 182 after parturition. To protect CLA supplements from rumen biohydrogenation, they were coated in hydrogenated vegetable fats, consisting of palmitic acid and stearic acid, both linked to glycerine (PAPPRITZ et al. 2011a).

Water was available ad libitum and water consumption was automatically recorded. (Insentec B.V., Marknesse, Netherlands).

Table 2 – Components and chemical composition of concentrates and partial mixed ration (PAPPRITZ et al. 2011b);

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentrate</th>
<th>PMR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>38.50</td>
<td>38.50</td>
</tr>
<tr>
<td>Dried sugar beet pulp</td>
<td>29.00</td>
<td>29.00</td>
</tr>
<tr>
<td>Rapeseed meal</td>
<td>20.00</td>
<td>20.00</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>6.50</td>
<td>6.50</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Mineral feed*</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>CLA supplement</td>
<td>–</td>
<td>2.50</td>
</tr>
<tr>
<td>Control fatty acid supplement</td>
<td>2.50</td>
<td>–</td>
</tr>
<tr>
<td>Dry matter (g/kg)</td>
<td>889 ± 10</td>
<td>887 ± 11</td>
</tr>
<tr>
<td>Nutrients (g/kg DM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total ash</td>
<td>71 ± 5</td>
<td>74 ± 3</td>
</tr>
<tr>
<td>Crude protein</td>
<td>187 ± 1</td>
<td>187 ± 5</td>
</tr>
<tr>
<td>Ether extract</td>
<td>59 ± 3</td>
<td>53 ± 4</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>88 ± 6</td>
<td>89 ± 4</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>123 ± 12</td>
<td>124 ± 10</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>258 ± 10</td>
<td>256 ± 5</td>
</tr>
<tr>
<td>Energy ** (MJ NEL/kg DM)</td>
<td>8.8</td>
<td>8.8</td>
</tr>
<tr>
<td>Trans-10,cis-12 CLA*** (g/kg DM)</td>
<td>0.02</td>
<td>2.25</td>
</tr>
</tbody>
</table>

Notes: *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, 5 mg Co, 1,000,000 IU vitamin A, 100,000 IU vitamin D3, 1500 mg vitamin E; **Calculation based on nutrient digestibilities measured with wethers (GfE 1991); ***Calculation based on analysed concentrations in concentrates and silage; Means ± SD.
Table 3 – fatty acid profile of fat supplements (PAPPRITZ et al. 2011b);

FA = fatty acids, FAME = fatty acid methyl esters, other CLA = conjugated linoleic acid isomers, CON = control concentrate without conjugated linoleic acids, CLA = fat supplement containing conjugated linoleic acids

<table>
<thead>
<tr>
<th>Fatty acid [% of total FAME]</th>
<th>CON</th>
<th>CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁₆:0</td>
<td>10.89</td>
<td>10.89</td>
</tr>
<tr>
<td>C₁₈:₀</td>
<td>87.30</td>
<td>50.31</td>
</tr>
<tr>
<td>C₁₈:₁ cis-9</td>
<td>&lt; 0.01</td>
<td>10.66</td>
</tr>
</tbody>
</table>

Conjugated linoleic acid

| C₁₈:₂ cis-9,trans-11        | 0.06  | 11.99 |
| C₁₈:₂ trans-10,cis-12       | 0.02  | 11.88 |
| Other CLA                   | 0.15  | 0.95  |
| Other FA                    | 1.58  | 3.32  |

According to GFE (2001) suggestions, which were used for calculation of the daily ration during this trial, dry and lactating dairy cows should receive 500 mg vitamin E per day (≈ 725 IU/day).

Diets should contain at least 0.2 mg selenium per kilogram dry matter of feed, to meet requirements of two milligram selenium per day in the antepartum period and up to five milligram per day during lactation, depending on milk yield (KAMPHUES et al. 2004). Dietary vitamin E and selenium contents are listed in Table 4.

Table 4 – Vitamin E and selenium content of different feeds (DM = dry matter, dry TMR = total mixed ration animals received during dry period, lactation PMR = partial mixed ration animals received during lactation)

<table>
<thead>
<tr>
<th></th>
<th>Daily amount (kg)</th>
<th>Vitamin E (mg/kg DM)</th>
<th>Selenium (mg/kg DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry TMR</td>
<td>restricted</td>
<td>44.8 (≈ 66.9)</td>
<td>1.488</td>
</tr>
<tr>
<td>Lactation PMR</td>
<td>ad libitum</td>
<td>36.7 (≈ 54.7)</td>
<td>0.398</td>
</tr>
</tbody>
</table>

Concentrate

<table>
<thead>
<tr>
<th></th>
<th>Daily amount (kg)</th>
<th>Vitamin E (mg/kg DM)</th>
<th>Selenium (mg/kg DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>14.6 (≈ 21.8)</td>
<td>0.947</td>
</tr>
<tr>
<td>CLA 50</td>
<td>4</td>
<td>15.8 (≈ 23.5)</td>
<td>0.986</td>
</tr>
<tr>
<td>CLA 100</td>
<td>4</td>
<td>16.9 (≈ 25.2)</td>
<td>1.024</td>
</tr>
</tbody>
</table>
3.3 Sampling

Cows were milked twice daily, milk yield was recorded at each milking time and milk constituents were analysed twice weekly. After milking, cows were weighed automatically. Blood samples were taken at day (d) -21 before calving and on d 1, 21, 70, 105, 140, 182, 224, and 252 after parturition from the jugular vein into a 10 ml serum tube and a 4 ml heparin-coated tube. Blood was centrifuged immediately and aliquots of serum or plasma were stored at -80° C.

3.4 Biochemical methods

3.4.1 Blood metabolites

3.4.1.1 Protein content

The Protein content was measured in both serum and plasma samples by the biuret method (GORNALL et al. 1949) using commercial assay kits (Liquick Cor – Total Protein, Ref 2 - 237, PZ Cormay S.A., Lublin, Poland).

3.4.1.2 Serum cholesterol

The serum cholesterol concentrations were analysed in an automated procedure, using a Cobas Mira Plus System (Roche Diagnostic, Mannheim, Germany), based on a spectrophotometric assay. The system was calibrated with „Multikalibrator” (LT-Sys, Berlin, Ref Nr: LT-Cal 052) and the control was Seronorm™ Human (Sero AS, Norway; bought at invicon, Munich, Ref Nr 110001). The assay was run with an inter-assay coefficient of variation of 1.6 % at the laboratory of the clinic for cattle in Hanover.

Further metabolic blood parameters (concentrations of glucose, NEFA, and β-hydroxybutyrate), as well as production parameters, were assessed and already presented by PAPPRITZ et al. (2011b).
3.5 Antioxidative status

3.5.1 Alpha-tocopherol and retinol
Alpha-tocopherol and retinol were analysed using a HPLC system (LC10-AT and RF-551, Shimadzu, Japan). Serum was diluted with bi-distilled water and vitamins were stabilised using ascorbic acid (Kat Nr 10168, Grüssing GmbH, Filsum, Germany). Alpha-tocopherol and retinol were esterified and extracted using potassium hydroxide (CAS Nr 1310-58-3, Grüssing GmbH, Filsum, Germany) and a mixture of n-hexane, methanol and ethanol (all Carlo Erba Reagents, France). The n-hexane mixture was evaporated and the dried α-tocopherol and retinol were dissolved in pure methanol for HPLC. Two standards were used, Level I and Level II (Chromosystems Instruments & Chemicals GmbH, Germany) and the inter-assay coefficient of variation was 7.2 % (n = 15) and 9.8 % (n = 14) for retinol and α-tocopherol, respectively. Both parameters were expressed as mg/l.

Tocopherol isomers, including α-tocopherol, are carried in the blood stream bound to lipoproteins, containing cholesterol. Low cholesterol concentrations in the blood stream, due to effects of lactation stage and low dietary fat, may result in a shortage of carrier molecules for α-tocopherol, dissembling low α-tocopherol concentrations in the blood stream. To avoid this dissemblance, a mass ratio was calculated for α-tocopherol and cholesterol, according to HERDT and SMITH (1996).

3.5.2 Ferric reducing ability of plasma (FRAP)
The total antioxidative capacity was determined based on the FRAP, slightly modified from the method described by BENZIE and STRAIN (1996). The reducing ability of plasma is influenced to different degree by plasma content of ascorbic acid, α-tocopherol, uric acid (60 % contribution to total FRAP), bilirubin, protein and other co-antioxidants. Undiluted plasma (25 µl) was added to 2.25 ml “working agent”, made up of 300 mmol/l acetate buffer (pH 3.6; Cat.No 577970115, POCh S.A., Poland), 10 mmol/l 2,4,6-tri-pyridyl-s-triazine (TPTZ, Sigma, Germany) mixed in 40 mmol/l HCl (Cat.No 575313163, POCh S.A., Poland), and 20 mmol/l FeCl₃ x 6H₂O (Cat.No 904180113, POCh S.A., Poland) dissolved in distilled water – mixed at a ratio of 10:1:1. Absorbance was measured at 593 nm, the change in absorbance over ten minutes was directly related to the total reducing capacity of antioxidants in the plasma sample, the working agent alone served as control. A standard curve was
Materials and Methods

Prepared with ten different dilutions of Fe (II) between 0 and 1000 µmol/l and the intra- and inter-assay coefficients of variation were 8.8 % and 8.5 % (n = 10), respectively. Results were expressed as µmol/l.

3.6 Oxidative status

3.6.1 Amount of hydroperoxides in the sample

Oxygen-centred radicals abstract hydrogen atoms from neighbouring molecules, leading to formation of alcohols and hydroperoxides (ALBERTI et al. 2000). Hydroperoxides decompose to form alkoxy and peroxyl radicals (HIATT and IRWIN 1968). For this indirect measurement of hydroperoxides in the sample, described by ALBERTI et al. (2000) the serum was diluted five times and 20 µl of the dilution were added to one millilitre of acetate buffer (pH 4.8) and 10 µl of a 0.37 mol/l solution of DEPPD (Cat 16.834-3, Sigma-Aldrich, Steinheim, Germany). The solution was vortexed and incubated at 37°C for 1.5 hours. The alkoxy and peroxyl radicals present in the sample turn the DEPPD into RC-DEPPD, which has a strong pink colour. Absorbance was read at 505 nm against a control sample, in which distilled water replaced the serum. A standard curve was prepared for calculations with six different dilutions of H₂O₂ and results were expressed as µmol/l. The intra- and inter-assay coefficients of variation were 9.1 % and 8.9 % (n = 10), respectively.

3.6.2 Lipid peroxidation

3.6.2.1 Thiobarbituric acid reactive substances

For the determination of TBARS the method described by LEDWOZYW et al. (1986) was used. Five hundred microlitres of serum sample was added to 2.5 ml of trichloroacetic acid (Cat.No 577970115, POCh S.A., Poland) dissolved in 0.6 mmol/l HCl (Cat.No 575313163, POCh S.A., Poland) and vortexed immediately. After ten minutes incubation at room temperature, 1.5 ml of TBA (0.67 % in 1 mol/l NaOH; Cat.No 576500116, POCh S.A., Poland) were added, the solution was vortexed again and incubated in a boiling water bath for twenty minutes. During the heating process a pink chromogen is formed between TBA and MDA, the [TBA]₂-MDA adduct, and possibly between TBA and other aldehydes. The test tubes were cooled, 4 ml of butanol-2 (CAS 78-92-2, Chempur®, Poland) were added, and the solution was shaken vigorously for three minutes and centrifuged at 1500 x g for ten minutes.
The upper layer was refilled into cuvettes and absorbance was measured immediately at 532 nm. Malondialdehyde served as standard and results were expressed as nmol/l. The intra- and inter-assay coefficients of variation were 9.4 % and 9.1 %, respectively.

3.6.3 Protein peroxidation

3.6.3.1 Sulfhydryl groups

The level of SH groups was measured according to the method described by RICE-EVANS et al. (1991). Plasma was diluted five times. For this determination 300 µl of diluted plasma were added to 300 µl of 10 % sodium dodecyl sulphate (SDS; L-5750, Sigma-Aldrich, Steinheim, Germany) and thoroughly vortexed to unfold protein chains. After mixing, 2.4 ml of 10 mmol/l sodium phosphate buffer (pH 8.0; POCh S.A., Poland) were added. Three hundred microlitres of 5,5'-dithiobis(2-nitrobenzoic) acid solution (DTNB; D8130-1G, Sigma-Aldrich, Steinheim, Germany), 20 mg dissolved in 50 ml buffer, were added to the samples. No DTNB was added to control tubes, which contained plasma and SDS in same amounts as sample tubes and 2.7 ml of buffer to reach an equal volume in all tubes.

Absorbance was measured immediately at 412 nm (Ultrospec 2000, Pharmacia, Sweden). All samples were incubated for one hour at 37° C, after incubation absorbance was read again. The difference between absorbance (after subtracting absorbance of control) was used to calculate the content of SH groups, with a standard curve, prepared with different solutions of GSH (Sigma, Poland) and the intra- and inter-assay coefficients of variation were 6.9 % and 7.1 % (n = 10), respectively. Results were expressed as mmol/g protein.

3.6.3.2 N'-Formylkynurenine and bityroline

The two indicators of protein peroxidation used in this study, bityroline and N'-formylkynurenine, were both determined by spectrofluorometry, as described by RICE-EVANS et al. (1991). Plasma samples were diluted 50 times and, in case of bityroline, excited by light at 325 nm and emission was read at 420 nm. For measurement of N'-formylkynurenine excitation wavelength was 360 nm and emission was read at 454 nm. The spectrofluorometer (Jasco, Tokyo, Japan) was standardised to 100 deflections with 0.1 µg/ml chinine sulphate dissolved in 0.1 mol H₂SO₄. Results were expressed as mg/g protein. The intra- and inter-assay coefficients of variation for bityroline were 5.9 %
and 6.0 % (n = 10), respectively and for N'-formylkynurenine 6.3 % and 6.5 % (n = 10), respectively.

3.7 Statistical analysis

Statistics were calculated with SAS version 9.3 TS for Windows (SAS Institute, Inc., Cary, NC). Results were tested for normal distribution using the PROC UNIVARIATE procedure. If data deviated significantly from normal distribution the logarithm to the base 10 was used for further statistical evaluation, as was the case for FRAP, α-tocopherol, TBARS, SH groups and the α-tocopherol:cholesterol mass ratio. The transition period (d -21 until d 105), referred to as period one, and the mid-lactation period (d 105 until d 252), referred to as period two, were analysed separately.

Data was evaluated by analysis of variance for repeated measurements (days in milk, DIM; PROC MIXED), with treatment group (G; control, CLA 50, CLA 100) and lactation number (pluriparous, primiparous) as fixed effects and the individual animal as random effect. Interactions of effects (G x DIM, G x Lact) were also considered in the model. Multiple comparisons of means were performed using the pdiff-option.

Results are presented as least square means (LS means) with standard error (SE) of means, or in the case of logarithmic data as medians with P 5 and P 95 percentiles. A p-value < 0.05 was considered significant; p-values between 0.05 and 1 were referred to as a trend.
4 Results

4.1 Antioxidative status

4.1.1 Ferric reducing ability

Days in milk (DIM) had a significant influence on FRAP in both periods, one and two (P 1 and P 2: p < 0.001), with significantly lower concentrations at d 1 compared to antepartum concentrations at d -21 (p = 0.007), recovering at d 70 (p < 0.001, compared to d 1 and d 21). Concentrations reached a peak at d 140 and decreased significantly towards d 224 (p < 0.001).

In period one, groups (G) tended to have different FRAP concentrations (p = 0.068; Table 5). There were no significant differences between groups in period two (p = 0.17). In both periods one and two, no significant influence was observed for the factor of number of lactations (Lact) and no significant G x DIM and G x Lact interactions could be determined (Table 6).

Table 5 – Medians of plasma FRAP concentrations in μmol/l (with five per cent and ninety-five per cent percentiles) for dairy cows in different stages of lactation, receiving a control fat supplement (n = 14), 50 g CLA (CLA 50; n = 15) or 100 g CLA supplement (CLA 100; n = 16) between d 1 and d 182, depletion period was between d 182 and d 252. For results of statistical evaluation refer to Table 6. DIM = days in milk

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</table>
RESULTS

Table 6 – p-values of fixed effects and their interactions of plasma FRAP concentrations during periods one (d -21 – d 105) and two (d 105 – d 252) of lactation, of dairy cows and heifers separated in three groups, receiving a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

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</tr>
<tr>
<td>G x Lact</td>
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<td>0.56</td>
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4.1.2 Alpha-tocopherol

Serum α-tocopherol varied substantially with DIM in periods one and two (P 1 and P 2: p < 0.001) with a steep decrease between d -21 and d 1, towards parturition (p < 0.001). After parturition serum concentrations increased and recovered to antepartum values at d 70 (Table 7). Alpha-tocopherol concentrations decreased significantly in the depletion period, between d 182 and d 252 (p = 0.025).

No significant differences were observed in periods one and two between groups, G x DIM interaction and G x Lact interaction (Table 9).

Heifers showed significantly higher serum α-tocopherol concentrations than pluriparous cows in period one (p = 0.012) and in period two (p < 0.001; Table 8; Table 9).
RESULTS

Table 7 – Median serum α-tocopherol concentrations in mg/l (five per cent and ninety-five per cent percentiles) for dairy cows in different stages of lactation. Animals were separated in three groups, receiving a control fat supplement (Control; n = 14), 50 g CLA (CLA 50; n = 15) or 100 g CLA supplement (CLA 100; n = 16) between d 1 and d 182, depletion period was between d 182 and d 252. Results of statistical evaluation refer to Table 9. DIM = days in milk

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<th>70</th>
<th>105</th>
<th>140</th>
<th>182</th>
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<td>(P95)</td>
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Table 8 – Median serum α-tocopherol concentrations in mg/l (five per cent and ninety-five per cent percentiles) for dairy cows (n = 32) and heifers (n = 13) in different stages of lactation. Results of statistical evaluation refer to Table 9. DIM = days in milk

<table>
<thead>
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<th>Groups</th>
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</table>
RESULTS

Table 9 – p-values of fixed effects and their interactions for serum α-tocopherol concentrations during periods one (d -21 - d 105) and two (d 105 – d 252) of lactation, of dairy cows (n = 32) and heifers (n = 13) in three groups, fed a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

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4.1.3 Cholesterol

Days in milk had a significant influence on serum cholesterol concentrations in both periods one and two (P 1 and P 2: p < 0.001; Figure 2), with a significant decrease towards parturition (p < 0.001), followed by a subsequent increase between d 1 and d 21 (p < 0.001) and between d 21 and d 70 (p < 0.001). After the end of supplementation period, the serum cholesterol concentrations significantly decrease during depletion period (p < 0.001).

Figure 2 – LS Means (± SE) of serum cholesterol concentrations in mmol/l for dairy cows in different stages of lactation. Animals were separated in three groups, receiving a control fat supplement (Control; n = 14), 50 g CLA (CLA 50; n = 15) or 100 g CLA supplement (CLA 100; n = 16) between d 1 and d 182, depletion period was between d 182 and d 252. Results of statistical evaluation refer to Table 10.
RESULTS

There were no significant differences between groups or cows and heifers in periods one and two (Table 10). In period one heifers had lower serum cholesterol concentrations than pluriparous cows ($p = 0.027$; Figure 3). Group x DIM and G x Lact interactions exhibited no significant effects (Table 10).

**Figure 3** – LS means ($\pm$ SE) of serum cholesterol concentrations in mmol/l for dairy cows ($n = 32$) and heifers ($n = 13$) in different stages of lactation. Results of statistical evaluation refer to Table 10.

**Table 10** – p-values of fixed effects and their interactions for serum cholesterol concentrations during periods one (d -21 - d 105) and two (d 105 – d 252) of lactation, of dairy cows ($n = 32$) and heifers ($n = 13$) separated in three groups, receiving a control fat supplement ($n = 14$), 50 g CLA ($n = 15$) or 100 g CLA supplement ($n = 16$) between d 1 and d 182, depletion period was between d 182 and d 252.

$G =$ group; $\text{DIM} =$ days in milk; $\text{Lact} =$ number of lactations (primiparous vs. pluriparous)

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4.1.4 Alpha-tocopherol:cholesterol mass ratio

The mass ratio between α-tocopherol and cholesterol varied with DIM during period one (p < 0.001), but not in period two. A sharp decrease between d -21 and d 1 was observed (p < 0.001; Table 11).

During lactation a steady level was kept, which was significantly higher for heifers than cows in both periods one and two (P 1 and P 2: p < 0.001; Table 12; Table 13).

There were no significant differences for groups or any G x DIM and G x Lact interactions.

Table 11 – Median serum α-tocopherol:cholesterol mass ratio (x 10⁻³) (five per cent and ninety-five per cent percentiles) for dairy cows in different stages of lactation. Animals were separated in three groups, receiving a control fat supplement (Control; n = 14), 50 g CLA (CLA 50; n = 15) or 100 g CLA supplement (CLA 100; n = 16) between d 1 and d 182, depletion period was between d 182 and d 252. Results of statistical evaluation refer to Table 13. DIM = days in milk

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<th>Median</th>
<th>(P5 – P95)</th>
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<th>Median</th>
<th>(P5 – P95)</th>
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</tr>
<tr>
<td></td>
<td>-21</td>
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<td>(1.31 – 2.42)</td>
<td>1.99</td>
<td>(1.43 – 3.47)</td>
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<td>(1.90 – 4.52)</td>
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<td>(1.15 – 3.36)</td>
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<td>(1.34 – 5.54)</td>
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<td>(1.30 – 3.37)</td>
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<td>(1.90 – 4.52)</td>
<td>1.97</td>
<td>(0.93 – 4.79)</td>
<td>1.94</td>
<td>(1.15 – 3.36)</td>
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<td>(1.34 – 5.54)</td>
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<td>140</td>
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<td>1.97</td>
<td>(0.93 – 4.79)</td>
<td>1.94</td>
<td>(1.15 – 3.36)</td>
<td>2.40</td>
<td>(1.34 – 5.54)</td>
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<td></td>
<td>182</td>
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<td>1.97</td>
<td>(0.93 – 4.79)</td>
<td>1.94</td>
<td>(1.15 – 3.36)</td>
<td>2.40</td>
<td>(1.34 – 5.54)</td>
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<tr>
<td></td>
<td>224</td>
<td>2.93</td>
<td>(1.90 – 4.52)</td>
<td>1.97</td>
<td>(0.93 – 4.79)</td>
<td>1.94</td>
<td>(1.15 – 3.36)</td>
<td>2.40</td>
<td>(1.34 – 5.54)</td>
</tr>
<tr>
<td></td>
<td>252</td>
<td>2.93</td>
<td>(1.90 – 4.52)</td>
<td>1.97</td>
<td>(0.93 – 4.79)</td>
<td>1.94</td>
<td>(1.15 – 3.36)</td>
<td>2.40</td>
<td>(1.34 – 5.54)</td>
</tr>
</tbody>
</table>
RESULTS

Table 12 – Median serum α-tocopherol:cholesterol mass ratio (x 10^{-3}) (five per cent and ninety-five per cent percentiles) for dairy cows (n = 32) and heifers (n = 13) in different stages of lactation. Results of statistical evaluation refer to Table 13.

DIM = days in milk

<table>
<thead>
<tr>
<th>Groups</th>
<th>DIM</th>
<th>Median (P5 – P95)</th>
</tr>
</thead>
<tbody>
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<td>Cows</td>
<td>-21</td>
<td>3.35 (1.48 – 4.52)</td>
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<tr>
<td></td>
<td>1</td>
<td>1.99 (1.31 – 3.42)</td>
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<td></td>
<td>21</td>
<td>1.77 (1.15 – 2.57)</td>
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<tr>
<td></td>
<td>70</td>
<td>1.80 (1.30 – 2.55)</td>
</tr>
<tr>
<td></td>
<td>105</td>
<td>1.77 (1.29 – 2.54)</td>
</tr>
<tr>
<td></td>
<td>140</td>
<td>1.68 (1.09 – 2.54)</td>
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<tr>
<td></td>
<td>182</td>
<td>1.72 (1.09 – 2.51)</td>
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<tr>
<td></td>
<td>224</td>
<td>1.69 (1.18 – 2.47)</td>
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<tr>
<td></td>
<td>252</td>
<td>1.69 (1.03 – 3.18)</td>
</tr>
<tr>
<td>Heifers</td>
<td>Median (P5 – P95)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.40</td>
<td>1.91 (1.90 – 3.26)</td>
</tr>
<tr>
<td></td>
<td>3.06</td>
<td>3.06 (2.52 – 3.58)</td>
</tr>
<tr>
<td></td>
<td>4.70</td>
<td>4.70 (3.00 – 4.07)</td>
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<tr>
<td></td>
<td>5.75</td>
<td>5.75 (3.85 – 4.07)</td>
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<tr>
<td></td>
<td>6.41</td>
<td>6.41 (3.85 – 6.92)</td>
</tr>
<tr>
<td></td>
<td>6.20</td>
<td>6.20 (3.69 – 6.44)</td>
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<tr>
<td></td>
<td>5.66</td>
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<tr>
<td></td>
<td>5.35</td>
<td>5.35 (2.79 – 7.23)</td>
</tr>
</tbody>
</table>

Table 13 – p-values of fixed effects and their interaction for serum α-tocopherol:cholesterol mass ratio (x 10^{-3}) during periods one (d -21 – d 105) and two (d 105 – d 252) of lactation, of dairy cows (n = 32) and heifers (n = 13) separated in three groups, receiving a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

<table>
<thead>
<tr>
<th>Effects</th>
<th>period 1</th>
<th>period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>0.65</td>
<td>0.28</td>
</tr>
<tr>
<td>DIM</td>
<td>&lt; 0.001</td>
<td>0.35</td>
</tr>
<tr>
<td>Lact</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>G x DIM</td>
<td>0.82</td>
<td>0.11</td>
</tr>
<tr>
<td>G x Lact</td>
<td>0.61</td>
<td>0.67</td>
</tr>
</tbody>
</table>

4.1.5 Retinol

Retinol varied with DIM in periods one and two (P 1 and P 2; p < 0.001), with a sudden decrease between d -21 and d 1 (p < 0.001), recovering until d 21 (Figure 4). There was a significant decrease during depletion period, between d 182 and d 252 (p = 0.038).

No significant differences were observed in periods one or two between groups, cows and heifers and for G x DIM interaction (Table 14). There was a trend for a G x Lact interaction (p = 0.060; Table 14).
RESULTS

Figure 4 – LS Means (± SE) of serum retinol concentrations in mg/l for dairy cows in different stages of lactation. Animals, separated in three groups, received a control fat supplement (Control; n = 14), 50 g CLA (CLA 50; n = 15) or 100 g CLA supplement (CLA 100; n = 16) between d 1 and d 182, depletion period was between d 182 and d 252. Results of statistical evaluation refer to Table 14.

Table 14 – p-values of fixed effects and their interactions for mean serum retinol concentrations in mg/ml during periods one (d -21 – d 105) and two (d 105 – d 252) of lactation, of dairy cows (n = 32) and heifers (n = 13) separated in three groups, receiving a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

<table>
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</thead>
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<td>&lt; 0.001</td>
<td>0.0003</td>
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<tr>
<td>Lact</td>
<td>0.46</td>
<td>0.44</td>
</tr>
<tr>
<td>G x DIM</td>
<td>0.36</td>
<td>0.91</td>
</tr>
<tr>
<td>G x Lact</td>
<td>0.16</td>
<td>0.060</td>
</tr>
</tbody>
</table>
4.2 Oxidative status

4.2.1 Amount of hydroperoxides in the sample

The radical reaction creating RC-DEPPD showed no significant difference between groups in periods one or two (Table 15), but a significant influence of DIM (P 1 and P 2: \( p < 0.001 \)) was observed (Figure 5). The observed increase between d -21 and d 1 was significant (\( p = 0.003 \)), as well as the increase after the end of supplementation at d 182, towards d 224 (\( p < 0.001 \)). No difference was observed between cows and heifers in period one, but in period two pluriparous cows exhibited higher concentrations of hydroperoxides (\( p = 0.005 \); Figure 6).

No significant interactions between G x DIM and G x Lact were observed in both period one or two (Table 15).

![Figure 5](image_url)

**Figure 5** – LS Means (± SE) of RC-DEPPD (mmol/l) formed during reaction of DEPPD with serum of dairy cows in different stages of lactation. Animals were separated in three groups, receiving a control fat supplement (Control; \( n = 14 \)), 50 g CLA (CLA 50; \( n = 15 \)) or 100 g CLA supplement (CLA 100; \( n = 16 \)) between d 1 and d 182, depletion period was between d 182 and d 252. Results of statistical evaluation refer to Table 15.
RESULTS

Figure 6 – LS Means (± SE) of RC-DEPPD (mmol/l) formed during reaction of DEPPD with serum of dairy cows (n = 32) and heifers (n = 13) in different stages of lactation. Results of statistical evaluation refer to Table 15.

Table 15 – p-values of fixed effects and their interactions for RC-DEPPD (mmol/l) formed during reaction of DEPPD with serum of dairy cows (n = 32) and heifers (n = 13) during periods one (d -21 – d 105) and two (d 105 – d 252) of lactation, separated in three groups: receiving a control fat (n = 14), 50 g CLA (n = 15) or 100 g CLA (n = 16) supplement between d 1 and d 182, depletion period was between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

<table>
<thead>
<tr>
<th>factor</th>
<th>period 1</th>
<th>period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>0.41</td>
<td>0.46</td>
</tr>
<tr>
<td>DIM</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lact</td>
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<td>0.005</td>
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<td>G x DIM</td>
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</tr>
<tr>
<td>G x Lact</td>
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</table>
RESULTS

4.2.2 Lipid peroxidation

4.2.2.1 Thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances varied significantly with DIM during periods one and two (P 1 and P 2: p < 0.001; Table 18), rising towards parturition (p = 0.014) and decreasing significantly after the periparturient period between d 21 and d 70 (p < 0.001) and further over the course of lactation. Serum TBARS concentrations of all groups increased, when supplementation was stopped at d 182, towards d 224 (p < 0.001; Table 16).

During period one there were no significant differences between cows and heifers (p = 0.50), whereas in period two, heifers had significantly lower serum TBARS concentrations (p = 0.013; Table 17).

No significant interactions were observed for G x DIM and G x Lact in both periods.

Table 16 – Median serum TBARS concentrations in nmol/l (five per cent and ninety-five per cent percentiles) for dairy cows in different stages of lactation. Animals were separated in three groups, receiving a control fat supplement (Control; n = 14), 50 g CLA (CLA 50; n = 15) or 100 g CLA supplement (CLA 100; n = 16) between d 1 and d 182, depletion period was between d 182 and d 252. Results of statistical evaluation refer to Table 18. DIM = days in milk

<table>
<thead>
<tr>
<th>Groups</th>
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<th>21</th>
<th>70</th>
<th>105</th>
<th>140</th>
<th>182</th>
<th>224</th>
<th>252</th>
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<tbody>
<tr>
<td>Control</td>
<td>Median</td>
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<td>9.31</td>
<td>8.64</td>
<td>8.67</td>
<td>7.34</td>
</tr>
<tr>
<td></td>
<td>(P5 -)</td>
<td>(6.94 -)</td>
<td>(6.44 -)</td>
<td>(7.78 -)</td>
<td>(5.78 -)</td>
<td>(6.50 -)</td>
<td>(7.00 -)</td>
<td>(5.89 -)</td>
</tr>
<tr>
<td></td>
<td>(P95)</td>
<td>11.44</td>
<td>13.50</td>
<td>15.67</td>
<td>15.67</td>
<td>10.72</td>
<td>13.11</td>
<td>9.94</td>
</tr>
<tr>
<td>CLA 50</td>
<td>Median</td>
<td>8.64</td>
<td>9.11</td>
<td>9.17</td>
<td>7.33</td>
<td>8.61</td>
<td>7.78</td>
<td>6.61</td>
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<tr>
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<td>(P5 -)</td>
<td>(6.06 -)</td>
<td>(6.39 -)</td>
<td>(7.00 -)</td>
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<td>11.78</td>
<td>12.83</td>
<td>10.17</td>
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<td>CLA 100</td>
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<td>7.44</td>
<td>7.58</td>
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<td></td>
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<td>(6.22 -)</td>
<td>(7.00 -)</td>
<td>(6.50 -)</td>
<td>(6.00 -)</td>
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<td>(6.17 -)</td>
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<td>(P95)</td>
<td>12.44</td>
<td>14.11</td>
<td>11.06</td>
<td>14.83</td>
<td>10.22</td>
<td>13.50</td>
<td>11.5</td>
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</table>
RESULTS

**Table 17** – Median serum TBARS concentrations in nmol/l (five per cent and ninety-five per cent percentiles) for dairy cows (n = 32) and heifers (n = 13) in different stages of lactation. Results of statistical evaluation refer to Table 18.

DIM = days in milk

<table>
<thead>
<tr>
<th>Groups</th>
<th>DIM</th>
<th>-21</th>
<th>1</th>
<th>21</th>
<th>70</th>
<th>105</th>
<th>140</th>
<th>182</th>
<th>224</th>
<th>252</th>
</tr>
</thead>
</table>

**Table 18** – p-values of fixed effects and their interactions for mean serum TBARS concentrations during periods one (d -21 - d 105) and two (d 105 - d 252) of lactation, of dairy cows (n = 32) and heifers (n = 13) separated in three groups, receiving a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

<table>
<thead>
<tr>
<th>factor</th>
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</thead>
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<tr>
<td>G</td>
<td>0.11</td>
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<tr>
<td>DIM</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lact</td>
<td>0.50</td>
<td><strong>0.013</strong></td>
</tr>
<tr>
<td>G x DIM</td>
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<td>0.49</td>
</tr>
<tr>
<td>G x Lact</td>
<td>0.61</td>
<td>0.27</td>
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</table>
4.2.3 Protein peroxidation

4.2.3.1 Protein content

Parameters concerning protein peroxidation were expressed per gram protein, thus the serum protein content will be illustrated shortly.

The serum protein content varied significantly with DIM (Figure 7) during periods one and two (P1 and P2 p < 0.001). Between d -21 and d 1 a significant decrease (p < 0.001) in serum concentrations was observed, followed by an increase towards d 21 (p < 0.001).

No significant differences were found between groups (Table 19), but there was a significant interaction between groups and DIM in both periods (P1 and P2 p = 0.006 and p = 0.021, respectively). During depletion period a significant increase was observed between d 182 and d 224 (p = 0.002), followed by a significant decrease between d 224 and d 252 (p = 0.003).

Figure 7 – LS Means (± SE) of serum protein content in g/l of dairy cows receiving a control fat (Control; n = 14), 50 g (CLA 50; n = 15) or 100 g (CLA 100; n = 16) dietary CLA supplement. Animals were in different stages of lactation; supplementation was between d 1 and d 182, depletion period between d 182 and d 252. Results of statistical evaluation refer to Table 19.
There was a significant difference in serum protein content with number of lactations, since cows had higher protein concentrations than heifers throughout the trial (P 1: \( p = 0.008 \) and P 2: \( p = 0.006 \); Figure 8).

**Figure 8** – LS Means (± SE) of serum protein content in g/l of dairy cows (n = 32) and heifers (n = 13) during different stages of lactation. Results of statistical evaluation refer to Table 19.

**Table 19** – \( p \)-values of fixed effects and their interaction for mean serum protein content during periods one (d -21 - d 105) and two (d 105 - d 252) of lactation, of dairy cows (n = 32) and heifers (n = 13) separated in three groups, receiving a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

\( G = \) group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

<table>
<thead>
<tr>
<th>effects factor</th>
<th>period 1</th>
<th>period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>0.13</td>
<td>0.19</td>
</tr>
<tr>
<td>DIM</td>
<td>(&lt; 0.001)</td>
<td>(&lt; 0.001)</td>
</tr>
<tr>
<td>Lact</td>
<td>0.008</td>
<td>0.006</td>
</tr>
<tr>
<td>G x DIM</td>
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<td>0.021</td>
</tr>
<tr>
<td>G x Lact</td>
<td>0.85</td>
<td>0.40</td>
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</tbody>
</table>
4.2.3.2 Bityrosine

Bityrosine was influenced by DIM in period one and two (P 1 and P 2: $p < 0.001$; Figure 9). A significant decrease in plasma concentration per gram protein towards parturition was observed ($p < 0.001$) with a subsequent increased after the periparturient period between d 21 and d 70 ($p < 0.001$).

At the end of supplementation, a decrease was observed between d 182 and d 224 ($p < 0.001$).

Bityrosine concentrations showed a group trend ($p = 0.054$) in period one, but not in period two, where the CLA 100 group exhibited the highest bityrosine concentrations.

There were no significant differences between cows and heifers or G x DIM and G x Lact interactions during both periods (Table 20).

**Figure 9** – LS Means ($\pm$ SE) of plasma bityrosine concentrations in mg/g protein of dairy cows in different stages of lactation. Animals received either a control fat supplement (Control; $n = 14$), 50 g CLA (CLA 50; $n = 15$) or 100 g CLA (CLA 100; $n = 16$) between d 1 and d 182, depletion period was between d 182 and d 252. Results of statistical evaluation refer to Table 20.
Table 20 – p-values of fixed effects and their interactions for plasma bityrosine concentrations of dairy cows (n = 32) and heifers (n = 13) during periods one (d~21 – d 105) and two (d 105 – d 252) of lactation, separated in three groups, receiving a control fat (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

| G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous) |
|---------------------------------|----------|---------|
| factor                          | period 1 | period 2 |
| G                               | 0.054    | 0.41    |
| DIM                             | < 0.001  | < 0.001 |
| Lact                            | 0.26     | 0.17    |
| G x DIM                         | 0.97     | 0.90    |
| G x Lact                        | 0.29     | 0.54    |

4.2.3.3 N’-Formylkynurenine

N’-Formylkynurenine was influenced by DIM in periods one and two (P 1 and P 2: p < 0.001; Figure 10). Plasma concentrations per gram protein decreased significantly towards parturition (p < 0.001) and increased after the periparturient period, between d 21 and d 70 (p < 0.001). After the end of supplementation, plasma concentrations decreased during depletion periods between d 182 and d 224 (p < 0.001).

There were no group effects and no significant differences between cows and heifers during both periods, as well as no interactions between G x DIM or G x Lact (Table 21).
RESULTS

Figure 10 – LS means (± SE) of plasma N’-formylkynurenine concentrations in µg/g protein of dairy cows in different stages of lactation, separated in three groups, receiving either a control fat supplement (Control; n = 14), 50 g CLA (CLA 50; n = 15) or 100 g CLA (CLA 100; n = 16) between d 1 and d 182, with a depletion period between d 182 and d 252. Results of statistical evaluation refer to Table 21.

Table 21 – p-values of fixed effects and their interactions for plasma N’-formylkynurenine concentrations in of dairy cows (n = 32) and heifers (n = 13) during periods one (d -21 – d 105) and two (d 105 – d 252) of lactation, separated in three groups, receiving either a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA (n = 16) between d 1 and d 182, with a depletion period between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)

<table>
<thead>
<tr>
<th>effects</th>
<th>period 1</th>
<th>period 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>0.38</td>
<td>0.58</td>
</tr>
<tr>
<td>DIM</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Lact</td>
<td>0.70</td>
<td>0.27</td>
</tr>
<tr>
<td>G x DIM</td>
<td>0.99</td>
<td>1.00</td>
</tr>
<tr>
<td>G x Lact</td>
<td>0.31</td>
<td>0.94</td>
</tr>
</tbody>
</table>
4.2.3.4 Sulphydryl groups

During periods one and two, SH groups were significantly influenced by DIM (P 1 and P 2: \( p < 0.001 \)) increasing between d -21 and d 1 (\( p = 0.038; \) Table 22) and decreasing again between d 1 and d 21 (\( p < 0.001 \)). During the course of lactation a steady decrease was observed. At the end of supplementation, between d 182 and d 224, a trend for a decrease was observed in the control group (\( p = 0.055 \)), whereas in the two CLA groups the decrease was significant (CLA 50: \( p = 0.012; \) CLA 100: \( p = 0.002 \)).

In period one there was a trend for a group difference (\( p = 0.085 \)), in period two there was a significant group difference (\( p = 0.021 \)), the CLA 100 group showed the highest concentrations of SH groups.

The difference between pluriparous cows and heifers was significant in both periods one and two (\( p = 0.024 \) and \( p = 0.004 \), respectively), with heifers exhibiting higher plasma concentrations of SH groups than pluriparous cows (Table 23).

No interactions between G x DIM and G x Lact could be observed (Table 24).

### Table 22 – Median plasma SH groups concentrations in mmol/g protein (five per cent and ninety-five per cent percentiles) for dairy cows in different stages of lactation, separated in three groups, receiving either a control fat supplement (Control; \( n = 14 \)), 50 g CLA (CLA 50; \( n = 15 \)) or 100 g CLA (CLA 100; \( n = 16 \)) between d 1 and d 182, with a depletion period (d 182 - d 252). Results of statistical evaluation refer to Table 24. DIM = days in milk

<table>
<thead>
<tr>
<th>Groups</th>
<th>DIM</th>
<th>-21</th>
<th>1</th>
<th>21</th>
<th>70</th>
<th>105</th>
<th>140</th>
<th>182</th>
<th>224</th>
<th>252</th>
</tr>
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<tr>
<td>Control</td>
<td>Median</td>
<td>8.83</td>
<td>9.44</td>
<td>8.54</td>
<td>7.59</td>
<td>7.18</td>
<td>6.95</td>
<td>6.27</td>
<td>6.09</td>
<td>6.64</td>
</tr>
<tr>
<td>CLA 50</td>
<td>Median</td>
<td>9.25</td>
<td>9.69</td>
<td>7.61</td>
<td>7.49</td>
<td>7.38</td>
<td>7.20</td>
<td>6.47</td>
<td>5.79</td>
<td>6.50</td>
</tr>
<tr>
<td>CLA 100</td>
<td>Median</td>
<td>9.44</td>
<td>9.83</td>
<td>8.56</td>
<td>8.42</td>
<td>7.69</td>
<td>7.68</td>
<td>6.59</td>
<td>6.31</td>
<td>7.12</td>
</tr>
</tbody>
</table>
RESULTS

**Table 23** – Median plasma SH groups concentrations in mmol/g protein (five per cent and ninety-five per cent percentile) for dairy cows (n = 32) and heifers (n = 13) in different stages of lactation. Results of statistical evaluation refer to Table 24.

DIM = days in milk

<table>
<thead>
<tr>
<th>Groups</th>
<th>DIM</th>
<th>21</th>
<th>70</th>
<th>105</th>
<th>140</th>
<th>182</th>
<th>224</th>
<th>252</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cows</td>
<td>Median</td>
<td>9.08</td>
<td>9.32</td>
<td>7.99</td>
<td>7.72</td>
<td>7.37</td>
<td>6.82</td>
<td>6.15</td>
</tr>
<tr>
<td></td>
<td>(P5 –)</td>
<td>(7.63 –)</td>
<td>(7.73 –)</td>
<td>(6.58 –)</td>
<td>(6.23 –)</td>
<td>(5.65 –)</td>
<td>(5.57 –)</td>
<td>(4.71 –)</td>
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<tr>
<td></td>
<td>P95</td>
<td>10.61</td>
<td>11.40</td>
<td>10.21</td>
<td>9.27</td>
<td>9.61</td>
<td>8.23</td>
<td>7.65</td>
</tr>
<tr>
<td></td>
<td>(P5 –)</td>
<td>(7.18 –)</td>
<td>(8.48 –)</td>
<td>(6.67 –)</td>
<td>(7.15 –)</td>
<td>(5.79 –)</td>
<td>(6.90 –)</td>
<td>(5.86 –)</td>
</tr>
<tr>
<td></td>
<td>P95</td>
<td>10.68</td>
<td>12.26</td>
<td>10.03</td>
<td>10.58</td>
<td>9.53</td>
<td>8.30</td>
<td>8.11</td>
</tr>
</tbody>
</table>

**Table 24** – p-values of fixed effects and their interaction for plasma SH groups concentrations in mmol/g protein of dairy cows (n = 32) and heifers (n = 13) during periods one (d -21 – d 105) and two (d 105 – d 252) of lactation, animals were separated in three groups, receiving a control fat supplement (n = 14), 50 g CLA (n = 15) or 100 g CLA supplement (n = 16) between d 1 and d 182, depletion period was between d 182 and d 252.

G = group; DIM = days in milk; Lact = number of lactations (primiparous vs. pluriparous)
5 Discussion

In the present study, we investigated effects of different doses of CLA, the periparturient period and parity, as well as possible interactions, on the antioxidative and oxidative status of dairy cows, in a thirty-nine week trial. Antioxidative and oxidative status of dairy cows in the present study was predominately affected by stage of lactation and parity, no effects were revealed for a supplementation with trans-10,cis-12 CLA and cis-9,trans-11 CLA between d 1 and d 182 of lactation.

5.1 Effect of lactational stage on antioxidative and oxidative status

5.1.1 Antioxidative status

The ferric reducing ability of plasma (FRAP) is an index of the antioxidative potential of plasma, influenced to 60 % by uric acid (BENZIE and STRAIN 1996), but also by various co-antioxidants in the plasma (BOUWSTRA et al. 2010a). Our results show a decrease in FRAP concentrations between d -21 and d 1 and steady concentrations between d 1 and d 21, which are low compared to mid-lactation values (refer to 4.1.1). These low peripartal FRAP levels were also seen in results of BOUWSTRA et al. (2010a) where levels values dropped during the dry period towards parturition and by GAAL et al. (2006), where FRAP levels were lower at the day of parturition compared to pre- and post-calving data. This data indicates that calving causes significant, but temporary changes in the antioxidant system of cows’ blood. However, GAAL et al. (2006) concluded that antioxidant parameters appear to change more rapidly in the plasma or serum than inside the cell. Accordingly, results from plasma FRAP may not fully deflect the intracellular antioxidative potential.

Blood antioxidants, such as α-tocopherol, the most potent vitamin E isomer, as well as vitamin A, including its precursors such as β-carotene (PALOZZA and KRINSKY 1992), may influence FRAP. However, plasma concentrations of both vitamins and β-carotene only influence overall FRAP to an extent of less than 10 % (BENZIE and STRAIN 1996) and were therefore addressed discretely in this study.

A shortage in α-tocopherol and other antioxidants weakens the immune defence system (HOGAN et al. 1992) and subsequently may explain a disposition for developing infectious
diseases, such as mastitis, in the periparturient period (LEBLANC et al. 2004). Dairy cows with a shortage of α-tocopherol, selenium or β-carotene are more prone to suffer from RFM (JULIEN et al. 1976a; JULIEN et al. 1976b; MICHAL et al. 1994). Fertility was improved by injection of α-tocopherol and selenium in dairy cows with and without RFM in study by ARÉCHIGA et al. (1994). Correlations between low α-tocopherol concentrations, udder oedema and ovarian cysts have been discussed (ALLISON and LAVEN 2000).

In this study, α-tocopherol and retinol serum concentrations decreased significantly during the dry period towards parturition and basal levels were regained at d 70 (refer to 4.1.2 and 0). Similar α-tocopherol and retinol patterns were observed in other studies (GOFF and STABEL 1990; POLITIS et al. 2012), who attributed the decreased peripartal serum concentrations of α-tocopherol and retinol to transfer of considerable amounts into colostrum.

As a lipid soluble molecule α-tocopherol is transported within the plasma mainly by lipoproteins, thus the total concentration of α-tocopherol in plasma is determined by the concentration of lipoprotein particles, as well as by the concentration of α-tocopherol within each particle. It was first described by HORWITT et al. (1972) that α-tocopherol concentrations should only be interpreted in combination with data on serum lipids, due to a strong relationship between the two. HERDT and SMITH (1996) recommended to index plasma vitamin E concentrations, based on other lipoprotein components, such as cholesterol, to compensate for the effect of variability in blood lipoprotein concentrations on serum vitamin E concentrations. The vitamin E:cholesterol ratio is a valid, relative estimator of the vitamin E concentration per lipoprotein particle (HERDT and SMITH 1996) and is calculated as ratio between vitamin E and total serum cholesterol concentrations.

A similar pattern as reported before (HERDT and SMITH 1996; ABENI et al. 2007; TURK et al. 2013) was found for mean serum cholesterol concentrations during the transition period in dairy cows of this study (refer to 4.1.3), showing similarities with the course of mean serum α-tocopherol concentrations. The decrease in mean serum cholesterol concentration towards and around parturition could also be explained by transfer into milk and a delayed increase in hepatic de-novo production after parturition (VITURRO et al. 2009; GRABER et al. 2010; SCHLEGEL et al. 2012).
At the end of the study period, after fat supplements were removed from the diet, mean serum cholesterol concentrations decreased. It is known that increased dietary fat proportions increase serum cholesterol content, as they are constituents of fat transporting chylomicrons (GRUMMER and CARROLL 1991). Decreased serum cholesterol concentrations at the end of lactation were also observed in a study of ABENI et al. (2007).

In cows of this study the α-tocopherol:cholesterol mass ratio decreased significantly at parturition by about 40 % compared to d -21 and thereafter remained almost stable until the end of the study period at d 252 (refer to 4.1.3). According to WEISS et al. (1992) this ante partum decrease of the α-tocopherol:cholesterol ratio, also found by other authors (HERDT and SMITH 1996; POLITIS et al. 2012), possibly indicates selective secretion of vitamin E into colostrum by the mammary gland.

After parturition, the stage of lactation had no effect on the α-tocopherol:cholesterol mass ratio, possibly due to stable milk yield and feeding conditions without major demands on the antioxidative defence system. However, the α-tocopherol:cholesterol mass ratio was low during the entire lactation in cows of the present study, compared to results of other studies (LEBLANC et al. 2002; 2004). This difference may be explained by the marginal dietary vitamin E supply (WEISS et al. 1997; NRC 2001) of cows in this trial.

5.1.2 Oxidative status
In this study hydroperoxides increased significantly between d -21 and d 1 (refer to 4.2.1). This observation is in accordance with results of KANKOFER et al. (2010), who used the same analytical method. POLITIS et al. (2012) measured hydroperoxides via the formation of the radical cation of N,N-dimethyl-para-phenylene diamine (DMPD). According to VERDE et al. (2002) this is an analytical technique which produces very reliable and reproducible results, which are comparable to the method for the detection of hydroperoxides used in the present study, described by ALBERTI et al. (2000). The authors mentioned above also found highest plasma concentrations of hydroperoxides in the transition period. Results indicate that dairy cows are exposed to increased oxidative stress at parturition. A possible explanation for this may be the considerably enhanced metabolism at the onset of lactation and an increased production of radicals by cytochrome P 450, amongst others (MILLER and BRZEZINSKA-SLEBODZINSKA 1993). The course of serum hydroperoxide concentrations and thus
DISCUSSION

oxidative stress, gives rise to the thought that it is parturition itself that is causing oxidative stress. The intensive muscular activity during labour may contribute to the production of ROS (CHIARADIA et al. 1998; POWERS and JACKSON 2008), thus increasing oxidative stress, as recently reported in a human trial (HUNG et al. 2011). The challenged immune system with an enhanced respiratory burst of immune cells may also contribute to increased ROS concentrations around parturition (BABIOR 1987).

5.1.2.1 Lipid peroxidation

The validity of the photometric TBA test, used for determination of TBARS in the present trial, has been thoroughly discussed. The major disadvantage of the test is that aldehydes other than MDA, which are generated during lipid peroxidation, can form adducts with TBA and are photometrically detectable at 532 nm (LYKKESFELDT and SVENDSEN 2007). However, although the photometric TBA test for assessment of MDA formation is less precise than HPLC-methods for MDA measurement (GUTTERIDGE and HALLIWELL 1990) it gives valid data for the comparison of groups of the same species (LYKKESFELDT 2001). The photometric TBA test, as used in this study, has been frequently used in recent studies (MUDRON et al. 2007; TURK et al. 2008; URBAN-CHMIEL et al. 2009; KANKOFER et al. 2010; SHARMA et al. 2011).

In animals of the present study, the mean TBARS concentrations followed a similar pattern to the hydroperoxide concentrations during transition period and lactation (refer to 4.2.2). Mean TBARS concentrations increased between d -21 and d 1, indicating increased lipid peroxidation, as reported before (BERNABUCCI et al. 2005; GAAL et al. 2006) and dropped over the further course of lactation. The increased peripartal lipid peroxidation was possibly due to the observed rise in hydroperoxides, which attack lipids and may induce the lipid peroxidation chain reaction (GIROTTI 1998).

The reduced availability of α-tocopherol and other antioxidants during this period, as demonstrated by low α-tocopherol:cholesterol mass ratio and FRAP, probably contributed to increased production of TBARS. However, the physiological relevance of these findings needs further evaluation since GAAL et al. (2006) concluded from their results, that the intracellular antioxidative defence system is more stable than the antioxidative defence in extracellular fluids, such as plasma or serum, which was determined in the present trial.
5.1.2.2 Protein peroxidation

5.1.2.2.1 Bityrosine and N′-formylkynurenine

During the transition period bityrosine and N′-formylkynurenine (refer to 4.2.3.2 and 0) followed a plasma concentration pattern nearly opposite to mean TBARS and hydroperoxide concentrations. Bityrosine crosslinks are formed by oxidative attack of hydroxide radicals or lipid hydroperoxides, formed during lipid peroxidation (WOLFF et al. 1986). N′-formylkynurenine is formed during oxidative attack of tryptophan by hydroxide radicals and other ROS (GIULIVI et al. 2003). Since the amount of hydroperoxides in the sample, as well as the serum TBARS concentration, increased around parturition, the decrease of plasma concentrations of bityrosine and N′-formylkynurenine around parturition with a subsequent increase towards mid of lactation was unexpected.

However, to compensate the effects of plasma protein fluctuation around parturition and during lactation on protein peroxidation values, results of these parameters are expressed as milligram per gram protein or microgram per gram protein, respectively.

Serum protein content varied significantly with DIM in cows of this study (see 4.2.3.1). Between d -21 and d 1 the protein content dropped significantly. Results of CASTILLO et al. (2005) showed a comparable decline in serum protein, suggesting that this is due to globulin secretion into colostrum (GOFF and HORST 1997). Postpartum, the protein content recovered to higher concentrations than antepartum values, which could be explained by protein requirements of the foetus in late gestation.

Due to adjustment of protein peroxidation results to mean protein concentration it appears unlikely that changes in plasma protein concentrations caused the unexpected concentration pattern of bityrosine and N′-formylkynurenine in cows of this study. It rather appears that despite the observed antepartum decline in serum α-tocopherol, retinol and FRAP concentrations (refer to 4.1) the antioxidative defence system is still strong enough to protect tryptophan and tyrosine residues from oxidative attack. DAVIES et al. (1987) showed that uric acid could effectively protect tryptophan and, to some extent, prevent bityrosine formation. Uric acid is the dominant factor contributing to overall FRAP, a measure of the total plasma antioxidative capacity.
KANKOFER et al. (2010) observed an increase of FRAP between two weeks and five days antepartum, a significant decrease at partus and an increase towards one week postpartum. Together with results of SORDILLO et al. (2007), KANKOFER et al. (2010) concluded that the antioxidant defence mechanisms in the plasma of cows are sufficient to cope with peripartal oxidative stress. Due to sampling interval, these changes were not observed in the present trial, but bityroside and N'-formylkynurenine concentrations also suggest that the antioxidative defence system was sufficient to protect proteins from oxidative damage.

5.1.2.2.2 Sulfhydryl groups

In the plasma, sulfhydryl groups appear as part of proteins, such as albumin or glutathione, the amino acid cysteine and its derivatives homocysteine and t-cysteine (BERNABUCCI et al. 2005). To avoid fluctuation of mean plasma protein concentrations influencing the SH group concentrations, results were expressed as mmol/g protein. Plasma SH groups are the most abundant low molecular weight plasma antioxidant and represent an important extracellular antioxidant defence (UELAND et al. 1996).

In period one of the present study a significant increase of SH groups was found between d -21 and d 1 (refer to 4.2.3.4), as observed in other trials (BERNABUCCI et al. 2002; POLITIS et al. 2012). These changes in SH group concentrations during the dry period may be interpreted as decreased oxidative stress and accordingly high concentrations of reduced SH groups (KANKOFER et al. 2010).

On the other hand, an increase in plasma SH group concentrations may indicate a compensatory increase of SH groups, due to increased oxidative stress (BERNABUCCI et al. 2002; POLITIS et al. 2012). Increased oxidative stress at calving was observed in the present study, in terms of increased mean TBARS and hydroperoxide concentrations. A better insight in these processes would be gained if ratios between redox partners were determined, such as GSH and GS-SG. This was done by SORDILLO et al. (2007) and a low GSH:GS-SG ratio was found at calving, indicating high oxidative stress. Due to measurement of SH group concentration in this study, rather than GSH concentrations, determination of disulphide bridges as the corresponding redox partner, was not possible.

Plasma SH group concentrations significantly decreased after parturition between d 1 and d 21, which could be due to an initial depletion of the antioxidant system, due to high...
DISCUSSION

oxidative stress in the peripartal period (SORDILLO et al. 2007). On the other hand, the
decrease in plasma SH groups continued throughout lactation, as observed for concentrations
of TBARS and hydroperoxides, thus the decreased SH groups could also be due to an
adaptation to decreased oxidative stress.

5.2 CLA effects on antioxidative and oxidative status

5.2.1 Antioxidative status

In the present trial no significant effects of CLA supplementation on the antioxidative status
could be determined.

The FRAP is an index for reductive potential, influenced by co-antioxidants, possibly
including CLA. From the beginning of period one, before CLA supplementation started, mean
FRAP plasma values of CLA 50 cows were lower than in other groups while mean plasma
FRAP in control and CLA 100 cows were almost equal (refer to 4.2.1). Thus, the statistical
trend for a group effect in period one should be interpreted carefully and cannot be seen as an
indication for a CLA effect on oxidative status.

The conjugated double bonds of the CLA isomers, used in the present study, are assumed to
provide antioxidative, radical scavenging properties with an antioxidative potential possibly
even better than vitamin E (HA et al. 1990). Radical quenching properties much weaker than
those of vitamin E or vitamin C were revealed in vitro for a mixture of equal amounts of
trans-10,cis-12 and cis-9,trans-11 CLA (YU 2001). To identify the properties of the different
CLA isomers, in vitro experiments were carried out with highly purified cis-9,trans-11 and
trans-10,cis-12 CLA by LEUNG and LIU (2000). Results showed that cis-9,trans-11 CLA
acted as a weak antioxidant in low concentrations but as pro-oxidant in high concentrations;
trans-10,cis-12 CLA acted as an antioxidant at all concentrations, in low concentrations even
better than vitamin E. A similar experiment, carried out to detect free radical scavenging
properties of the same two isomers, revealed a dose dependent antioxidative effect for both
isomers, with the trans-10,cis-12 CLA isomer showing a greater initial scavenging velocity
and the cis-9,trans-11 CLA isomer quenching more radicals. Both isomers had a synergistic
effect (YU et al. 2002). However, VAN DEN BERG et al. (1995) used a mixture of
trans-10,cis-12, cis-9,trans-11, cis-11,trans-13 and cis-8,trans-10 CLA in a phospholipid
model system and revealed neither antioxidative nor oxidative properties of this CLA mixture.

In the present study equal amounts of trans-10,cis-12 CLA and cis-9,trans-11 CLA were fed to the animals. The missing effect of CLA supplementation on FRAP in this study may be due to too low bioavailability of CLA after supplementation or opposing effects of the two isomers on the antioxidative status.

Since no significant differences in serum α-tocopherol and retinol concentrations between groups (see 4.1.2 and 0) could be determined, changes in parameters, determining the antioxidative or oxidative status, due to the antioxidant effects of α-tocopherol and retinol, can be precluded.

5.2.2 Oxidative status

There were no significant differences between groups for mean serum hydroperoxides or TBARS concentrations (refer to 4.2.1 and 4.2.2.1), indicating that supplementation of cis-9,trans-11 CLA and trans-10,cis-12 CLA had no significant effect on oxidative stress and lipid peroxidation in dairy cows.

Supplementation of mixtures of CLA isomers reduced lipid peroxidation as measured by plasma or hepatic TBARS in rats (IP et al. 1991; SANTOS-ZAGO et al. 2007), broiler chicks (ZHANG et al. 2008) and laying hens (QI et al. 2011). In contrast, in healthy humans (BASU et al. 2000b) and men with abdominal obesity (BASU et al. 2000a) CLA mixture supplements even increased lipid peroxidation as demonstrated by an increase in urinary excretion of 8-iso-prostaglandin F2α, a major isoprostane, and 15-oxo-dihydro-prostaglandin 2α, a major metabolite of prostaglandin F2α. Both are indicators of non-enzymatic and enzymatic arachidonic acid oxidation, which is a long chain polyunsaturated fatty acid prone to oxidation. Increased hepatic TBARS concentrations were also observed in rats (YAMASAKI et al. 2000) after CLA supplementation. In other studies no effects of CLA mixtures on TBARS were reported in rat hepatocytes (CANTWELL et al. 1999), hepatoma cell lines (IGARASHI and MIYAZAWA 2001), and bovine endothelial cells (LAI et al. 2005). Both, antioxidative and pro-oxidative properties of isolated CLA isomers on human low density lipoproteins, depending on CLA concentration, were shown in vitro (FLINTOFF-DYE and OMAYE 2005).
IGARASHI and MIYAZAWA (2001) detected reduced hydroperoxide concentrations in human hepatoma membrane phospholipids after CLA application. On the other hand, CLA supplementation induced increased concentrations of lipid peroxides in rat liver (YAMASAKI et al. 2000). CLA treatment of human colon cancer cells (PIERRE et al. 2013) and isolated macrophages (STACHOWSKA et al. 2008; RYBICKA et al. 2011) induced increased formation of intracellular ROS. In summary, currently reported effects of CLA supplements on lipid peroxidation and formation of reactive oxygen species are inconclusive.

BANNI et al. (1998) and BANNI et al. (1999) negate any specific antioxidative or oxidative effects of CLA isomers. The authors assume that CLA behave as any other polyunsaturated fatty acid (PUFA) in biological systems. Dietary CLA isomers are incorporated into body lipids and are as all other PUFA prone to oxidation, depending on the status of the antioxidative defence system. Furthermore, incorporation of CLA induces changes in lipid fatty acid profiles, depending on the dietary CLA content, thus reducing proportions of other lipid PUFA in particular arachidonic acid (CANTWELL et al. 1999). Decreasing proportions of PUFA, which are readily oxidised, may result in decreased risk for oxidative lipid damage, dissembling an antioxidative effect of CLA (LIVISAY et al. 2000). MDA is formed from oxidation of fatty acids with three or more double bonds, including arachidonic acid, among other physiological reactions (JANERO 1990). A decreased proportion of these PUFA, would reduce the absolute amount of MDA produced by lipid peroxidation but without reducing relative lipid peroxidative activity (LIVISAY et al. 2000).

In studies with rats (BANNI et al., 1999; LIVISAY et al. 2000) and laying hens (QI et al. 2011), which revealed a significant change in fatty acid profiles of body lipids due to CLA supplementation, supplementation resulted in a dietary CLA content of about 2%. In the present study supplementing 5 and 10 g of each CLA isomer in the CLA 50 and CLA 100 group, respectively, dietary content was less than 0.05 % of each isomer assuming an average dry matter intake of about 20 kg per cow and day (PAPPRITZ et al. 2008). Supplementing the same commercial CLA product in comparable dosages had almost no effects on fatty acids profiles in various organ lipids in heifers, suggesting that CLA bioavailability was too low to cause an effect on arachidonic acid and other PUFA proportions and thus TBARS concentrations (KRAMER et al. 2013).
For protein peroxidation, in terms of mean plasma bityrosine concentrations, a group trend was observed during period one; with CLA 100 exhibiting the highest bityrosine concentrations (refer to 4.2.3.2). Considering that no significant differences in plasma N\(^\prime\)-formylkynurenine were found between groups (refer to 0) and that the CLA 100 group had numerically higher bityrosine concentrations from the beginning of the trial, before the CLA supplementation started, the observed statistical trend in period one is probably not indicative for CLA effects on the oxidative status of cows in this study.

A similar trend as observed for bityrosine concentrations was revealed for mean plasma SH group concentrations during period one; highest plasma concentrations were detected in the CLA 100 group. During period two they were significantly higher in the CLA 100 group (refer to 4.2.3.4). However, these results should be interpreted carefully, since mean plasma SH group concentrations of the CLA 100 group were already higher before supplementation started, compared to the other groups.

5.2.3 CLA dosage and bioavailability

A possible explanation for the little observed effects of the CLA supplement on the antioxidative and oxidative status of dairy cows is the CLA dosage used in the study. Animals of the CLA 50 and the CLA 100 groups were supposed to receive 5 g and 10 g of each of the trans-10,cis-12 CLA and cis-9,trans-11 CLA isomer, respectively. According to the analysed dietary CLA content and the consumed amounts of concentrate, containing the CLA, the total CLA intake was 20% lower than expected, 4 and 8 g of each CLA isomer (PAPPRITZ et al. 2011b). However, even though CLA intake was lower than expected, it was still within the same range as in comparable trials on effects of CLA supplementation on production parameters and energy metabolism in dairy cows (PERFIELD II et al. 2002; BERNAL-SANTOS et al. 2003; DE VETH et al. 2004; MOORE et al. 2004; HARVATINE and BAUMAN 2011).

Both CLA isomers supplemented in this study are also microbiologically produced in the rumen during the natural biohydration process of dietary unsaturated fatty acids. Butyrivibrio fibrosolvens is known to produce cis-9,trans-11 CLA and vaccenic acid, a trans-11 18:1 fatty acid, under regular ruminal fermentation conditions, as expected in this study according to the composition of the diet. Trans-10 18:1 fatty acids and trans-10,cis-12 CLA are produced by
Megasphaera elsdeni under mildly acidotic ruminal fermentation conditions (KIM et al. 2002). Absorbed vaccenic acid can be converted back into cis-9,trans-11 CLA by Δ⁹-desaturase, found in the intestine, mammary gland and adipose tissue of ruminants. Mammals do not have Δ¹²-desaturase, so it is impossible for them to synthesize trans-10,cis-12 CLA from absorbed trans-10 18:1 fatty acids (CHURRUCA et al. 2009).

In a follow up study with heifers conducted by KRAMER et al. (2013), the same commercial CLA product was supplemented to heifers over a 105 day period in a dose of 5.7 g/d of each CLA isomer, using the same supplementation technique as in the present study. The authors found numerically small but statistically significant increases in the proportions of cis-9,trans-11 CLA and trans-10,cis-12 CLA in milk lipids after CLA supplementation while no significant effects were seen on the proportions of these CLA isomers in lipids of hepatic, muscular and mammary gland tissues. The proportions of trans-10 and trans-11 C18:1 fatty acids in milk lipids remained unaffected after CLA supplementation, but a significant increase in trans-10 C18:1 was detected in lipids of hepatic and mammary gland tissues of supplemented compared to control heifers. Other studies supplementing comparable amounts of the same CLA product as in this study also revealed little effects on the proportion of trans-10 and trans-11 fatty acids in lipid membranes of erythrocytes and leucocytes of dairy cows (DÄNICKE et al. 2012; RENNER et al. 2012). Thus, the chosen dosage of CLA supplements may have been too low to increase the bioavailability of CLA isomers above the level of naturally occurring CLA and thereby to induce detectable changes in oxidative status of dairy cows of the present study.

The commercial CLA supplement used in the present study was enclosed in a lipid capsule, providing protection against ruminal biohydrogenation, according to the manufacturers' information. Formation of calcium salts, cross-linkage to formaldehyde and linkage by amide bonds are also used as rumen protection of CLA supplements (JENKINS and BRIDGES 2007). PERFIELD II et al. (2004) found no difference between effects of CLA supplements on milk fat depression, which were protected either by amide-bonds or lipid-encapsulation. Results of PERFIELD II et al. (2004) were also comparable to results obtained by abomasally infused CLA, which avoids exposure of the CLA supplement to rumen bacteria (BAUMGARD et al. 2001), or feeding calcium salts of CLA (PERFIELD II et al. 2002;
Thus, results on the effects of CLA on milk fat depression appear not to be affected by the type of rumen protection used. The lipid capsule of CLA supplement used in the present study consisted of hydrogenated vegetable fats, containing palmitic and stearic acid. PAPPRITZ et al. (2011a) used an equal dose of the same commercial, lipid-encapsulated CLA supplement as in the present trial to determine duodenal availability of CLA isomers, which was only 16% in the CLA 50 group and five per cent in the CLA 100 group. Considering dietary CLA intake of cows in the present study (approximately 4 g and 8 g of each CLA isomer in the CLA 50 and CLA 100 group, respectively (PAPPRITZ et al. 2011b)) mean duodenal availability of each CLA isomer ranged from 0.50 to 0.70 g per day in both groups (PAPPRITZ et al. 2011a). Results also revealed that an average of 35 to 50% of the available trans-10, cis-12 CLA were transferred into milk fat in the CLA supplemented groups. Consequently only very little amounts of the supplemented CLA could be detected in the plasma or different tissues of animals in a follow-up study, using the same commercial CLA supplement (VON SOOSTEN et al. 2013), which supports results of KRAMER et al. (2013).

The presented results suggest that despite rumen protection using hydrogenated vegetable fats a high proportion of the supplemented CLA was hydrogenated in the rumen by bacteria and therefore not available to affect oxidative and anti-oxidative status in cows of this study. This assumption is supported by findings of PAPPRITZ et al. (2011a), who detected increasing amounts of 18:1 fatty acids, the hydrogenated form of 18:2 CLA, in the faeces of CLA supplemented animals. The effects of CLA supplements on milk fat depression, irrespective of the type of their rumen protection (PERFIELD II et al 2004) may be explained by the fact that both trans-10, cis-12 CLA and the hydrogenated form trans-10 C18-1 fatty acids induce a reduction in milk fat proportions (GRIINARI et al. 1998; BAUMAN and GRIINARI 2003; HARVATINE and BAUMAN 2011).

5.2.4 Lipid peroxidation at the end of supplementation
Concentrations of TBARS and hydroperoxides increased significantly in serum of all groups after supplementation was stopped at d 182 (refer to 4.2.1 and 4.2.2.1). Possible explanations for this increase include heat stress (BERNASUCCI et al. 2002; DI TRANA et al. 2006) or herding and rearrangements in hierarchy, resulting in increased muscular activity (POWERS
and JACKSON 2008; RADAK et al. 2008). None of the above occurred at the end of the CLA supplementation in this study, but the transponder controlled feeding of CLA or control concentrate was stopped and the amount of concentrate in the TMR was adjusted to late lactational milk yield. Due to reduction of the dietary concentrate proportion, metabolism, lipomobilisation in particular, was enhanced, indicated by rising plasma NEFA concentrations (unpublished data), which may have led to increased production of ROS, hydroperoxides and thus TBARS.

5.3 Differences in antioxidative and oxidative status between cows and heifers

5.3.1 Antioxidative status
Cows and heifers differed significantly in serum α-tocopherol concentrations (Table 9), with primiparous animals exhibiting constantly higher values, in contrast to results of BOUWSTRA et al. (2010b), where pluriparous cows had significantly higher values. In the study mentioned above, recent changes in feeding strategies for milking cows, but not for the young stock, are mentioned as a possible reason for these discrepancies. These changes in feeding strategies involve high vitamin E supplementation for mastitis prevention in milking cows but not in the young stock. This theory is underlined by the fact that antepartum serum α-tocopherol values for primiparous animals in the present trial (Table 8: 3.36 mg/l at three weeks antepartum) are comparable to mean antepartum concentrations of 9.1 μmol/l (= 3.92 mg/l) in heifers obtained by BOUWSTRA et al. (2010b). Primiparous heifers were pasture fed for about three months until enrolment for this study at d -21. In contrast, pluriparous cows were kept on pasture only for a short period of two to five weeks between dry off and study start. Pasture feed results in higher serum vitamin E concentrations feeding silage to housed animals (LEIBER et al. 2003). Thus, the different feeding strategies before study start are most likely to explain the differences in plasma α-tocopherol levels of primiparous and pluriparous cows.

The longer pasture feeding period also explains the observed higher α-tocopherol:cholesterol mass ratio indicating a better α-tocopherol supply in heifers compared to pluriparous cows, since plasma cholesterol concentrations were not statistically different between primiparous and pluriparous cows in this study (refer to 4.1.3). In contrast to the results of the present
study, LEBLANC et al. (2002) reported that heifers had a tendency to exhibit lower α-tocopherol:cholesterol mass ratio than animals in the third parity, which may also be due to higher vitamin E supply to pluriparous cows compared to the young stock in that study.

It should be noted, that despite sufficient dietary vitamin E supply, according to GFE (2001) recommendations, the pluriparous animals in this trials were α-tocopherol deficient during the transition period. Primiparous animals were above the lower reference values, apart from the temporary decrease between d 1 and d 21.

Based on recommendations of WEISS (1998) on vitamin E requirements of dairy cows BOUWSTRA et al. (2010b) suggested to set a lower reference value for serum vitamin E concentrations at 7.4 µmol/l (= 3.187 mg/l). Pluriparous animals in this trial had a median α-tocopherol value of 3.28 mg/l, some considerably lower (P5 = 1.42 mg/l). On d 1 and d 21 median serum α-tocopherol concentrations were clearly below the lower reference value (Table 8).

Similar results were obtained, looking at the α-tocopherol:cholesterol mass ratio, LEBLANC et al. (2002) refers to animals with a α-tocopherol:cholesterol ratio of less than 2.5 x 10⁻³, as vitamin E-deficient animals. In a consecutive study LEBLANC et al. (2004) found that animals with a ratio of less than 2.0 x 10⁻³ had a higher odds ratio of suffering from retained foetal membranes. Pluriparous animals in this study started with a α-tocopherol:cholesterol mass ratio of 3.35 x 10⁻³, but exhibited average values of less than 2.0 x 10⁻³ throughout the rest of the study.

Commonly, studies on requirements and effects of vitamin E in dairy cows (WEISS and WYATT 2003; LEBLANC et al. 2004; BROZOS et al. 2009; BOUWSTRA et al. 2010b; POLITIS et al. 2012) refer to NRC (2001) guidelines, suggesting a daily intake of 2.6 IU (= 1.74 mg) vitamin E per kilogram body weight (1150 mg per cow of 650 kg body weight). Taking results of the present study into consideration, indicating marginal or even insufficient α-tocopherol supply in dairy cows fed 500 mg vitamin E per cow per day according to GFE (2001) suggestions; GFE (2001) recommendations should be reconsidered and possibly adjusted to NRC (2001) guidelines or even higher.
5.3.2 Oxidative status

In period two of the present study, pluriparous cows had significantly higher mean serum concentrations of TBARS (refer to 4.2.2) compared to heifers, indicating that heifers experienced less lipid peroxidation during lactation. The amount of hydroperoxide in the serum was also significantly lower in heifers than in cows in period two (refer to 4.2.1). An explanation for this may be the significantly higher α-tocopherol (refer to 4.1.2) concentrations in serum of heifers and the higher α-tocopherol:cholesterol mass ratio, indicating a more effective protection of lipids against oxidation (BOWEN and OMAYE 1998). On the other hand, FREI et al. (1988) found that no lipid peroxidation was detectable in fresh human plasma as long as ascorbic acid was available. Only when ascorbic acid was completely utilised, lipid peroxidation was slowly traceable, even though other antioxidants, including vitamin E, were still available. A possible explanation for this is the ability of ascorbic acid to regenerate α-tocopherol from the tocopheroxyl radical (BURTON and TRABER 1990). It appears likely, that the longer period on pasture of heifers with consumption of fresh instead of conserved feed before the study start resulted in higher ascorbic acid availability in heifers (DESCALZO et al. 2005) and thereby improved antioxidative defence status compared to pluriparous cows (FREI et al. 1988). On the other hand, it has been reported that dietary vitamin C was completely destroyed in the rumen and had no effect on plasma concentrations (NOCKELS 1988), thus further research on ascorbic acid uptake and its role in the antioxidative defence in dairy cows appears to be necessary.

There were no significant differences between cows and heifers considering protein peroxidation products, bityrosine and N'-formylkynurenine. Plasma SH groups on the other hand, were significantly higher in heifers than in cows (Table 23). This possibly contributed to a better antioxidative defence in heifers (KANKOFER et al. 2010), measured as lower hydroperoxide concentrations than in cows.
5.4 Conclusion

Results of the present study revealed no clear effect of supplementation with 50 g or 100 g of a commercial CLA product on the antioxidative and oxidative status of dairy cows. The CLA supplement contained 10% of each of the cis-9,trans-11 CLA and trans-10,cis-12 CLA isomer and was fed between the day of parturition and day 182 postpartum. The absence of the expected effect of CLA on antioxidative and oxidative status may have been either due to opposing and thus mutually eliminating effects of both CLA isomers, too low a dose of CLA or poor rumen protection of the CLA product leading to low biological availability of the different CLA isomers.

During the transition period the typical alterations in oxidative status, a decreased antioxidative defence and increased oxidative stress around parturition, were observed. In this study, heifers exhibited higher α-tocopherol serum concentrations, a higher α-tocopherol:cholesterol mass ratio and higher plasma concentrations of sulphhydryl groups compared to pluriparous cows. Better antioxidative defence status in heifers compared to pluriparous cows resulted in lower TBARS and hydroperoxide serum concentrations, indicating less oxidative stress in heifers. Since the observed differences in antioxidative and oxidative status between cows and heifers are assumed to be mainly caused by differences in dietary vitamin E supply before the study, results of the study suggest the need for critical reconsideration of German recommendations for dietary vitamin E supply in dairy cows (GFE 2001).
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6 Zusammenfassung

Nina Hanschke

Der Effekt einer Langzeitsupplementation von konjugierten Linolsäuren auf den oxidativen und antioxidativen Status von Milchkühen während des peripartalen Zeitraumes und der darauffolgenden Laktation


Diese Studie wurde am Institut für Tierernährung des Friedrich-Löffler-Institutes in Braunschweig durchgeführt. Dort wurden 32 Milchkühe und 13 Färsen in drei Gruppen eingeteilt. Die Kontrollgruppe erhielt Stearinsäure als Supplement, die CLA 50 Gruppe 50 g und die CLA 100 Gruppe 100 g einer kommerziellen Mischung unterschiedlicher CLA Isomere, welche zu 10 % trans-10,cis-12 CLA und zu 10 % cis-9,trans-11 CLA enthielt. Zum Schutz vor ruminaler Biohydrierung befand sich die CLA Mischung in einer Kapsel aus an Glycerin gebundener Palmitin- und Stearinsäure.
Das Fettgemisch wurde in pelletiertes Kraftfutter eingearbeitet, welches vom Tag 1 (ein Tag post partum) bis Tag 182 an Transponder gesteuerten Kraftfutterstationen gefüttert wurde. Mais- und Grassilage sowie die übrigen Futterkomponenten wurden in der Laktation ad libitum und in der Trockenstehzeit bedarfsgerecht als Mischration angeboten. Den Tieren wurden an den Tagen -21, 1, 21, 70, 105, 140, 182, 224 und 252 (im Verhältnis zur Abkalbung) Blutproben aus der Vena jugularis entnommen.

Die statistische Auswertung erfolgte varianzanalytisch für wiederholte Messungen im gemischten Modell mit den fixen Effekten Fütterungsgruppe, Laktationstag und Laktationsnummer (Kuh vs. Färse) sowie deren Interaktionen sowie Tier als Zufallseffekt. Die Versuchszeit wurde in zwei zeitliche Abschnitte geteilt, die getrennt ausgewertet wurden: Phase eins (Tag -21 bis Tag 105) und Phase zwei (Tag 105 bis Tag 252).


Die Laktationsphase nahm stets einen signifikanten Einfluss auf den antioxidativen Status in Versuchsphase eins und zwei. Während der Phase eins konnte ein Abfall der Parameter FRAP (p < 0,001), α-Tocopherol (p < 0,001), Retinol (p < 0,001) und des α-Tocopherol:Cholesterin Massenverhältnisses (p < 0,001) in Bezug auf die herannahende Abkalbung nachgewiesen werden, gefolgt von einem anschließenden Anstieg der Blutwerte. In Phase zwei hingegen nahmen die Blutkonzentrationen von FRAP (p < 0,001), α-Tocopherol (p < 0,001) und
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Retinol (p < 0,001) signifikant ab, das α-Tocopherol:Cholesterin Massenverhältnis blieb jedoch weitgehend konstant (p = 0,35).

Neben dem antioxidativen Status war auch bezüglich des oxidativen Status eine deutliche Variation mit dem Laktationsverlauf zu beobachten. In Phase eins stiegen die Konzentrationen der Hydroperoxide (p < 0,001), der Thiobarbitursäure-reaktiven Substanzen (TBARS; p < 0,001) und SH-Gruppen (p < 0,001) zur Abkalbung hin signifikant an und fielen in der frühen Laktation wieder ab. Sie zeigten somit den umgekehrten Verlauf zu den Antioxidantien. In Phase zwei folgte ein signifikanter, fortschreitender Abfall der oben genannten Substanzen, gefolgt von einem Anstieg nach Absetzen des Supplements; Hydroperoxide (p < 0,001), TBARS (p < 0,001) und SH-Gruppen (p < 0,001).

Peroxidative Schäden an Proteinmakromolekülen, gemessen an Plasmakonzentrationen von N'-Formylkynurenin (p < 0,001) und Bityrosin (p < 0,001), nahmen in der ersten Phase zur Abkalbung hin signifikant ab, um nach dem erfolgten Partus in der Frühlaktation wieder anzusteigen. In Phase zwei konnte bei beiden Parametern ein signifikanter, fortlauender Abfall bis zu Ende des Versuchszeitraumes beobachtet werden; N'-Formylkynurenin (p < 0,001) und Bityrosin (p < 0,001).

Basierend auf den Ergebnissen dieses Fütterungsversuches, konnten keine signifikanten Unterschiede im antioxidativen oder oxidativen Status zwischen den Tieren der unterschiedlichen Gruppen festgestellt werden. Die einzige Ausnahme bildeten die Plasmakonzentrationen der SH-Gruppen, welche in Phase eins bei den Tieren der Gruppe CLA 100 tendenziell höher waren (p = 0,085). Diese Tendenz verdeutlichte sich in Phase zwei, dort wurden bei den Tieren der Gruppe CLA 100 signifikant höhere SH-Gruppen Konzentrationen im Plasma festgestellt (p = 0,021). Da bereits zu Versuchsbeginn die Tiere in der CLA 100 Gruppe im Mittel die höchsten Plasmakonzentrationen der SH-Gruppen aufwiesen, erscheint ein Effekt durch die CLA Supplementation jedoch unwahrscheinlich.

Zwischen Kühen und Färsen gab es einen signifikanten Unterschied in Bezug auf Serum α-Tocopherol Konzentrationen in Phase eins (p < 0,001) und in Phase zwei (p < 0,001), wobei Färsen stets höhere Serumkonzentrationen aufwiesen, gleiches galt für das α-Tocopherol:Cholesterin Massenverhältnis (in Phase eins und zwei: p < 0,001). In Phase zwei wurde des Weiteren deutlich, dass Färsen geringere Serumkonzentrationen von
Hydroperoxiden \((p = 0,005)\) und TBARS \((p = 0,013)\) aufwiesen. Die Konzentration der SH-Gruppen war in Phase eins \((p = 0,024)\) sowie in Phase zwei \((p = 0,004)\) im Plasma der Färsen höher als in dem der Kühe. Vermutliche Ursache hierfür erscheint eine längere Weideperiode der Färsen unmittelbar vor der Studienaufnahme und somit eine bessere Vitamin E-Versorgung als bei den Kühen zu sein, welche vor Studienbeginn nur wenige Wochen auf der Weide gehalten wurden.

7 Summary

Nina Hanschke

The effect of graded conjugated linoleic acid (CLA) supplementation on the oxidative and antioxidative status of periparturient and lactating dairy cows

Enhanced metabolism, as present in the periparturient period in dairy cows, might result in an increase of reactive oxygen species (ROS), which are thought to play an important role in various production diseases, such as mastitis. Since antioxidative properties of conjugated linoleic acids (CLA) are discussed controversially, the aim of the presented study was to investigate effects of supplementation of conjugated linoleic acids (CLA) to dairy cows and heifers on their antioxidative and oxidative status. Conjugated linoleic acids are widely used in dairy cattle feeding, to stabilise metabolism in the transition period, by reducing milk fat synthesis.

This study was carried out with German Holstein cows \((n = 32)\) and heifers \((n = 13)\), randomly assigned to three groups, receiving a control fat \((n = 14)\), 50 g \((n = 15)\) or 100 g \((n = 16)\) of a mixture of CLA isomers, containing 10 \% trans-10,cis-12 CLA and 10 \% cis-9,trans-11 CLA. Stearic acid was used as control fat. The CLA mixture was encapsulated with palmitic acid and stearic acid, both linked to glycerine to protect CLA supplements from rumen biohydrogenation. Blood samples were taken from the jugular vein at day -21, 1, 21, 70, 105, 140, 182, 224 and 252 relative to calving. Animals were supplemented between d 1 and d 182. Statistical analysis between groups was done separately for period one (d -21 until d 105) and period two (d 105 until d 252). Differences with days in milk (DIM) and between cows and heifers of all groups were also evaluated.

In the obtained blood samples the antioxidative and oxidative statuses of these animals were determined. For identification of the antioxidative status the ferric reducing ability of plasma was determined, as well as serum concentrations of \(\alpha\)-tocopherol and retinol. The latter were determined to rule out the possibility that differences in antioxidative or oxidative statuses
were caused by nutritional antioxidants. Serum cholesterol concentrations were determined for the calculation of the α-tocopherol:cholesterol ratio.

The oxidative status was determined by measurement of the amount of hydroperoxides in the serum and lipid peroxidation end products, using the thiobarbituric acid (TBA) test. Peroxidative damage to proteins was also determined by means of N′-formylkynurenine and bityrosine. Plasma sulphhydryl groups (SH groups) were determined as an indicator of oxidative stress.

Stage of lactation influenced parameters of the antioxidative status over both periods one and two. During period one the antioxidative parameters significantly decreased towards parturition with a subsequent increase; FRAP (p < 0.001), retinol (p < 0.001), α-tocopherol (p < 0.001) and α-tocopherol:cholesterol mass ratio (p < 0.001). During period two, the antioxidative status significantly decreased towards the end of the trial, including FRAP (p < 0.001), retinol (p < 0.001) and α-tocopherol (p < 0.001), but not the α-tocopherol:cholesterol mass ratio, which remained constant (p = 0.35).

Oxidative parameters were significantly influenced by stage of lactation over both periods. During period one, the amount of hydroperoxides (p < 0.001), TBARS (p < 0.001) and plasma SH groups (p < 0.001) showed the inverse pattern of the antioxidative substances, with a significant increase towards parturition and a subsequent decrease. The decrease continued through to period two, with an increase during depletion period for amount of hydroperoxides (p < 0.001), TBARS (p < 0.001) and plasma SH groups (p < 0.001). In period one protein peroxidation parameters, bityrosine (p < 0.001) and N′-formylkynurenine (p < 0.001) declined towards parturition and increased during early lactation, whereas in period two both parameters declined towards the end of the trial; bityrosine (p < 0.001) and N′-formylkynurenine (p < 0.001).

There were no significant differences in the determined antioxidative or oxidative parameters between animals of different groups, apart from SH group concentrations. The cows of the CLA 100 group tended to have higher SH group concentrations than the other groups during period one (p = 0.085) and had significantly higher SH group concentrations during period two (p = 0.021). Since animals of the CLA 100 group exhibited the highest average plasma
concentrations of SH groups before the CLA supplementation started, it is unlikely that the observed group effect for SH groups was due to the CLA supplementation.

Differences between cows and heifers were detected for serum α-tocopherol concentrations during periods one (p = 0.012) and period two (p < 0.001), with heifers exhibiting higher α-tocopherol concentrations and a higher α-tocopherol:cholesterol mass ratio (both periods p < 0.001). During period two, heifers were detected with lower serum concentrations of hydroperoxides in the sample (p = 0.005) and TBARS (p = 0.013). Plasma SH group concentrations were significantly higher in heifers than in cows in periods one (p = 0.024) and two (p = 0.004). A likely explanation for the observed differences is the longer pasture feed period before the study start for the heifers of the present study (about three months) compared to pluriparous cows, which were only on pasture for up to five weeks, between dry off and enrolment for the present study, leading to a higher vitamin E supply in heifers.

Overall, antioxidants were diminished during the periparturient period in dairy cows and heifers of this trial, while oxidants, such as hydroperoxides, and lipid peroxidation were markedly increased. Even though antioxidants were reduced, the antioxidant defence system could effectively protect proteins from peroxidation. The results of this study also revealed that long term supplementation of a commercial mixture of CLA isomers, containing equal amounts of trans-10,cis-12 CLA and cis-9,trans-11 CLA, had no effect on the antioxidative and oxidative status of dairy cows during the periparturient period and on-going lactation. This was possibly due to the too low CLA dosage combined with insufficient protection of the CLA supplement against ruminal biohydration, leading to poor bioavailability of the CLA isomers. Results suggest further that vitamin E requirements of pregnant and lactating dairy cows exceed the German recommendations published by GFE (2001) by far.
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9 Acknowledgements

Table 25 – Least square means (LS means) and standard error (SE) for serum retinol, cholesterol and protein concentrations of dairy cows in different stages of lactation, separated in three groups, receiving either a control fat supplement (Control; \( n = 14 \)), 50 g CLA (CLA 50; \( n = 15 \)) or 100 g CLA (CLA 100; \( n = 16 \)) between d 1 and d 182, with a depletion period between d 182 and d 252.

DIM = days in milk

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<th>Variable</th>
<th>Groups</th>
<th>DIM</th>
<th>-21</th>
<th>1</th>
<th>21</th>
<th>70</th>
<th>105</th>
<th>140</th>
<th>182</th>
<th>224</th>
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<td>Retinol</td>
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<td>LS Means</td>
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<td>(mg/ml)</td>
<td>CLA 50</td>
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<td>0.415</td>
<td>0.202</td>
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<td>CLA 100</td>
<td>LS Means</td>
<td>0.398</td>
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<td>(mmol/l)</td>
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Table 26 – Least square means (LS means) and standard error (SE) for serum radical cation formed (RC-DEPPD), plasma bityrosine and \(N\)-formylkynurenine of dairy cows in different stages of lactation, separated in three groups, receiving either a control fat supplement (Control; \(n=14\)), 50 g CLA (CLA 50; \(n=15\)) or 100 g CLA (CLA 100; \(n=16\)) between d 1 and d 182, with a depletion period between d 182 and d 252

DIM = days in milk

<table>
<thead>
<tr>
<th>Variable</th>
<th>Groups</th>
<th>DIM</th>
<th>-21</th>
<th>1</th>
<th>21</th>
<th>70</th>
<th>105</th>
<th>140</th>
<th>182</th>
<th>224</th>
<th>252</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC-DEPPD (mmol/l)</td>
<td>Control</td>
<td>LS Means</td>
<td>10.73</td>
<td>12.34</td>
<td>11.18</td>
<td>8.87</td>
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<td>7.63</td>
<td>6.50</td>
<td>10.43</td>
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<td>0.91</td>
<td>0.90</td>
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<td>0.90</td>
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<tr>
<td></td>
<td>CLA 50</td>
<td>LS Means</td>
<td>9.56</td>
<td>10.81</td>
<td>9.82</td>
<td>8.29</td>
<td>7.60</td>
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<tr>
<td></td>
<td>CLA 100</td>
<td>LS Means</td>
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<td>12.78</td>
<td>10.11</td>
<td>9.05</td>
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<tr>
<td>Bityrosine (mg/g)</td>
<td>Control</td>
<td>LS Means</td>
<td>0.399</td>
<td>0.313</td>
<td>0.339</td>
<td>0.377</td>
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<td>0.370</td>
<td>0.368</td>
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<td>LS Means</td>
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<td>0.313</td>
<td>0.317</td>
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<tr>
<td>(N)-formylkynurenine (µg/g)</td>
<td>Control</td>
<td>LS Means</td>
<td>120</td>
<td>98</td>
<td>104</td>
<td>116</td>
<td>117</td>
<td>110</td>
<td>107</td>
<td>93</td>
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<td>CLA 50</td>
<td>LS Means</td>
<td>121</td>
<td>97</td>
<td>97</td>
<td>116</td>
<td>117</td>
<td>111</td>
<td>100</td>
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</table>
Table 27 – Least square means (LS means) and standard error (SE) for serum retinol, cholesterol and protein concentrations of dairy cows (n = 32) and heifers (n = 13) in different stages of lactation

DIM = days in milk

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<tr>
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<tbody>
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<td>serum protein</td>
<td>Cows</td>
<td>LS Means</td>
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<td>(g/l)</td>
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<td>SE</td>
</tr>
<tr>
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<td>Heifers</td>
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<td>(mg/l)</td>
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</tr>
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<td>Cholesterol</td>
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<td>(mg/l)</td>
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</tr>
<tr>
<td></td>
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</tr>
</tbody>
</table>
Table 28 – Least square means (LS means) and standard error (SE) for serum radical cation formed (RC-DEPPD), plasma bityrosine and \(N^\prime\)-formylkynurenine of dairy cows (n = 32) and heifers (n = 13) in different stages of lactation

**DIM = days in milk**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
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<tbody>
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<td>Heifers</td>
<td>LS Means</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SE</td>
</tr>
<tr>
<td><strong>(N^\prime)-formylkynurenine</strong></td>
<td>Cows</td>
<td>LS Means</td>
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<td>LS Means</td>
</tr>
<tr>
<td></td>
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<td>SE</td>
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</tbody>
</table>